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COMMENTS:

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April 30, 2010

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Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
Attn: TSCA Section 8(e) Coordinator
Ariel Rios Building
1200 Pennsylvania Avenue, NW
Washington, DC 20004

8EHQ-0410-17925A

Re: TSCA Section 8(e) Notification of Substantial Risk
Octamethylcyclotetrasiloxane, Decamethylcyclopentasiloxane,
Dodecamethylcyclohexasiloxane and Octamethyltrisiloxane

Dear TSCA Section 8(e) Coordinator:

In accordance with the provisions of Section 8(e) of the Toxic Substances Control Act (TSCA), as interpreted in the TSCA Section 8(e) Policy Statement and Guidance, Fed. Reg. 33129 (June 3, 2003) and other Agency guidance, Dow Corning is submitting information from an ongoing program of sediment studies with cyclic and linear methylsiloxanes. Enclosed are copies of 11 final reports. Dow Corning has not made a determination at this time that any significant risk of injury to human health or the environment is presented by these findings. Dow Corning does not believe this information triggers reporting obligations but lacking relevant guidance from EPA, Dow Corning is submitting the information under Section 8(e) of TSCA as a precautionary measure.

Chemical Substances

CAS No. 556-67-2	Octamethylcyclotetrasiloxane (D4)
CAS No. 541-02-6	Decamethylcyclopentasiloxane (D5)
CAS No. 540-97-6	Dodecamethylcyclohexasiloxane (D6)
CAS No. 107-51-7	Octamethyltrisiloxane (L3)

DCN: 88100000254

Summary of Results

1) **Bioaccumulation Studies with *Lumbriculus variegatus***
   - Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment.
     This study was performed using artificial sediment prepared based on OECD Guideline 218. Two treatment groups were exposed and a negative control was performed concurrently. The mean measured sediment concentrations were 0.86 and 4.06 mg/Kg (based on dry weight). The biota-sediment accumulation factor (BSAF) values for the low and high level treatment groups were 28 and 9.5, respectively. The bioaccumulation factor (BAF) values were 20 and 6.7, and the kinetic bioaccumulation factor (BAFK) values were 20 and 6.7 also.

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- D5: A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. Two treatment groups were exposed and a negative control was performed concurrently. The mean measured sediment concentrations were 20.8 and 336 mg/Kg (based on dry weight). The BSAF values for the low and high level treatment groups were 6.9 and 0.74, respectively. The BAF values were 4.3 and 0.46, and the BAFK values were 4.3 and 0.46 also.

- Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. Two treatment groups were exposed and a negative control was performed concurrently. The mean measured sediment concentrations were 28.1 and 484 mg/Kg (based on dry weight). The BSAF values for the low and high level treatment groups were 0.92 and 0.097, respectively. The BAF values were 0.66 and 0.070, and the BAFK values were 0.67 and 0.070.

2) Prolonged Toxicity Studies with *Lumbriculus variegates*

- Octamethylcyclotetrasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 0.73 to 38 mg/Kg (based on dry weight). The 28-day median lethal concentration (LC50) was calculated as 9.32 mg/Kg. The lowest-observed-effect concentration (LOEC) was determined to be 0.73 mg/Kg. A no-observed-effect-concentration (NOEC) was not determined in this study.

- D4 – Sediment-Water Lumbriculus Toxicity using Spiked Natural Sediment, Following OECD Guideline 225.
  This study was performed using natural sediment collected from Glen Charlie Pond, Wareham, MA. The mean measured sediment concentrations ranged from 1.2 to 32 mg/Kg (based on dry weight). The 28-day LC50 was calculated to be >32 mg/Kg. The LOEC was determined to be 19 mg/Kg and the NOEC was 13 mg/Kg.

- D5: A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 24 to 1272 mg/Kg (based on dry weight). The 28-day LC50 was estimated to be >1272 mg/Kg. The LOEC was determined to be >1272 mg/Kg and the NOEC was 1272 mg/Kg, the highest concentration tested.

- Octamethyltrisiloxane (L3, CAS No 107-51-7): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 0.65 to 17 mg/Kg (based on dry weight). The 28-day LC50 was estimated to be >17 mg/Kg. The LOEC was determined to be 1.6 mg/Kg and the NOEC was 1.1 mg/Kg.

3) **Prolonged Toxicity Studies with *Chironomus riparius***

- D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 6.5 to 355 mg/Kg (based on dry weight). The 28-day median effect concentration (EC50) was calculated to be 114 mg/Kg. The LOEC was determined to be 131 mg/Kg and the NOEC was 44 mg/Kg.

- D5: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 35 to 759 mg/Kg (based on dry weight). The 28-day EC50 was calculated to be 257 mg/Kg. The LOEC was determined to be 160 mg/Kg and the NOEC was 70 mg/Kg.

- D6: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment. (*Final study report to be submitted under separate cover when available*)
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 22 to 684 mg/Kg (based on dry weight). The 28-day EC50 was calculated to be 37 mg/Kg. The LOEC was determined to be 22 mg/Kg. A NOEC value was not determined in this study.

  A follow-up study is planned with D6 and *Chironomus riparius* using natural sediment, which may be completed as early as Q4 2010. The bioavailability of D6 in natural sediment is more representative of the situation in the real environment, which should yield a more realistic measure of toxicity. A copy of this study report will be forwarded to OPPT when it becomes available.

- Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment.
  This study was performed using artificial sediment prepared based on OECD Guideline 218. The mean measured sediment concentrations ranged from 7.4 to 435 mg/Kg (based on dry weight). The 28-day EC50 was calculated to be 166 mg/Kg. The LOEC was determined to be 84 mg/Kg and the NOEC was 39 mg/Kg.

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TSCA Section 8(e) Coordinator
US Environmental Protection Agency
April 30, 2010

4) Prolonged Toxicity Study with *Hyalella azteca*

- 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to D5 Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735.
  This study was performed using natural sediment collected from Glen Charlie Pond, Wareham, MA. The mean measured sediment concentrations ranged from 18 to 460 mg/Kg (based on dry weight). The 28-day LC50 was calculated to be 310 mg/Kg. The LOEC was determined to be 230 mg/Kg and the NOEC was 130 mg/Kg.

Summary

A description of all the results is available in the enclosed reports and final reports from studies in progress will be submitted to EPA as studies are completed. Dow Corning has not made a determination at this time that any significant risk of injury to human health or the environment is presented by these findings.

Actions

Dow Corning Corporation will notify EPA of any further relevant information that may be developed concerning these materials. If you have any questions concerning this submission, please contact me at (989) 496-8046, Kathy.plotzke@dowcorning.com, or at the address provided herein.

Sincerely,

Kathleen P. Plotzke

Kathleen P. Plotzke, Ph.D.
Director, Health and Environmental Sciences
(989) 496-8046

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OCTAMETHYLCYCLOTETRASILOXANE (D4): A BIOACCUMULATION TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-111

ASTM Standard E 1706-00

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

AUTHORS:

Henry O. Krueger, Ph.D.
Susan T. Thomas, B.S.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: July 13, 2007

STUDY COMPLETION DATE: August 14, 2008

SUBMITTED TO:

Centre Européen des Silicones
European Chemical Industry Council (Cefic)
Avenue E. van Nieuwenhuyse 4
B-1160 Brussels, Belgium

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
U.S.A
(410) 822-8600

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Centre Européen des Silicones European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-111

STUDY COMPLETION: August 14, 2008

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exception:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods

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

STUDY DIRECTOR:

Henry O. Krueger, Ph.D — Date: 8/14/08
Director of Aquatic , Plant and Insect Toxicology

SPONSOR APPROVAL:

Sponsor Representative — Date: 8/20/08

Applicant/Submitter — Date

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM(98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: STUDY DIRECTOR	MANAGEMENT:
Protocol	August 10, 2007	August 10, 2007	August 21, 2007
Test Substance Preparation	September 14, 2007	September 14, 2007	October 8, 2007
Dissolved Oxygen Measurements	November 7, 2007	November 7, 2007	November 12, 2007
Biological Data and Draft Report	March 31 – April 3, 2008	April 3, 2008	April 14, 2008
Analytical Data and Draft Report	March 24 – 28, 2008	March 28, 2008	April 1, 2008
Final Report	August 14, 2008	August 14, 2008	August 14, 2008

All inspections were study-based unless otherwise noted.

Michael D. Greene
Quality Assurance Representative

8/14/08
Date

REPORT APPROVAL

SPONSOR: Centre Européen des Silicones European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-111

STUDY DIRECTOR:

Henry O. Krueger, Ph. D.
Director of Aquatic , Plant and Insect Toxicology

Date 8/14/08

PRINCIPAL INVESTIGATORS:

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Date 8/14/08

Willard B. Nixon for Timothy Z. Kendall

Timothy Z. Kendall, M.S.
Supervisor

Date 8/14/08

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

Willard B. Nixon, Ph. D.
Director of Chemistry

Date 8/14/08

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SUMMARY

SPONSOR: Centre Européen des Silicones European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-111

TEST DATES:		
	Study Initiation:	July 13, 2007
	Experimental Start (OECD):	September 12, 2007
	Experimental Start (EPA):	September 17, 2007
	Biological Termination:	November 7, 2007
	Experimental Termination:	December 5, 2007

LENGTH OF EXPOSURE: 51 Days (28 days Uptake; 23 days Depuration)

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
1423 N. 8th St. Suite 118
Superior, Wisconsin 54880
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured in Sediment
Negative Control	<LOQ
5.0 mg a.i./Kg	0.86 mg a.i./Kg
20 mg a.i./Kg	4.06 mg a.i./Kg

RESULTS:

	0.86 mg a.i./Kg	4.06 mg a.i./Kg
BAF[1]	19.6	6.7
k_1[2]	4.0	1.3
k_2[3]	0.21	0.20
BAFK[4]	19.7	6.7
$t_{1/2}$ (days)[5]	3.4	3.5
t_{90} (days)[6]	11.2	11.5

[1] BAF = Bioaccumulation Factor
[2] k_1 = Kinetic Uptake Rate
[3] k_2 = Depuration Rate
[4] BAFK based on Whole Tissue Concentrations (BAFK = Kinetic Bioaccumulation Factor)
[5] $t_{1/2}$ = Calculated time for 50% clearance of D4 in tissue
[6] t_{90} = Calculated time to reach 90% of steady-state in tissues.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Centre Européen des Silicones European Chemical Industry Council (Cefic) at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from September 17, 2007 to November 7, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 570A-111 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the bioaccumulation potential of sediment-incorporated D4 in the oligochaete, *Lumbriculus variegatus* during a 28-day exposure period under flow-through test conditions followed by a depuration period of at least 14 days in untreated sediment. The sediment used in the test had an organic content of approximately 3%. A 28-day ration of food was dry mixed into the test chambers after the sediment had been added, but immediately before water was added to the test compartment, and 48 hours before the organisms were added. The measured endpoints of the test were tissue concentrations after 14 and 28 days of uptake and during depuration.

EXPERIMENTAL DESIGN

The test consisted of a 28-day uptake phase followed by a 23-day depuration phase. During the uptake phase, oligochaetes were exposed to two sub-lethal test concentrations and a negative control. Eight replicate test chambers were maintained in each treatment and control group. Seven replicates were initiated with approximately one gram of tissue in each replicate. One replicate was used for analytical sampling of sediment on Day 0 and did not contain organisms. Each test chamber contained a quantity of sediment and overlying water.

Nominal test concentrations selected in consultation with the sponsor were 5.0 and 20 mg a.i./Kg of sediment based on the dry weight of the sediment. The results of the study are based on the mean measured test concentrations. Sediment samples were collected and analyzed from one sacrificed replicate of each treatment group and control on days 0, 14 and 28 of the uptake phase. Results of the analyses were used to verify the exposure over time. Tissue samples were collected from the culture on Day 0 and from one sacrificed replicate from each treatment group and control on days 14 and 28 of the uptake phase and day 14 of the depuration phase. An additional replicate was sacrificed at the end of the depuration phase from each treatment group and control for the determination of lipid content in the

tissue. The tissue concentrations were used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioaccumulation factor (BAFK).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "D4: A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et. Al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (1); the ASTM E 1706-00 Guideline: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (2); the OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (3); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (4), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (5).

Test Substance

The test substance was received from Dow Corning on June 22, 2007 and assigned Wildlife International identification number 8160. The test substance, a liquid, was identified as: Dow Corning® 244 Fluid; CAS Number 556-67-2; Batch No. LL084732. The reported purity was 99.75%, with an expiration date of January 19, 2009. The test substance was stored under ambient conditions.

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and was assigned Wildlife International, Ltd. identification number 7566 upon receipt. The standard, described as a liquid, was identified on the label as: Tetrakis (trimethylsilyloxy) silane; 97%; Lot #: 13222MB; CAS #: 3555-47-3. The standard had a reported purity of 97%. The standard was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guidelines 218 (6) was used. The sediment was composed of approximately 10% peat moss, 70% sand and 20% silt and clay (kaolin caly). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the

peat, since the peat was added later (Appendix 2). The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry constituents of the final mixture were stored under ambient conditions until used. The results of periodic analyses performed to measure the concentrations of selected contaminants in the formulated sediment used by Wildlife International, Ltd. are presented in Appendix 3. Samples of the sediment used in the test were sent to Agvise Laboratories, Northwood, ND and summaries of the sediment characterizations are found in Appendix 8. The percent organic carbon of the sediment used during the uptake phase and depuration phase was found to be 3.2 and 3.4%, respectively.

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 measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The study was conducted in a temperature-controlled room. Each test chamber was a 9-L aquaria (approximately 15 x 30 x 20cm high) holding approximately 5 liters of water and 1liter of sediment. Aquaria were arranged in groups of four according to treatment group.

A continuous-flow diluter was used to provide dilution water (well water) to each aquaria so that there were two volume additions per day. The flow of dilution water was controlled using rotameters. The rotameters were calibrated prior to the start of the test and approximately weekly during the test. The diluter system delivered dilution water to a mixing chamber from which one line was split into four separate supply lines that deliver water to each aquaria in the group. The proportion of water split to each replicate was checked prior to the test and approximately weekly

during the test to ensure that flow rates varied by no more that 10% of the mean flow rate of the replicates. The general operation of the diluter was checked visually two times per day during the test and at least one time at the beginning and end of the test.

Preparation of Test Concentrations

Concentrations of the test substance were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment). Nominal test concentrations were 5.0 and 20 mg a.i./Kg. An aliquot of 400 g of peat (75% moisture content) was weighed into 24 Nalgene bottles. Eight bottles were used to prepare the 5.0 mg a.i./Kg treatment level and eight were used to prepare the 20 mg a.i./Kg treatment level. The remaining eight bottles were used to prepare the negative control. Neat test material was then added to the peat and mixed overnight on a rotary mixer. After approximately 18 hours of mixing the treatment groups were completely processed one at a time. Approximately 900.0g of the sand and clay mixture was added to each of the 24 Nalgene bottles (eight at a time) and mixed on the rotary mixer for approximately 40 - 60 minutes each.

Eight replicate test chambers were prepared for each treatment and control group for use in the uptake phase. For each replicate, 1000 mL of sediment was placed in a 9L glass aquaria. Next, a pre-weighed amount of food (Salmon Starter), enough for 28 days, was added to each chamber that would contain organisms during the test and was mixed into the dry sediment using a glass stir rod. Finally, approximately 5000 mL of overlying water (well water) was poured into the chamber over a baffle to minimize the disturbance to the sediment. Test chambers were indiscriminately positioned in an environmentally controlled room in groups of four according to treatment group approximately 48 hours prior to test initiation to condition the sediment prior to introduction of organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Three replicate test chambers were prepared for each treatment and control group for use in the depuration phase. For each replicate, 1000 mL of untreated sediment was placed in 9L glass aquaria. The untreated sediment used in the depuration phase of the study was prepared similarly to the treated sediment used in the uptake phase of the study, however 286.0 g of peat (with a 65% moisture content) were mixed with 900.0g of the sand/clay mixture. The reason the weight of peat added to the mixture changed between the sediment prepared for the uptake phase and the sediment prepared for the depuration phase is due to the fact that the moisture content of the peat was different. When corrected

for the percent moisture, the same amount of peat was added to each mixture. The percent organic carbon for the sediment used in the uptake phase and in the depuration phase was 3.2 and 3.4% respectively, showing that the final mixtures were comparable

A pre-weighed aliquot of salmon starter was mixed into the dry sediment prior to adding the 5000mL of overlying water. The chambers were allowed to settle in the environmental chamber approximately three days prior to transferring the test organisms from the uptake test chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 4-day holding period immediately preceding the test, water temperature ranged from 21.5 to 25.8°C, while the pH ranged from 8.0 to 8.6 and dissolved oxygen ranged from 6.7 to 7.7 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, approximately one gram (wet weight) of oligochaetes was impartially distributed to transfer chambers, which were then impartially assigned to test chambers and the organisms were transferred to the test chambers below the air/water interface. Observations of mortality and abnormal behavior were made daily during the test. Seven replicate test chambers in each treatment and control group contained organisms, while one test chamber per group was maintained without organisms for day 0 analytical purposes.

Oligochaetes were fed a mixture of yeast, cereal grass and trout chow (YCT) during the holding period and salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the test. A 200 mg aliquot of food was weighed for each test chamber that contained organisms during the test and was added to the test chamber after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. An additional 200 mg aliquot of salmon starter was added to the

test chambers prepared for the depuration phase of the test after adding the sediment, but prior to adding the overlying water and organisms.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 457 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test chamber daily during the test using a hand-held liquid-in-glass thermometer. Temperature also was measured continuously in a beaker of water adjacent to the test chambers using a Fulscope ER/C Recorder. The continuous recorder was verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a sample of overlying water collected from one alternating replicate test chamber from each treatment and control group at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (7). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test chambers at the beginning of the test, an additional 1 gram organisms were impartially selected at the beginning of the test and measured for dry weight. The weight of the subset of organisms was used to determine the ratio of organic carbon in the sediment to the dry weight of organisms. The test chambers were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). The test was divided into two phases: uptake and depuration. The duration of the uptake phase was 28 days, and the depuration phase was 23 days. On Day 28 of the uptake phase, the organisms from three replicates of each treatment group were sieved from the sediment, enumerated and transferred to new test chambers containing untreated sediment and clean water for the duration of the test. At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group was measured.

Statistical Analyses

The equations used for BAF (bioaccumulation factor) and BAFK (kinetic bioaccumulation factor) calculations are described in draft OPPTS 850.1730 Guidance Document (8). The kinetic uptake rate (k_1) and depuration rate (k_2) were calculated for oligochaete tissue and then used to calculate a kinetic bioconcentration factor (BAFK = k_1/k_2). These rate constants were also used to calculate both the half-life for clearance in tissue ($t_{½}$) and time to reach 90% of steady-state (t_{90}).

Analytical Sampling

Sediment samples were collected from one test chamber of each treatment group at the beginning of the test, on Day 14 and Day 28 of the uptake phase. The replicate used for sample collection on Day 0 did not contain oligochaetes, whereas at test initiation oligochaetes were placed in the replicates used for sample collection on Days 14 and 28. The samples were analyzed on the day of collection. Tissue samples were collected on Day 0 from the culture, on Days 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Tissue samples were also collected on Day 23 of the depuration phase from each treatment group for the determination of lipid content. The depuration phase was continued past Day 14 to Day 23 so that the analytical results of tissue concentrations could be evaluated. Tissue samples were collected by sieving the sediment to remove organisms. The organisms were rinsed free of as much sediment as possible and placed in a tared liquid scintillation vial. The water was removed from the vial (as much as possible) and a wet weight was obtained.

Analytical Method

The methods used for the sediment and tissue analyses of the D4 samples were developed by Wildlife International, Ltd. Sediment was sampled through overlying water from the test beakers and approximately 5 mL of sediment was added to each centrifuge tube. To each sample, 10.0 mL of HPLC-grade bottled water and 5 µL of internal standard was added. Samples were vortexed and shaken after the addition of approximately 20 mL of ethyl acetate and the samples were centrifuged for approximately five minutes at 60% speed. The top layer was transferred into a 50 mL volumetric flask containing 2.5 g of sodium sulfate. Samples were extracted with an additional 20 mL of ethyl acetate. The extracts (1.00 mL) were transferred into autosampler vials and 50.0 µL N-methyl, N-trimethylsilyl trifluoroacetamide was added to each vial. For the tissue analysis, the method consisted of extracting the samples with tetrahydrofuran (THF). Samples were then submitted for analysis by GC/MS or GC/FID.

Concentrations of D4 in the sediment and tissue samples were determined using an Agilent 6890 Gas Chromatograph (GC) equipped with an Agilent Model 5975 Mass Selective Detector operated in SIM mode. Chromatographic separations were achieved using a Phenomen ZB-5 Column (0.25 mm x 30 m, 0.25 µm film thickness). An Agilent 6890 Gas Chromatograph (GC) equipped with Flame Ionization Capture Detector (FID) for tissue samples was also used. Chromatographic separations were achieved using a J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness). Method flowcharts for the analyses of D4 in sediment and tissue are provided in Appendices 6.1 and 6.2, respectively. The instrumental parameters for the analyses are provided in Appendix 6.4 and 6.5, respectively.

For the sediment analyses, calibration standards of D4 analytical standard, ranging in concentration from 0.0500 to 2.50 mg a.i./L were prepared using stock solutions of D4 analytical standard in 80% dimethylformide (DMF) : 20% toluene. The 0.0500 mg a.i./L standard was fortified with 50.0 µL of the internal standard prior to final volume. The standards, ranging from 0.500 to 2.50 mg a.i./L were fortified with 45.0 µL of the internal standard prior to final volume (Appendix 6.6). For the tissue analyses, calibration standards of D4 analytical standard, ranging in concentration from 0.0500 to 0.500 mg/L and 1.00 to 70.0 mg/L were prepared using stock solutions of D4 analytical standard in tetrahydrofuran. All standards for the tissue analyses were fortified with 100 µL of the internal standard prior to final volume (Appendix 6.6). Fresh calibration standards were prepared and

analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of D4 in the samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation. An example of the calculations for a representative sediment sample is included in Appendix 6.7.

For sediment and tissue, the method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (0.0500 mg a.i/L for sediment and 0.0500 mg/L for tissue) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample. No interferences were observed at or above the LOQ during the sample analyses (Appendices 6.8 and 6.9).

Sediment samples were fortified at 5.0 and 20 mg a.i./Kg using stock solutions of D4 in 80% DMF : 20% toluene (Appendix 6.6), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 85.4 to 108% of nominal concentrations (Appendix 6.8).

Tissue samples were fortified at 2.50, 25.0 and 250 mg a.i./Kg, using stock solutions of D4 in THF (Appendix 6.6). The internal standard was fortified at 250 mg a.i./L at the instrument. The measured concentrations for the matrix fortification samples were 90.6 to 97.7% (Appendix 6.9).

Lipid Analyses

Vials containing tissue samples for lipid analysis were removed from the freezer and allowed to thaw. Tare weights of the vials had been pre-determined. Bottled water (10 mL) was added to each sample and samples were homogenized for approximately one minute using a hand-held tissue homogenizer. The homogenizer was rinsed with the appropriate solvent(s) in between samples. Each homogenate was transferred to a 250-mL separatory funnel containing 25 mL of chloroform and 50 mL of methanol. Each vial was rinsed with an additional 10 mL of bottled water and the rinse was poured into its respective separatory funnel. The funnels were then shaken with venting for approximately one minute, and 50 mL of chloroform followed by 50 mL of saturated sodium chloride was added to each separatory funnel. Funnels were swirled briefly with venting and the phases were

allowed to separate. For each sample, the chloroform layer was drained through a powder funnel packed with Teflon® wool and anhydrous sodium sulfate into a 250-mL roundbottom flask. An additional 50-mL aliquot of chloroform was added to each separatory funnel and the extraction and draining procedures were repeated. The extracts were rotary-evaporated to near dryness in a waterbath maintained at approximately 40°C. Each sample was then transferred to a pre-weighed, labeled scintillation vial. Each 250-mL roundbottom flask was rinsed with a small volume of chloroform and the rinse was transferred to its respective scintillation vial. The remaining solvent in each vial was evaporated under a gentle stream of nitrogen or clean dry air. Each vial was reweighed and the weight recorded. The lipid content was calculated from the difference in the vial tare weight and the vial weight containing the lipid. Percent lipids were calculated from the ratio of lipid content to total tissue sample weight. Lipid content determinations are presented in Table 4, and a method flowchart is presented in Appendix 6.3.

A representative calibration curve for sediment is presented in Appendix 6.10. Representative chromatograms of low and high-level calibration standards for sediment are presented in Appendices 6.11 and 6.12, respectively. A representative chromatogram of a matrix fortification sample is presented in Appendix 6.13. A representative chromatogram of a test sample is presented in Appendix 6.14.

A representative calibration curve for tissue is presented in Appendix 6.15. Representative chromatograms of low and high-level calibration standards are presented in Appendices 6.16 and 6.17, respectively. A representative chromatogram of a tissue matrix fortification sample is presented in Appendix 6.18. A representative chromatogram of a tissue test sample is presented in Appendix 6.19.

RESULTS AND DISCUSSION

Measurement of Test Concentrations in Sediment

Results of analyses to measure concentrations of D4 in samples collected during the definitive test are presented in Tables 1-4 and Appendix 6. Nominal concentrations selected for use in this definitive test were 5.0 and 20 mg a.i./Kg.

Samples of sediment were collected from the negative control and each of the test concentrations on Day 0, Day 14 and Day 28. The purpose of these samples was to verify the

concentrations in sediment at test initiation and to monitor the test substance in the sediment over the course of the study. Results are based on mean measured concentrations. Negative control samples on Days 0, 14, and 28 were all below the limit of quantitation (LOQ).

Mean measured concentrations of D4 in sediment from the 5.0 and 20 mg a.i./Kg nominal test concentrations on Day 0 were 0.908 and 6.17 mg a.i./Kg, respectively (Table 1). Mean measured concentrations of D4 in sediment from the 5.0 and 20 mg a.i./Kg nominal test concentrations on Day 14 were 0.818 and 3.97 mg a.i./Kg, respectively. Mean measured concentrations of D4 in sediment from the 5.0 and 20 mg a.i./Kg nominal test concentrations on Day 28 were 0.845 and 2.04 mg a.i./Kg, respectively. All concentrations showed a low but constant trend in the sediment throughout the study. The mean measured concentrations for the 5.0 and 20 mg a.i./Kg nominal treatment groups were 0.86 and 4.06 mg a.i./Kg, or 17 and 20 % of nominal, respectively.

Measurement of D4 in Tissue

Tissue samples were collected from the negative control and each of the test concentrations on Days 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Results of the analyses were used to verify the exposure over time. Negative control samples were below the limit of quantitation (LOQ) at each sampling interval.

The concentrations of D4 in tissues of oligochaetes exposed to 0.86 mg a.i./Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 1. Measured concentrations of D4 in tissues of oligochaetes from the 0.86 mg a.i./Kg test concentration on Days 14 and 28 were 16 and 17 mg a.i./Kg. The BAF and BAFK values for oligochaete tissue were 19.6 and 19.7 respectively (Table 5). The estimated time to reach 90% of steady state (t_{90}) in oligochaete tissue was 11.2 days (Table 5). The concentration of D4 depurated quickly in oligochaete tissue and the mean measured concentration of D4 in oligochaete tissue for the 0.86 mg a.i./Kg treatment group by Day 14 of depuration was 0.96 mg a.i./Kg, which is approximately 5.7% of the mean tissue concentration at Day 28 of uptake (Figure 1). The estimated time to reach 50% clearance ($t_{1/2}$) for oligochaete tissue was 3.4 days (Table 5).

The concentrations of D4 in tissues of oligochaetes exposed to 4.06 mg a.i./Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 2. Measured concentrations of D4 in tissues of oligochaetes from the 4.06 mg a.i./Kg test concentration on Days 14 and 28 were 66 and 27 mg a.i./Kg. The BAF and BAFK values for oligochaete tissue were 6.7 and 6.7, respectively (Table 5). The calculation to estimate time to reach 90% of steady state in oligochaete tissue was 11.5 days (Table 5). As in the 0.86 mg a.i./Kg treatment group, the concentration of D4 depurated quickly in oligochaete tissue and the mean measured concentration of D4 in oligochaete tissue by Day 14 of depuration was 1.6 mg a.i./kg, which is approximately 6.0% of the mean tissue concentration at Day 28 of uptake (Figure 2). Estimate of time to reach 50% clearance for oligochaete tissue was 3.5 days (Table 5).

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 6 through 11. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 69% (5.9 mg/L) of saturation throughout the test. Measurements of pH ranged from 8.1 to 8.3 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 12 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatments and control group and no treatment-related signs of toxicity. At test termination, there was an increase in the numbers of oligochaetes present in the test chambers from the number of worms originally placed in each replicate, indicating that reproduction had occurred.

At the beginning of the test, a one-gram (wet weight) aliquot of organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the dry weight of those organisms was 0.1463 g. It was determined that the formulated sediment had an organic content of 3%; therefore, the ratio of organic carbon to the dry weight of organisms was 30 g organic carbon : 0.1463 grams of dry tissue.

CONCLUSIONS

A kinetic BAF value for D4 in oligochaetes (*Lumbriculus variegatus*) was calculated for both the 0.86 and 4.06 mg a.i./Kg treatment groups. The BAF and BAFK values for oligochaete tissue in the 0.86 mg a.i./Kg treatment group were 19.6 and 19.7, respectively. The BAF and BAFK values for oligochaete tissue in the 4.06 mg a.i./Kg treatment group were 6.7 and 6.7, respectively. During depuration, D4 was eliminated quickly and the Day 14 depuration tissue concentrations for the two treatment groups were 5 7 - 6% of the Day 28 uptake tissue concentrations. The estimated time to reach 50% clearance for oligochaete tissue concentrations was 3.4 days for the 0.86 mg a.i./Kg treatment group, and 3.5 days for oligochaete tissue for the 4.06 mg a.i./Kg treatment group.

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Table 1

Measured Concentrations of D4 in Sediment Samples

Nominal Test Concentration (mg a.i./Kg)	Sample Number (570A-111-)	Sampling Time (Hours)	Measured Concentration (mg a.i./Kg)	Percent of Nominal [1]
Negative Control (0.0)	1A	0	< 0.835	--
	1B	0	< 0.770	--
	4A	14	< 0.397	--
	4B	14	< 0.402	--
	7A	28	< 0.454	--
	7B	28	< 0.520	--
5.0	2A	0	0.750	15.0
	2B	0	0.903	18.1
	2C	0	1.07	21.5
	5A	14	0.941	18.8
	5B	14	0.920	18.4
	5C	14	0.594	11.9
	8A	28	0.823	16.5
	8B	28	0.724	14.5
	8C	28	0.988	19.8
20	3A	0	6.31	31.6
	3B	0	5.72	28.6
	3C	0	6.47	32.3
	6A	14	3.98	19.9
	6B	14	4.49	22.4
	6C	14	3.45	17.2
	9A	28	2.71	13.5
	9B	28	1.53	7.66
	9C	28	1.89	9.46

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

Table 2

Measured Concentrations of D4 in Tissue Samples During the Uptake Phase

Nominal Test Concentration (mg/Kg)	Sample Number (570A-111-)	Sampling Time (Day)	Measured Concentration (mg/Kg)[1]	Mean Measured (mg/Kg)
Negative Control (0.0)	T1	0	< 0.420	--
	T2	14	< 0.373	
	T5	28	< 0.492	
5.0	T3	14	16.4	16.6
	T6	28	16.8	
20	T4	14	66.1	46.7
	T7	28	27.2	

[1] The LOQ was calculated as the product of the low calibration standard (0.0500 mg/L for tissue) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample.

Table 3

Measured Concentrations of D4 in Tissue Samples During the Depuration Phase

Nominal Test Concentration (mg/Kg)	Sample Number (570A-111-)	Sampling Time (Day)	Measured Concentration (mg/Kg)
Negative Control (0.0)	T8	14	< 0.388
5.0	T9*	14	0.959
20	T10*	14	1.64

* Samples reanalyzed.

Table 4

Tissue Lipid Content

Sample I.D. (570A-111-)	Tissue Weight (grams)	Lipid Weight (grams)	Percent Lipids (%)
L-1	1.1345	0.0256	2.26
L-2	1.0453	0.0214	2.05
L-3	0.9681	0.0241	2.49
		Mean lipid percentage	2.26

Table 5

BAF Values

Mean Measured Test Concentration (mg a.i./Kg)	BAF[1]	BAFK[1]	k_1	k_2
0.86	19.6	19.7	4.02	0.21
4.06	6.7	6.7	1.35	0.20

[1] Equations for BAF, BAFK, k_1 and k_2:

BAF = (Day 28 tissue conc) ÷ (mean sediment conc)

BAFK = $(k_1) \div (k_2)$

$k_1 = (k_2 \times \text{Day 28 uptake tissue concentration}) \div (\text{mean sediment conc} \times (1 - e(-k_2 t)))$

$k_2 = (\ln(\text{Day28uptake tissue conc}) - \ln(\text{Day14depuration tissue conc})) \div (\text{14 days of depuration})$

Estimated Time to Reach 50 and 90% of Steady State

Mean Measured Test Concentration (mg a.i./Kg)	$t_{1/2}$ (days)	t_{90} (days)
0.86	3.4	11.2
4.06	3.5	11.5

Table 6

Temperature of Overlying Water in the Test Chambers During the Uptake Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)														
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	G	A	B
Negative Control		23.0	22.7	22.9	22.7	22.8	23.0	23.0	22.7	22.7	22.9	23.0	23.0	22.8	22.8	22.7
0.86		22.6	22.5	22.6	22.6	22.6	22.8	22.9	22.6	22.5	22.6	22.8	22.9	22.7	22.8	22.4
4.06		23.0	22.8	22.8	22.8	22.7	22.7	22.7	22.6	22.7	22.7	22.9	22.8	22.5	22.7	22.6

[1] Temperature measured continuously in a beaker of water adjacent to the test chambers ranged from approximately 22.5 to 23.5°C during the test.

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	C	C	D	E	A	B	C	D	E	A	B	C	D	E
Negative Control		22.8	23.0	22.9	22.9	23.1	23.2	23.1	23.0	23.0	22.7	22.5	22.6	23.1	23.1
0.86		22.7	22.8	22.7	22.7	23.0	23.0	22.9	22.8	22.9	22.6	22.1	22.0	22.7	22.9
4.06		22.7	22.8	22.8	22.7	23.1	23.0	23.0	22.9	22.8	22.7	22.3	22.6	23.2	23.0

Table 7

Temperature of Overlying Water in the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control		23.0	23.1	23.2	23.1	23.1	23.1	23.0	23.0	23.2	23.0	22.9	23.2	22.5	22.3
0.86		22.8	23.0	23.2	23.0	23.0	23.0	22.6	22.8	23.0	22.7	22.5	22.8	22.2	22.0
4.06		23.0	23.1	23.2	23.3	23.0	23.0	23.0	22.9	23.0	23.1	22.7	22.8	22.5	22.0

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)								
		15	16	17	18	19	20	21	22	23
	Replicate:	A	B	A	B	A	B	A	B	A
Negative Control		22.9	23.1	23.5	23.1	22.9	23.2	22.7	23.2	22.8
0.86		22.7	22.7	23.2	22.7	22.3	22.5	22.5	22.4	22.0
4.06		23.0	22.8	23.1	22.8	22.6	22.6	22.6	22.5	22.2

Table 8

Dissolved Oxygen in the Overlying Water in the Test Chambers During the Uptake Phase

Mean Measuredl Test Concentration (mg a.i./Kg)	Day	0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	G	A	B
		Dissolved Oxygen (mg/L)														
Negative Control		7.9	7.9	7.5	7.5	7.6	7.5	7.9	7.7	7.4	7.7	7.5	7.4	7.9	8.2	7.9
0.86		7.9	7.5	7.4	7.4	7.6	7.4	7.6	7.5	7.4	7.5	7.6	7.6	7.5	8.2	7.8
4.06		7.7	7.7	7.5	7.2	7.0	7.3	7.6	7.6	7.6	7.4	7.5	7.4	7.6	8.3	7.9

[1] A dissolved oxygen concentration of 5.1mg/L represents 60% saturation at 23°C.

Mean Measured Test Concentration (mg a.i./Kg)	Day	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	C	C	D	E	A	B	C	D	E	A	B	C	D	E
		Dissolved Oxygen (mg/L)													
Negative Control		7.7	7.5	7.9	7.9	7.5	7.5	7.1	7.5	7.8	7.5	7.4	7.2	7.9	7.8
0.86		7.4	7.3	7.9	7.9	7.5	7.5	7.2	7.5	7.7	7.4	7.5	7.5	8.1	7.9
4.06		7.3	7.5	7.6	7.7	7.7	7.8	7.5	7.6	7.2	7.6	7.7	7.5	7.7	6.9

Table 9

Dissolved Oxygen in the Overlying Water of the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	Dissolved Oxygen (mg/L)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control		7.8	7.4	7.7	7.3	7.6	7.2	7.6	7.0	7.5	7.9	7.6	7.9	8.1	8.3
0.86		8.0	7.0	7.9	7.6	7.8	7.7	7.4	5.9	7.4	7.7	6.6	6.9	8.1	7.2
4.06		7.4	6.7	6.3	6.2	6.6	6.9	7.3	6.6	6.9	7.6	7.6	7.9	8.0	8.2

Mean Measured Test Concentration (mg a.i./Kg)	Day	Dissolved Oxygen (mg/L)								
		15	16	17	18	19	20	21	22	23
	Replicate:	A	B	A	B	A	B	A	B	A
Negative Control		8.0	8.1	8.0	8.0	8.1	7.8	7.7	7.7	8.3
0.86		8.1	7.4	8.0	7.4	8.2	7.7	7.8	7.1	8.2
4.06		7.9	8.1	7.9	7.9	8.0	7.6	7.7	7.4	8.2

Table 10

pH of Overlying Water in the Test Chambers During the Uptake Phase

Mean Measured Test Concentration (mg a.i./Kg)	Replicate:	pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
		A	B	C	D	E
Negative Control		8.2	8.2	8.1	8.2	8.2
0.86		8.2	8.2	8.1	8.2	8.3
4.06		8.2	8.2	8.2	8.3	8.2

Table 11

pH of Overlying Water in the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	pH			
	Day 7	Day 14	Day 21	Day 23
Replicate:	A	B	A	B
Negative Control	8.2	8.3	8.3	8.3
0.86	8.2	8.2	8.3	8.3
4.06	8.2	8.3	8.3	8.3

Table 12

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Chambers During the Test

Mean Measured Test Concentration (mg a.i./Kg)	Day 0 (Uptake)				Day 23 (Depuration)			
	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)
Negative Control	140	186	330	<0.17[1]	140	180	330	<0.17
0.86	144	180	340	<0.17	140	180	330	<0.17
4.06	148	182	340	<0.17	144	182	340	<0.17
Replicate	A				B			

[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Figure 1.

Concentrations of D4 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 0.86 mg a.i./Kg

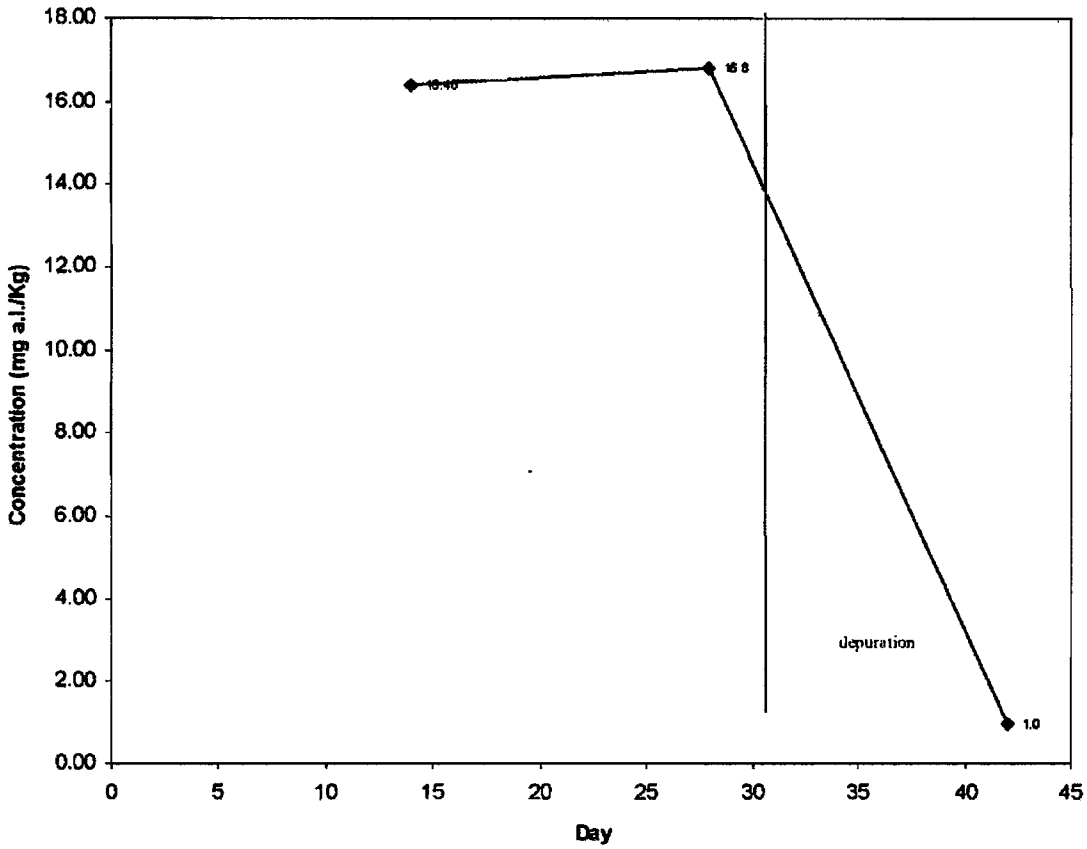
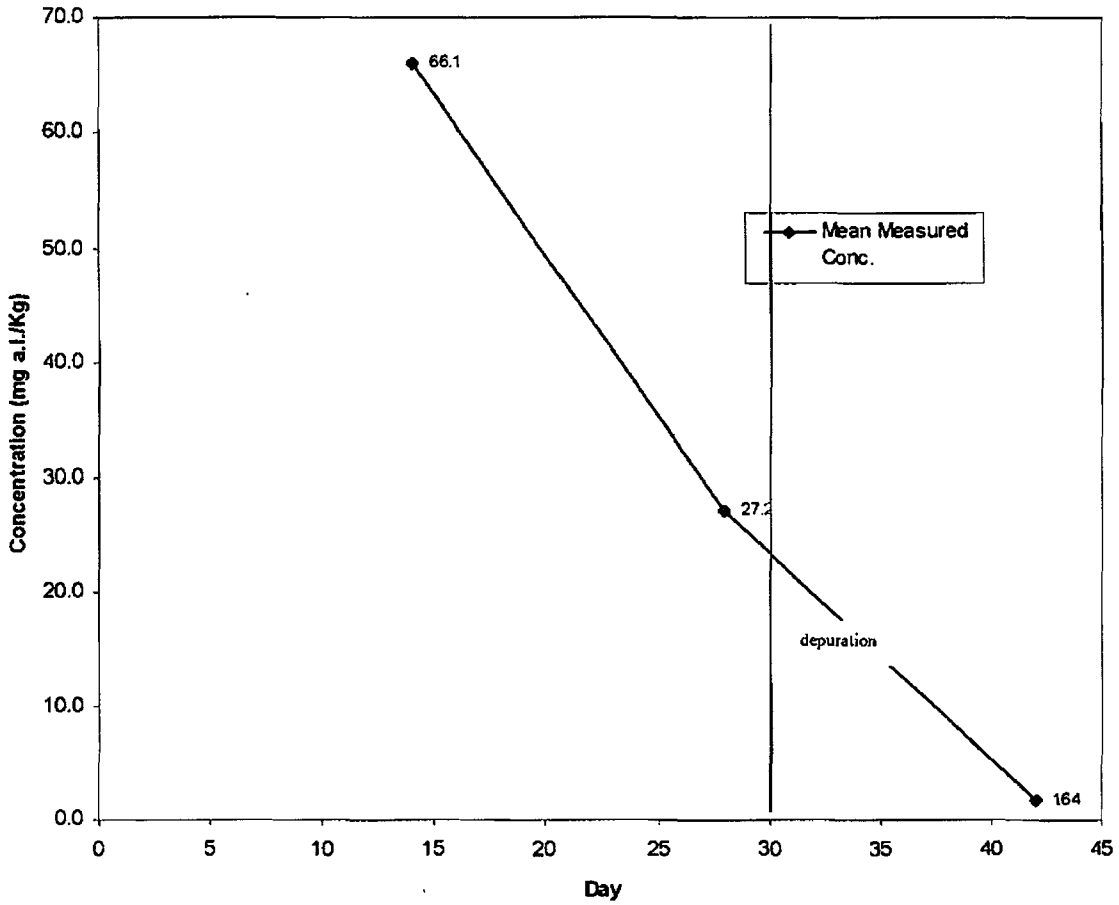


Figure 2.

Concentrations of D4 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 4.06 mg a.i./Kg



Appendix 1

Study Protocol, Amendments and Deviations

PROTOCOL

OCTAMETHYLCYCLOPENTASILOXANE (D_4): A BIOACCUMULATION TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

ASTM Standard E 1706-00

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
570A-111

SPONSOR

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

PERFORMING LABORATORY

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

July 6, 2007

Wildlife International, Ltd.

OCTAMETHYLCYCLOPENTASILOXANE (D_4): A BIOACCUMULATION TEST
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

SPONSOR: Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

SPONSOR'S REPRESENTATIVE: Kurt R. Drottar
Dow Corning Corporation
Health and Environmental Sciences
2200 West Salzburg Road
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TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
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STUDY DIRECTOR: Henry O. Krueger Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants and Insects
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Willard B. Nixon, Ph. D.
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 7/13/07

LABORATORY MANAGEMENT — DATE 7/18/07

SPONSOR'S REPRESENTATIVE — DATE 7/6/07

Wildlife International, Ltd.

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FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: _____ Experimental Termination Date: _____

Project No.: 570A-111

Test Concentrations: _____

Test Substance No.: _____ Reference Substance No. (if applicable): _____

INTRODUCTION

Wildlife International, Ltd. will conduct a bioaccumulation test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1); the U.S. EPA 600/R-99/064 *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.) (2), the ASTM Standard E 1706-00: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (5), and the Federal Environment Agency: Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (6). Raw data for all work performed at Wildlife International, Ltd. and 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. A copy of the final report will be archived at Dow Corning.

OBJECTIVE

The objective of this study is to determine the bioaccumulation potential of a sediment-incorporated test substance in the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions followed by a depuration period of at least 14 days in untreated sediment. The sediment used in the test will have an organic content of approximately 5%. A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after the sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding organisms. The measured endpoints of the test are tissue concentrations after 14 and 28 days of uptake and during depuration

EXPERIMENTAL DESIGN

Oligochaetes will be exposed to two test concentrations (100 and 1000 mg a.i./kg) and a negative control. Each group will consist of eight replicate test chambers. Seven replicates will contain up to approximately one gram of tissue (approximately 300 organisms) in each chamber. One

replicate will be used for analytical sampling of sediment on Day 0. The test will be divided into two phases: uptake and depuration. During the uptake phase, oligochaetes in the treatment groups will be exposed to sublethal concentrations of sediment incorporated D-4, while oligochaetes in the control group will be exposed only to untreated sediment. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Days 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Each test chamber will contain a quantity of sediment and overlying water. Only sediment will be analyzed in test systems, since the water solubility of D4 is 56.2 µg/L (7). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of D4 in water. Therefore, only sediment samples from the analytical sampling test chambers will be collected from each control and treatment group at specified intervals for analysis of the test substance. No oligochaetes will be placed in "analytical" replicate(s) sampled at the beginning of the test. However, the "analytical" replicate(s) sampled after the beginning of the test (e.g., on Day 14 and 28) will contain oligochaetes. Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be 100 and 1000 mg a.i./kg. Results of the analyses will be used to verify the exposure over time.

To control bias, oligochaetes will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to

GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	Octamethylcyclotetrasiloxane (D4), (Supplied as Dow Corning® 244 Fluid)
Lot No.:	LL084732
Expiration Date:	January 19, 2009
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	556-67-2
Physical Description:	Colorless liquid
Chemical Stability:	Stable
Purity:	99.75 ± 0.003 area percent purity
Solubility:	Soluble in common organic solvents such as acetone
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1.5 kg batch of sediment to 5%. The peat and test substance will be mixed overnight on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data. Approximately 1 - 2 L of the dry mix will then be transferred to each of eight test chambers. The same volume of sediment will be added to each chamber. The transfer time will be kept to a

minimum (approximately 10-20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance.

After all 8 chambers have been filled with sediment, a 28 day ration of food (approximately 200 mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 5 L of well water will be added to the chambers. Test chambers will be maintained in an environmentally controlled room. Dilution water will be delivered to each test chamber at a rate that will result in two turnovers of dilution water per day. After approximately 48 hours of acclimation and settling, organisms will be added to the chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study. Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd. Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Organisms will be fed YCT during holding. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test. Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (8) will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon.

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductanec, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report.

Test Apparatus

The study will be conducted in a temperature-controlled room. Each test chamber will be a 9-L aquarium (approximately 15 X 30 X 20 cm high) holding approximately 5 liters of water. Each aquarium will be filled with the same amount of sediment (1 to 2 L). Aquaria will be set out in groups of four.

A continuous-flow diluter will be used to provide dilution water (well water) to each aquaria so that there are two volume additions per day. The flow of dilution water will be controlled using rotameters. The rotameters will be calibrated prior to test initiation and at approximately weekly intervals thereafter. The diluter system delivers dilution water to a mixing chamber from which one line is split into four separate supply lines that deliver water to each aquaria in a group. The proportion of water split to each replicate will be checked prior to the test and at approximately weekly intervals thereafter to ensure that these flow rates vary by no more than ±10% of the mean flow rate of the replicates. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured daily using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured daily in the overlying water from one alternating replicate of each experimental group. Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week.

Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a sample of the overlying water from one replicate of each experimental group at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (9). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, approximately 1 g (approximately 300) adult oligochaetes will be impartially distributed to transfer containers (e.g., glass beakers) until each container holds its complement of approximately 1 g. The organisms in each transfer container then will be transferred below the air/water interface to the test chambers (glass aquaria). An additional batch of individuals (~1 g) will be impartially selected at the beginning of the test and will be measured for dry weight. The weight of the subset of organisms will be used to determine the ration of organic carbon in the sediment to the dry weight of organisms. After completing the transfer of organisms, the test chambers will be maintained in a temperature-controlled room. Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming). At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group will be measured.

The test will be divided into two phases: uptake and depuration. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Day 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from one test chamber of each concentration at the beginning of the test (no more than one hour after completing the addition of organisms to the test system), at 14 days and at the end of the uptake phase (Day 28). The overlying water will be removed from the test chamber. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below:

Phase	Sample Interval	Sediment[1]	Oligochaetes[2]	Action
Uptake	0 hours	X	X[3]	Organisms added
	14 days	X	X	
	28 days	X	X	Organisms removed And purged
Depuration[4]	14 days		X	

[1] There will be two concentration tested (100 and 1000 mg a.i./kg dry weight). Sufficient sediment will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[2] Approximately 1g of oligochaetes will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[3] One sample of approximately 1g of oligochaetes from the culture will be used to determine background levels in oligochaetes.

[4] Additional depuration samples will be added if necessary pending the outcome of the Day 14 samples.

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

The kinetic uptake rate (k_1) and depuration rate (k_2) will be calculated for oligochaete tissue using the calculations described in draft OPPTS 850.1730 Guidance Document (10). These rate constants will be used to calculate a kinetic bioconcentration factor (BCFK = k_1/k_2) and also to calculate the half-life for clearance in tissue ($t_{1/2}$), time to reach 90% of steady-state (t_{90}), and BCFK values using the equations and graphical methods outlined in the draft OPPTS 850.1730 Guidance Document.

The BCFK of D4 can be defined as the ratio of the uptake rate constant, k_1 to the elimination (depuration) rate constant, k_2. That is,

$$BCFK = \frac{k_1}{k_2}. \quad \text{(Eq. 1).}$$

This kinetic definition of the BCFK is valid regardless of whether a steady-state concentration of a chemical is achieved during uptake as long as first-order kinetics can be assumed for uptake and elimination processes. Both the uptake rate, k_1, and elimination rate, k_2, of the chemical can be estimated by measuring concentrations of the chemical in water and fish tissues at several points in time.

If elimination follows first order kinetics, the decline in log concentration of the chemical in tissues over time is a straight line. The slope of this line is the elimination rate constant, k_2. Thus, if measurements of chemical concentration in tissues are made at only two time points, the estimate can be obtained using the simple algebraic relationship

$$k_2 = \frac{(\ln C_m - \ln C_t)}{t_e} \quad \text{(Eq. 2).}$$

where C_m is the concentration in fish tissue at the end of the uptake period and C_t is the concentration in the fish tissue after an elimination period of length t_e. Alternatively, k_2 can be estimated as the

slope calculated by linear regression if measurements are made at more than two time points.

Finally, the uptake rate is calculated as

$$k_1 = \frac{k_2 C_w}{C_w(1-e^{-k_2 t})} \quad \text{(Eq. 3).}$$

where t is the length of the uptake (exposure) period and C_w is the concentration of D4 in the water during the exposure period.

The half-life for clearance in tissue during the depuration phase was determined by the following equation:

$$t_{1/2} = (1/k_2)\ln 2$$

It is assumed that the two chamber, two-parameter model applies. This means that the time to reach 90% steady-state in the uptake phase is about equal to the time it takes to reach 90% of depuration in the depuration phase. Therefore, k_2, the depuration rate constant, was used in the following equation to determine the time to reach 90% steady-state for uptake:

$$0.9 = 1 - e^{-k2t}$$

or

$$t_{90} = 2.3/k_2$$

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Lumbriculus variegatus* culture records.
5. Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations.
6. Biological observations.
7. Test conditions (light intensity, photoperiod, etc.).

8. Water chemistry results (e.g., hardness and alkalinity).
9. Statistical calculations, if applicable.
10. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.

14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

REFERENCES

1 **Phipps, G.L., G.T. Ankley, D.A. Benoit, and V.R. Mattson.** 1993. Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants. Environ. Toxicol. Chem. 12:269-279.

2 **U.S. Environmental Protection Agency.** 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.). EPA 600/R-99/064

3 **ASTM Standard E 1706-00.** 2000. *Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates.* American Society for Testing and Materials.

4 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater.*

5 **European Chemicals Bureau.** 2003. *Technical Guidance Document on Risk Assessment, Part II.* Institute for Health and Consumer Protection.

6 **Federal Environment Agency.** 2001. *Validation of the Preliminary EU- Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances.* Institute of Hydrobiology Dresden University of Technology.

7 **Varaprath, S., C.L. Frye and J. Hamelink.** 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263 – 1265.

8 **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Organization for Economic Cooperation and Development. Adopted 13 April 2004.

9 **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.

10 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1730: *Fish BCF.*

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclopentasiloxane (D_4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOa/SUB570 **AMENDMENT NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-111

EFFECTIVE DATE: September 14, 2007

AMENDMENT: Page 3:

ADD:

Proposed Dates:	Experimental Start Date: September 17, 2007 Experimental Termination Date: October 29, 2007
Test Concentrations:	Negative Control, 5.0 and 20 mg a.i./Kg
Test Substance No.:	8160
Reference Substance No.:	NA

REASON: This information was not available at the time the protocol was signed by the Study Director.

AMENDMENT: Preparation of Test Concentrations, Page 6:

Sediment will be prepared in 1 kg batches instead of 1.5 kg batches.

REASON: Smaller batches will be prepared for ease of handling and to reduce the amount of sediment left over after preparation of test chambers.

STUDY DIRECTOR — DATE 9/14/07

LABORATORY MANAGEMENT — DATE 9/17/07

Wildlife International, Ltd. Project Number 570A-111

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D_4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOa/SUB570 **AMENDMENT NO.:** 2

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-111

EFFECTIVE DATE: October 12, 2007

AMENDMENT: Page 1 and 2:

CHANGE: **SPONSOR:** Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

TO: **SPONSOR:** Centre Européen des Silicones
European Chemical Industry Council (Cefic)
Avenue E. van Nieuwenhuyse 4
B-1160 Brussels, Belgium

REASON: Request of Sponsor.

STUDY DIRECTOR — DATE 10/16/07

LABORATORY MANAGEMENT — DATE 10/16/07

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D_4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOa/SUB570 **AMENDMENT NO.:** 3

SPONSOR: Centre Européen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 570A-111

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Page 1 and 2:

CHANGE: Octamethylpentasiloxane

TO: Octamethyltetrasiloxane

REASON: There was an error in the spelling of the test substance name. Octamethyltetrasiloxane is the correct spelling.

STUDY DIRECTOR — DATE 4-10-08

LABORATORY MANAGEMENT — DATE 4/10/08

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOa/SUB570 **DEVIATION NO.:** 1

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 570A-111

DATE OF DEVIATION: October 3, 2007

DEVIATION: Water quality measurements were measured in replicate C in all treatment groups on Day 15 and Day 16 instead of the next replicate in the sequence as indicated by the protocol.

REASON: Biologist oversight. The same replicate was inadvertently sampled two days in a row. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

STUDY DIRECTOR — DATE 4-10-08

LABORATORY MANAGEMENT — DATE 4/10/08

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D4): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOa/SUB570 **DEVIATION NO.:** 2

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 570A-111

DATE OF DEVIATION: November 5, 2007

DEVIATION: A second check on the operation of the diluter system was not performed on depuration Day 21 of the test (November 5, 2007).

REASON: Biologist oversight. The operation of the diluter system was inadvertently not checked a second time on depuration Day 21 of the test. Diluter checks on depuration Day 22 show the diluter system was working properly. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

STUDY DIRECTOR — DATE 4-10-08

LABORATORY MANAGEMENT — DATE 4/10/08

Appendix 2

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000[2]

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used.

[2] The amount of peat stated above is a theoretical value, based on 10% of the target final weight of the recipe. The peat was added to individual batches, based on the same percentage, and all ingredients were mixed on a rotary mixer.

Appendix 3

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Formulated Sediment w/Peat[1]

Page 1

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.88	Gamma BHC – Lindane	< 0.88
Alpha BHC	< 1.1	Gamma Chlordane	< 3.2
Alpha Chlordane	< 0.88	Guthion (Azinphos-methyl)	< 71
Beta BHC	< 2.1	HCB	< 0.88
Bolstar	< 71	Heptachlor	< 0.88
Chlordane	< 18	Heptachlor Epoxide	< 0.88
Coumaphos	< 71	Kepone	< 7.4
Delta BHC	< 0.88	Malathion	< 71
Demeton-O	< 71	Merphos	< 71
Demeton-S	< 71	Methoxychlor	< 8.8
Diazinon	< 71	Methyl Parathion	< 71
Dichlorvos	< 71	Mevinphos	< 71
Dieldrin	< 1.8	Mirex	< 1.8
Disulfoton	< 80	Naled	< 71
Dursban (Chlorpyrifos)	< 71	o,p-DDD	< 1.8
Endosulfan I	< 0.88	o,p-DDE	< 1.8
Endosulfan II	< 1.8	o,p-DDT	< 1.8
Endosulfan Sulfate	< 1.8	p,p-DDD	< 1.8
Endrin	< 1.8	p,p-DDE	< 1.8
Endrin Aldehyde	< 1.8	p,p-DDT	< 1.8
Endrin Ketone	< 1.8	Phorate	< 71
EPN	< 71	Ronnel	< 71
Ethion	< 71	Stirophos	< 71
Ethoprop	< 71	Telodrin	< 1.3
Ethyl Parathion	< 71	Tokuthion	< 71
Famphur	< 71	Toxaphene	< 35
Fensulfothion	< 71	Trichloronate	< 71
Fenthion	< 71	Trithion	< 71

[1]Analyses performed by Lancaster Laboratories on samples collected on December 19, 2007.

Appendix 3
(continued)

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Formulated Sediment w/Peat[1]

Page 2

Metals			
Component	Measured Concentration (mg/kg)	Component	Measured Concentration (mg/kg)
Aluminum	9720	Magnesium	3200
Antimony	< 2.04	Manganese	3.80
Arsenic	< 2.04	Mercury	< 0.104
Barium	22.6	Nickel	3.92
Beryllium	< 0.510	Nitrate by IC (solid)	< 1.6
Bromide by IC (solid)	< 5.3	Nitrite by IC (solid)	< 1.1
Cadmium	< 0.510	Potassium	73.1
Calcium	5470	Selenium	< 2.04
Chloride by IC (solid)	55.9	Silver	< 0.510
Chromium	9.31	Sodium	< 102
Cobalt	< 0.510	Sulfate by IC (solid)	82.9
Copper	1.43	Thallium	< 2.04
Fluoride by IC (solid)	2.5	Vanadium	2.89
Iron	344	Zinc	3.75
Lead	9.76		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 19, 2007.

Appendix 4

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

	Mean	Range
Specific Conductance (µmhos/cm)	315 (N = 4)	310 - 320
Hardness (mg/L as $CaC0_3$)	132 (N = 4)	128 - 136
Alkalinity (mg/L as $CaC0_3$)	180 (N = 4)	180 - 180
pH	8.2 (N = 4)	8.1 - 8.2

Appendix 5

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.020	Gamma BHC – Lindane	< 0.010
Alpha BHC	< 0.010	Gamma Chlordane	< 0.010
Alpha Chlordane	< 0.010	Guthion (Azinphos-methyl)	< 3.0
Beta BHC	< 0.020	HCB	< 0.010
Bolstar	< 2.0	Heptachlor	< 0.010
Chlordane	< 0.50	Heptachlor Epoxide	< 0.010
Coumaphos	< 3.0	Kepone	< 0.20
Delta BHC	< 0.010	Malathion	< 2.0
Demeton-O	< 2.0	Merphos	< 6.0
Demeton-S	< 2.0	Methoxychlor	< 0.10
Diazinon	< 3.0	Methyl Parathion	< 2.0
Dichlorvos	< 3.0	Mevinphos	< 3.0
Dieldrin	< 0.020	Mirex	< 0.11
Disulfoton	< 2.0	Naled	< 2.0
Dursban (Chlorpyrifos)	< 2.0	o,p-DDD	< 0.020
Endosulfan I	< 0.010	o,p-DDE	< 0.020
Endosulfan II	< 0.020	o,p-DDT	< 0.020
Endosulfan Sulfate	< 0.020	p,p-DDD	< 0.020
Endrin	< 0.020	p,p-DDE	< 0.020
Endrin Aldehyde	< 0.10	p,p-DDT	< 0.020
Endrin Ketone	< 0.020	Phorate	< 2.0
EPN	< 2.0	Ronnel	< 2.0
Ethion	< 2.0	Stirophos	< 2.0
Ethoprop	< 3.0	Telodrin	< 0.010
Ethyl Parathion	< 2.0	Tokuthion	< 2.0
Famphur	< 2.0	Toxaphene	< 3.0
Fensulfothion	< 3.0	Trichloronate	< 2.0
Fenthion	< 2.0	Trithion	< 2.0

[1]Analyses performed by Lancaster Laboratories on samples collected on December 19, 2007.

Appendix 5

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	14.6
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	6.97
Calcium	38.7	Selenium	< 0.0200
Chloride	4.2	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.8
Cobalt	< 0.0050	Sulfate	6.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	0.55	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 19, 2007.

Appendix 6

The Analysis of D4 in Sediment and Tissue

Appendix 6.1

Analytical Method Flowchart for the Analysis of D4 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF D4 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample sediment through the overlying water from the test beakers. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.

↓

Fortify recovery samples with the appropriate stock solution.

↓

Add 10.0 mL HPLC-grade bottled water and 5 µL of internal standard to each sample and QC.

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately 10 minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50 mL volumetric flask containing 2.5 g sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining the ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Transfer 1.00 mL of extracts into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit samples for analysis.

Appendix 6.2

Analytical Method Flowchart for the Analysis of D4 in Tissue

FLOWCHART FOR THE ANALYSIS OF D4 IN TISSUE

Rinse all glassware with THF.

↓

Whole samples will be extracted in scintillation vials as received. Add 10 mL of THF to each sample and matrix blank. Fortify quality control samples with appropriate stock solutions. Fortify all samples and QC samples with internal standard (250 µg a.i./L at instrument).

↓

Homogenize tissues with a spatula. Minimize the time the contents of the vial is open to air. Vortex for approximately two minutes. Sonicate for 10 minutes and vortex again briefly to wash the sides of the vial

↓

Transfer approximately 2 mL of the extract to a second vial and add approximately 500 mg of NA_2SO_4. Vortex for 15 seconds and allow to dry for approximately 15 minutes.

↓

Transfer an aliquot of the dried extract to a microcentrifuge tube and ultra-centrifuge each sample for approximately five minutes at 14,000 rpm.

↓

Transfer an aliquot of each extract to an autosampler vial. Submit samples for GC/MS or GC/FID analysis.

Appendix 6.3

Analytical Method Flowchart for the Analysis of Lipids in Tissue

METHOD OUTLINE FOR THE PROCESSING OF LIPIDS IN TISSUE FOR D4 CONCENTRATIONS

Remove vials to be analyzed from freezer and allow samples to thaw.

↓

To each sample, add 10 mL of HPLC-grade bottled water and homogenize for approximately one minute using a hand-held tissue homogenizer. Rinse the homogenizer wit the appropriate solvent(s) in between samples.

↓

Transfer each homogenate to a 250-mL separatory funnel that contains 25 mL of chloroform and 50 mL of methanol.

↓

Rinse each vial with an additional 10 mL of bottled water and pour rinse into respective separatory funnel.

↓

Shake each separatory funnel for approximately one minute with venting.

↓

Add 50 mL of chloroform followed by 50 mL of saturated sodium chloride to each separatory funnel.

↓

Briefly swirl each separatory funnel with venting.

↓

Allow the phases to separate. For each sample, drain the chloroform layer through a powder funnel packed with Teflon® wool and anhydrous sodium sulfate into a 250-mL round-bottom flask.

↓

Add an additional 50-mL aliquot of chloroform to each separatory funnel and repeat the extraction and draining procedures.

↓

Rotary evaporate the extracts in a water bath maintained at approximately 40°C to near dryness.

↓

Transfer each extract to a pre-weighed, labeled scintillation vial.

↓

Rinse each 250-mL round-bottom flask with a small volume of chloroform and transfer rinse to respective scintillation vial.

↓

Evaporate the remaining solvent in each vial under a gentle stream of nitrogen or clean dry air.

↓

Reweigh each vial and record weight.

Appendix 6.4

Typical Gas Chromatograph (GC) Operational Parameters for Sediment Analysis

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Agilent Model 5975 Mass Selective Detector operated in SIM mode
ANALYTICAL COLUMN:	Phenomen ZB-5 column (30 m x 0.25 mm, 0.25 µm film thickness)
INJECTOR TEMPERATURE:	280°C
RUN TIME:	15.25 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 8.00°C/minute Final temperature 1: 150°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 220°C Final hold time 2: 0.00 minute
DETECTOR TEMPERATURE:	310°C
INJECTION VOLUME:	1.0 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	Approximately 12 psi
APPROXIMATE D4 RETENTION TIME:	10.2 minutes

Appendix 6.5

Typical Gas Chromatograph (GC) Operational Parameters for Sediment/Tissue Analysis

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)	
DETECTOR:	Hewlett-Packard Flame Ionized Detector (FID)	
ANALYTICAL COLUMN:	J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness)	
INJECTOR TEMPERATURE:	280°C	
OVEN:	Initial temperature:	50°C
	Initial hold time:	1.00 minute
	Ramp 1:	8.00°C/minute
	Final temperature 1:	150°C
	Final hold time 1:	0.00 minute
	Ramp 2:	40.00°C/minute
	Final temperature 2:	220°C
	Final hold time 2:	0.00 minute
DETECTOR TEMPERATURE:	310°C	
INJECTION VOLUME:	1.0 µL	
CARRIER GAS:	Helium	
HEAD PRESSURE:	~12 psi	
APPROXIMATE D4 PEAK RETENTION TIME:	7.2 minutes	

Appendix 6.6

Analytical Stocks and Standards Preparation

For the sediment analysis, stock solutions of D4 were prepared by weighing 1.0002 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 80% DMF : 20% toluene. This primary stock solution (10.0 mg/mL) was diluted in 80% DMF : 20% toluene to produce 1.00 and 0.100 mg/mL stock solutions. The 1.00 and 0.100 mg/mL stock solutions were used to fortify the sediment quality control samples. Calibration standards were prepared in ethyl acetate using the 0.100 mg/mL stock solution. 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)
0.500*	10000	10.0	0.0500
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
0.100	250	10.0	2.50

A stock solution of Tetrakis (trimethylsilyloxy) silane (D4 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% DMF : 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% DMF : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to sediment samples and calibration standards (50 µL or 45 µL*).

For the tissue analyses, a stock solution of D4 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 tetrahydrofuran. This primary stock solution (1.00 mg/mL) was diluted in tetrahydrofuran to produce 0.100 and 0.0100 mg/mL stock solutions. The 1.00, 0.100 and 0.0100 mg/mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in tetrahydrofuran using the 0.100 and 1.00 mg/mL stock solutions. The following shows the dilution schemes for both sets of calibration standards for the tissue analyses:

Stock Concentration (mg/mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg/L)
0.0100	50.0	10.0	0.0500
0.0100	150	10.0	0.150
0.0100	250	10.0	0.250
0.0100	350	10.0	0.350
0.0100	500	10.0	0.500

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg/L)
0.100	100	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D4 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using tetrahydrofuran. This primary stock solution (1.00 mg a.i./mL) was diluted in tetrahydrofuran to prepare a 0.100 mg a.i./mL stock solution. The 1.00 and 0.100 mg a.i./mL stock solutions were added to all tissue samples and calibration standards (25 µL or 100 µL).

Appendix 6.7

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-111-4, with a nominal concentration of 5.0 mg a.i./Kg, were calculated using the following equations:

$$\text{D4 in sample (mg a.i./Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of D4 in sample (mg a.i./Kg)}}{\text{nominal concentration of D4 in sample (mg a.i./Kg)}} \text{ X } 100$$

Peak Area = 18968.0
Y-intercept = 6349.31341
Slope = 120825.71
Dilution factor = 8.65

$$\text{D4 in sample (mg a.i./Kg)} = \frac{18968.0 - 6349.31341}{120825.71} \text{ X } 8.65$$

Concentration of D4 in sample (mg a.i./Kg) = 0.903 mg a.i./Kg

$$\text{Percent of nominal concentration} = \frac{0.903 \text{ m a.i./Kg}}{5.0 \text{ mg a.i./Kg}} \text{ X } 100$$

Percent of nominal concentration = 18.1%

Appendix 6.8

Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (570A-111-)	Sampling Interval (Day)	Concentrations of D4		Percent Recovery[1]
		Fortified (mg a.i./Kg)	Measured (mg a.i./Kg)	
MAS-1	0	5.00	4.27	85.4
MAS-2	0	20.0	19.4	96.8
MAS-3	14	5.00	4.97	99.5
MAS-4	14	20.0	21.6	108
MAS-5	28	5.00	5.39	108
MAS-6	28	20.0	21.1	105
			Mean =	100
			Standard Deviation =	8.67
			CV =	8.67%

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

Appendix 6.9

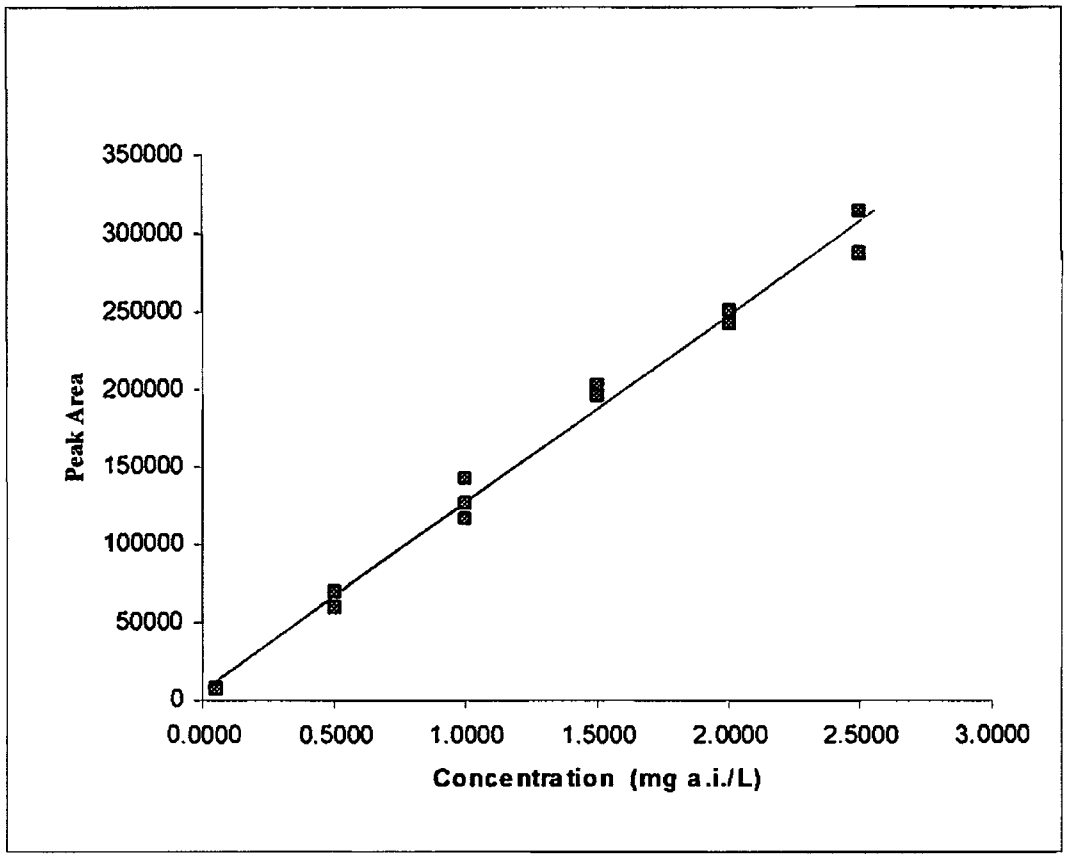
Matrix Fortifications Analyzed Concurrently During Sample Analyses in Tissue

Sample Number (570A-111-)	Sampling Interval (Day)	Concentrations of D4		Percent Recovery[1]
		Fortified (mg a.i./Kg)	Measured (mg a.i./Kg)	
TMAS-1	0	2.50	2.44	97.7
TMAS-2	14	25.0	23.3	93.1
TMAS-3	28	250	226	90.6
			Mean =	93.8
			Standard Deviation =	3.60
			CV =	3.84%

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

Appendix 6.10

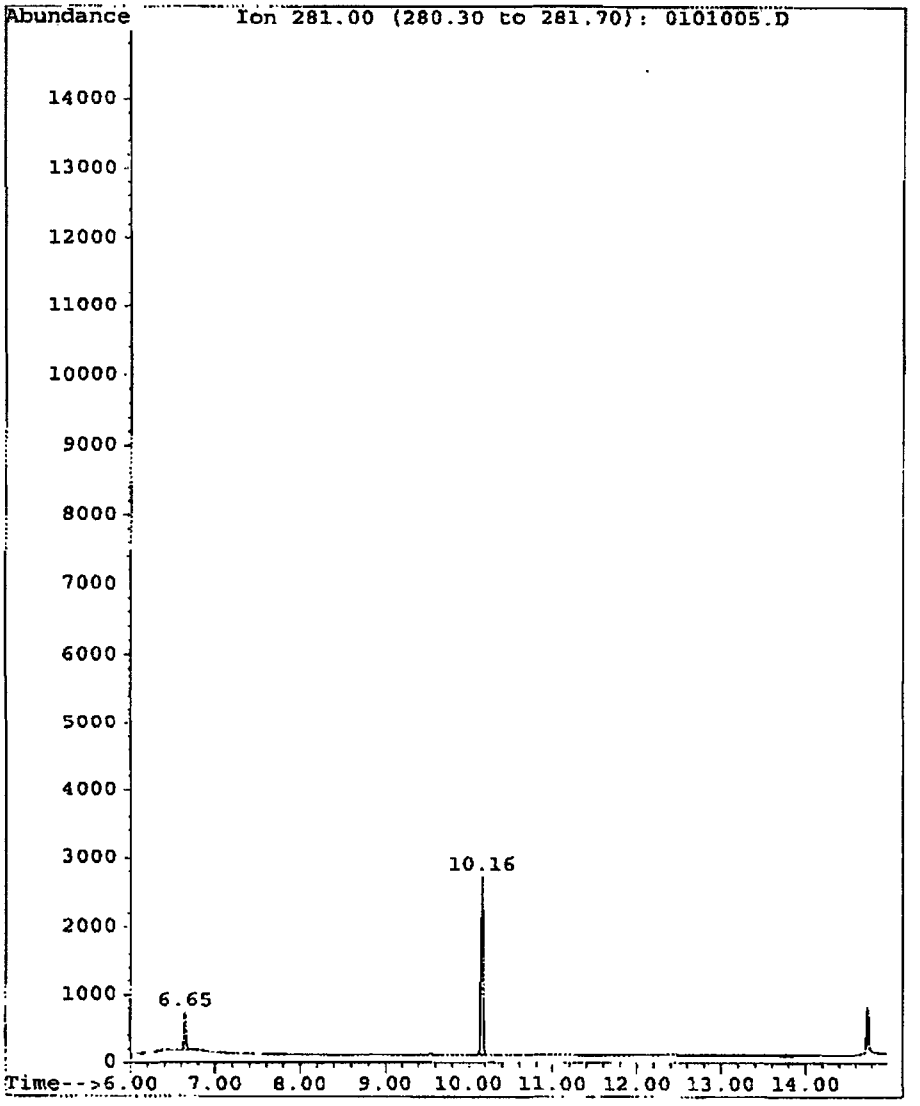
Representative Calibration Curve for D4 in Sediment



Slope = 120825.71; Y-Intercept = 6349.31341; $R^2 = 0.9899$

Appendix 6.11

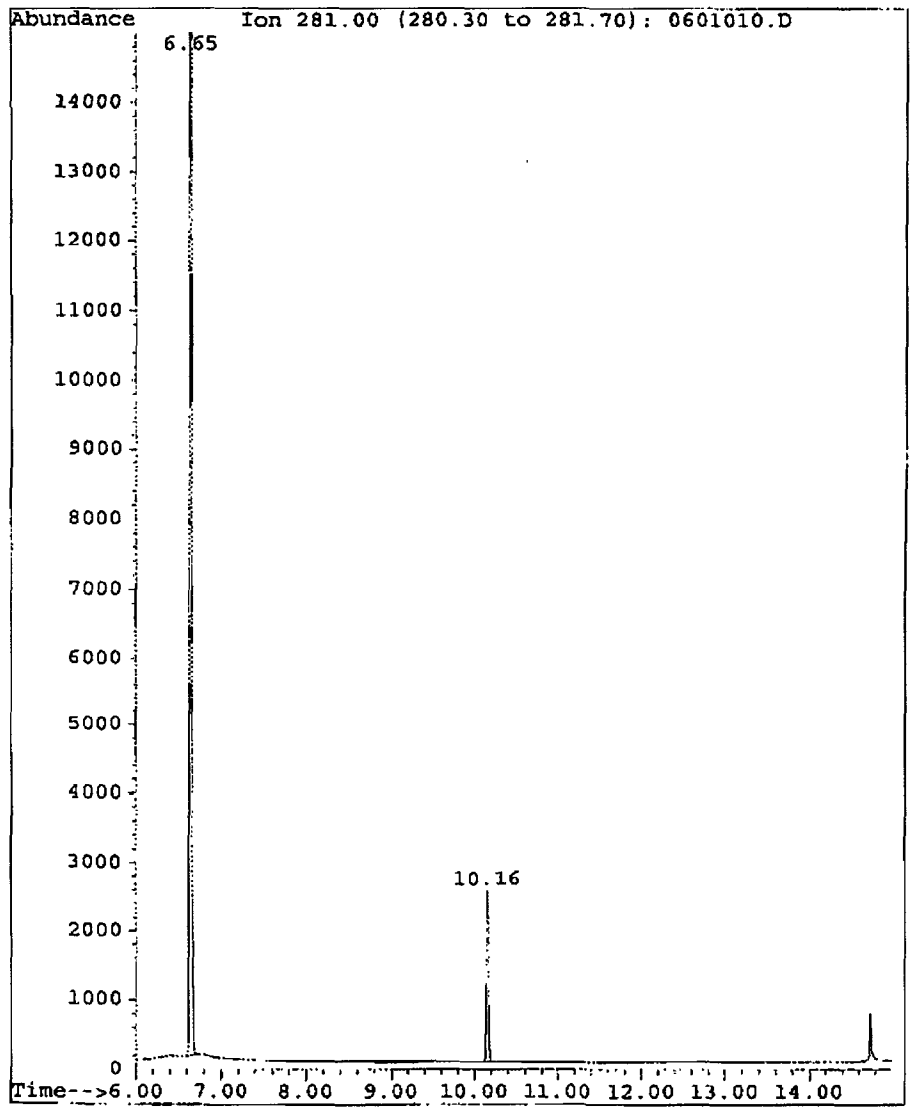
Representative Chromatogram of a Low-level D4 Sediment Calibration Standard



Nominal concentration: 0.0500 mg a.i./L

Appendix 6.12

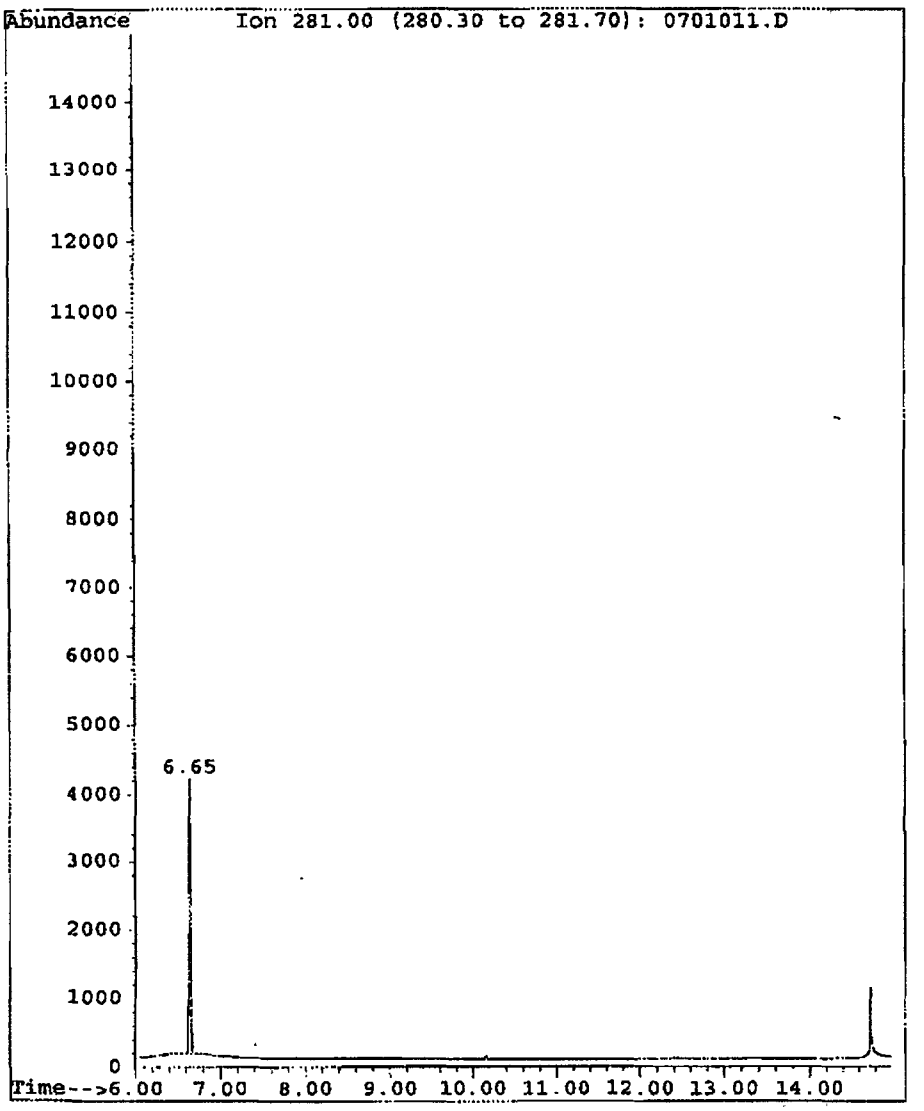
Representative Chromatogram of a High-level D4 Sediment Calibration Standard



Nominal concentration: 2.50 mg a.i./L

Appendix 6.13

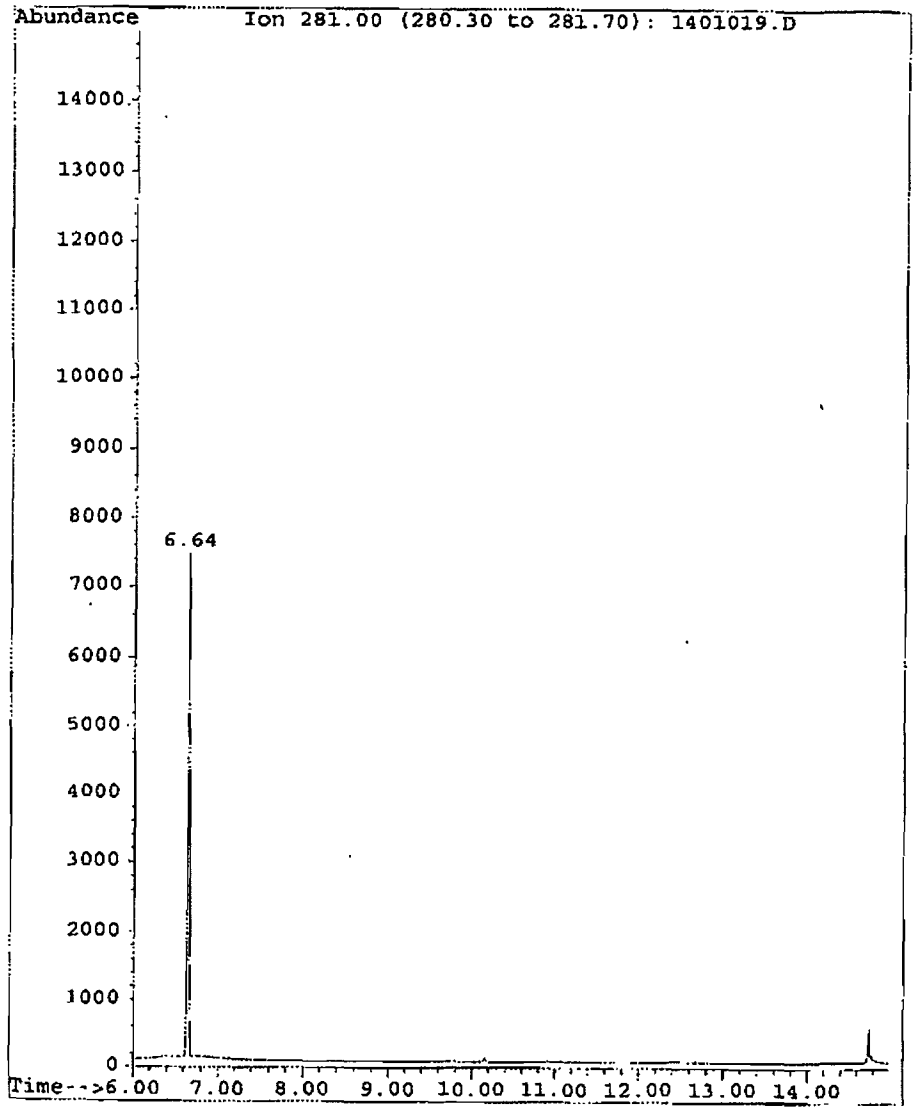
Representative Chromatogram of a Sediment Matrix Fortification Sample



Sample number 570A-111-MAS-1; 5.00 mg a.i./Kg, nominal concentration.

Appendix 6.14

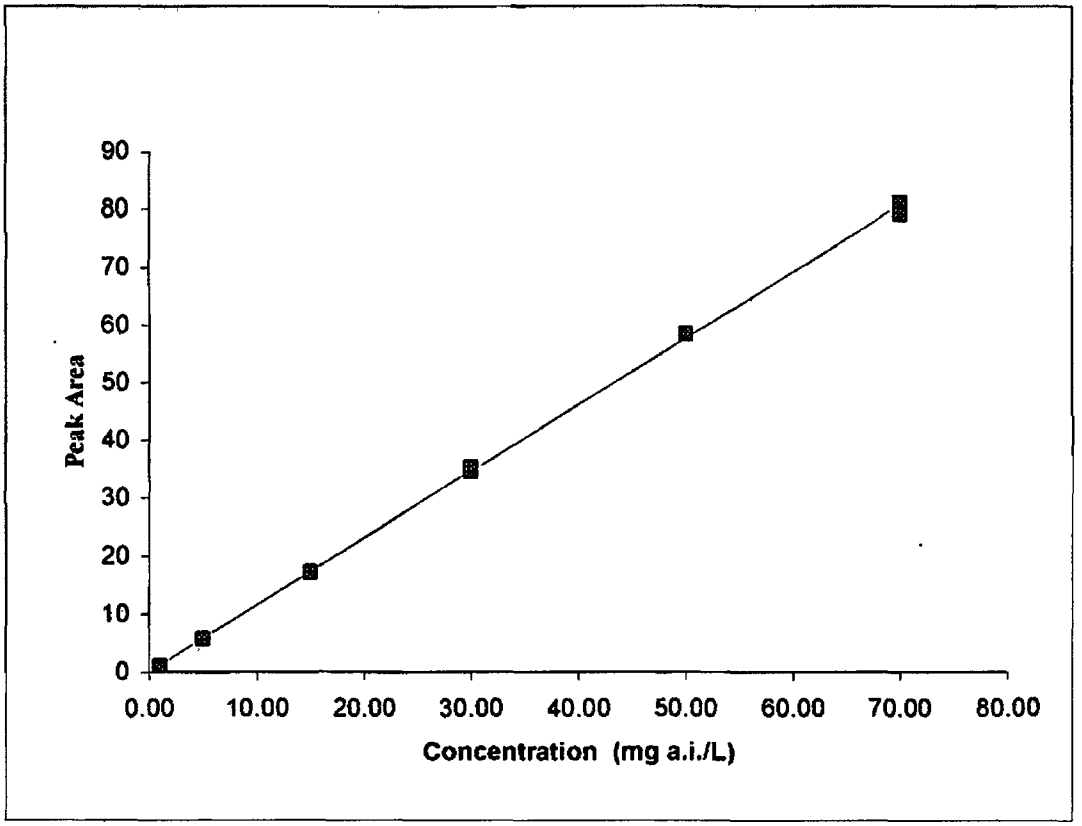
Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-111-6; 20 mg a.i./Kg nominal concentration

Appendix 6.15

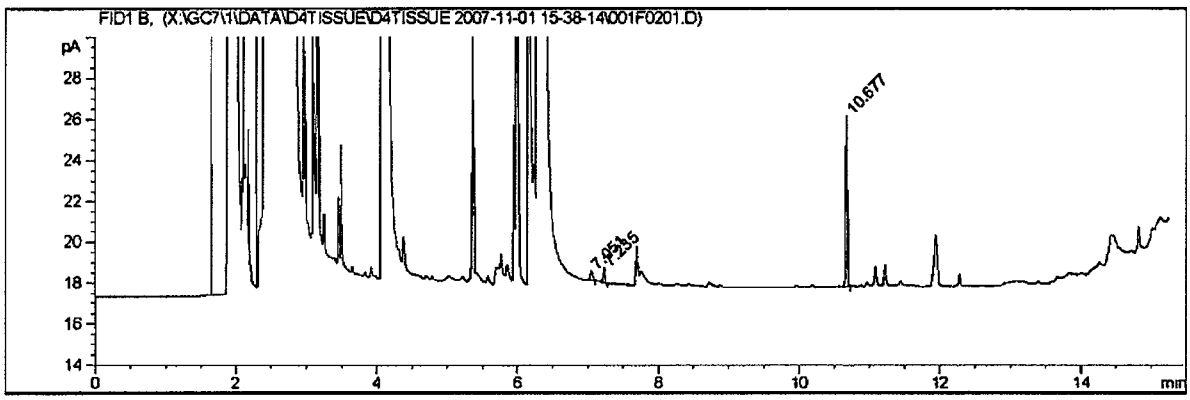
Representative Calibration Curve for D4 in Tissue



Slope = 1.15; Y-Intercept = 0.09554; $R^2 = 0.9996$

Appendix 6.16

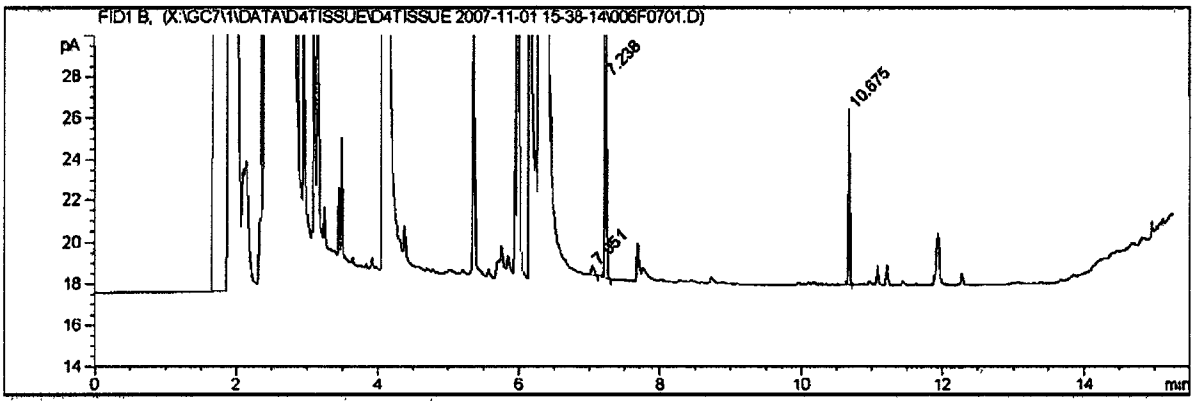
Representative Chromatogram of a Low-level D4 Calibration Standard for Tissue Analysis



Nominal concentration: 1.00 mg a.i./L

Appendix 6.17

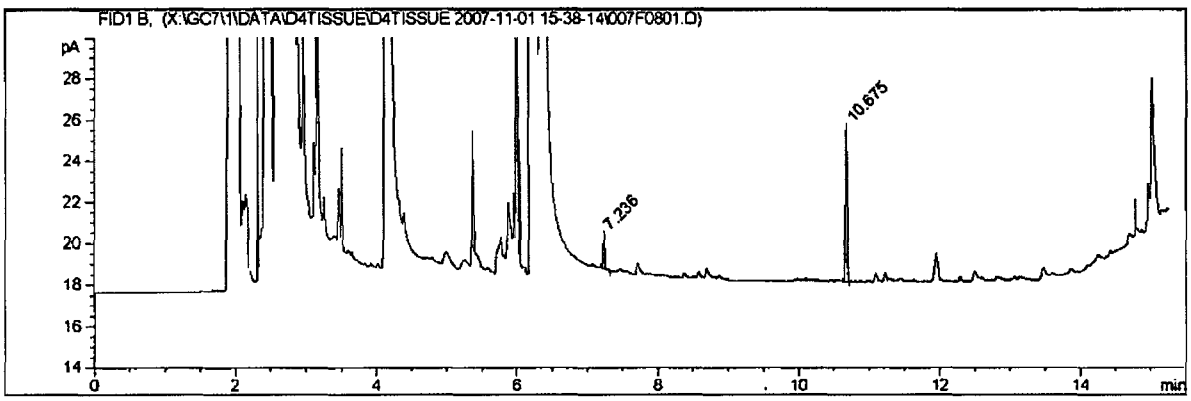
Representative Chromatogram of a High-level D4 Calibration Standard for Tissue Analysis



Nominal concentration: 70.0 mg a.i./L

Appendix 6.18

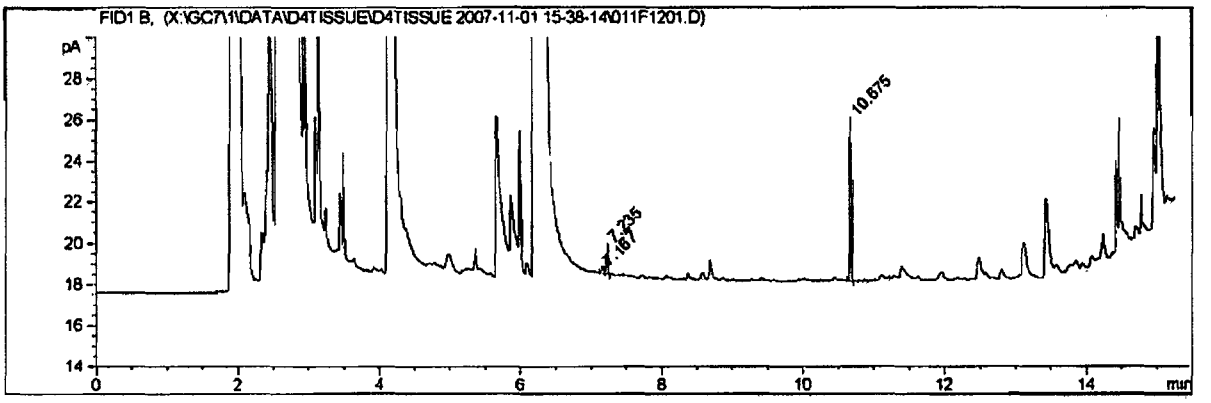
Representative Chromatogram of a Tissue Matrix Fortification



Sample number 570A-111-TMAS-2; 25.0 mg a.i./Kg, nominal concentration.

Appendix 6.19

Representative Chromatogram of a Day 28 Tissue Sample



Sample number 570A-111-T6; 5.0 mg a.i./Kg nominal concentration

Appendix 7

Cumulative Mortality and Treatment-Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	2G; rest AN	0	0	AN	0	0
7	AN	0	0	2G; rest AN	0	0
8	AN	0	0	1G; rest AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.

[2] The sediment was sieved using a 425 µm sieve. 346 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

[3] The sediment was sieved using a 425 µm sieve. 334 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	1G; rest AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	0

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 337 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 1.0176g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	1G; rest AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	[3]
15	AN	0	0	--	--	--
16	AN	0	0	--	--	--
17	AN	0	0	--	--	--
18	AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved using a 425 µm sieve. 1.3414g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	AN	0	0
8	AN	0	0
9	AN	0	0
10	1G; rest AN	0	0
11	1G; rest AN	0	0
12	AN	0	0
13	AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

0.86 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	1G; rest AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	1G; rest AN	0	0	1G; rest AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0.	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 324 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 252 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

0.86 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 293 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 1.1370g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

0.86 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	1G; rest AN	0	0	AN	0	0
7	1G; rest AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	[3]
15	AN	0	0	--	--	--
16	AN	0	0	--	--	--
17	AN	0	0	--	--	--
18	AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved using a 425 µm sieve. 1.3397g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

0.86 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	AN	0	0
8	AN	0	0
9	AN	0	0
10	AN	0	0
11	AN	0	0
12	AN	0	0
13	AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

4.06 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	1G; rest AN	0	0	1G; rest AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 222 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 268 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

4.06 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	1G; rest AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	1G; rest AN	0	0	AN	0	0
12	2G; rest AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 267 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 1.2914g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

4.06 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	1G; rest AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	1G; rest AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	[3]
15	AN	0	0	--	--	--
16	AN	0	0	--	--	--
17	AN	0	0	--	--	--
18	AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved using a 425 µm sieve. 1.5785 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

4.06 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	1G; rest AN	0	0
8	AN	0	0
9	AN	0	0
10	AN	0	0
11	AN	0	0
12	AN	0	0
13	AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment; H = organisms observed climbing the walls of the test chamber.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment-Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0	AN	0	0
9	2G; rest AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 361 organisms were found and used for lipid analysis on Depuration Day 23 (1.1345 g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 1.2893 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

0.86 mg a.i./Kg – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	2G; rest AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 337 organisms were found and used for lipid analysis on Depuration Day 23 (1.0453 g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 1.3623 g (wet weight of organism tissue) was collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

4.06 mg a.i./Kg – Depuration Phase

	Replicate A			Replicate B			Replicate C		
Day	Observations[1]	Cumulative Number Dead	Number Sampled	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	1G; rest AN	0	0	1G; rest AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	1G; rest AN	0	0	1G; rest AN	0	0
14	1G; rest AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	1G; rest AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 248 organisms were found and used for lipid analysis on Depuration Day 23 (0.9681 g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 1.2549 g (wet weight of organism tissue) was collected for analytical measurement.

Appendix 8

Agvisc Soil Characterization Report

AGVISE LABORATORIES

604 Highway 15 West
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Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013
email: agvise@polarcomm.com
Homepage: www.agvise.com

AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INT.
Protocol or Study No = VARIOUS
Sample ID. = 570A-111-DEP
Trial ID. = NEGATIVE CONTRO
Date Received = 12-21-07
Date Reported = 01-02-2008

AGVISE Lab No	07-1151
Percent Sand	71
Percent Silt	12
Percent Clay	17
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	0.86
Cation Exchange Capacity (meq/100 g)	9.7
% Moisture at 1/3 Bar	30.1
% Organic Carbon --Walkley Black	3.4
% Organic Matter--Walkley Black	5.8
pH in 1:1 soil:water ratio	7.4

Base Saturation Data

Cation	Percent	ppm
Calcium	45.6	883
Magneaium	43.2	502
Sodium	1.1	25
Potassium	0.3	11
Hydrogen	9.9	10

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

1/2/08
Date

AGVISE LABORATORIES

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AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INT.
Protocol or Study No = VARIOUS
Sample ID. = 570A-111
Trial ID. = NEGATIVE CONTRO
Date Received = 12-21-07
Date Reported = 01-02-2008

AGVISE Lab No	07-1150
Percent Sand	71
Percent Silt	10
Percent Clay	19
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	0.92
Cation Exchange Capacity (meq/100 g)	9.3
% Moisture at 1/3 Bar	29.5
% Organic Carbon --Walkley Black	3.2
% Organic Matter--Walkley Black	5.5
pH in 1:1 soil:water ratio	7.3

Base Saturation Data

Cation	Percent	ppm
Calcium	44.7	834
Magnesium	42.8	480
Sodium	1.1	23
Potassium	0.3	10
Hydrogen	11.1	10

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

1/2/08
Date

Appendix 9

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. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

ERROR: timeout
OFFENDING COMMAND: timeout

Study Title

D4 – Sediment-Water Lumbriculus Toxicity Test using Spiked Natural Sediment, Following OECD Guideline 225

Data Requirement

OECD Guideline 225

Author

Christian R. Picard

Study Completed On

27 August 2009

Submitted To

CES (Centre European des Silicones, European Chemical Industry Council (Cefic))
Avenue E. van Nieuwenhuyse 4
B1160 Brussels, Belgium

Performing Laboratory

Springborn Smithers Laboratories
790 Main Street
Wareham, Massachusetts 02571-1037

Laboratory Project ID

Springborn Smithers Study No. 13937.6103

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The data and report presented for **"D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225"** were produced and compiled in accordance with all pertinent OECD (OECD, 1998) Good Laboratory Practice regulations with the following exception: routine water contaminant screening analyses were conducted using standard U.S. EPA procedures by GeoLabs, Inc., Braintree, Massachusetts and are considered facility records under Springborn Smithers Laboratories' SOP 7.92. Since the analyses were conducted following standard validated methods, this exception had no impact on the study results. The internal standard, tetrakis(trimethylsiloxy)silane, was purchased from a commercial supplier and was not characterized under GLPs.

SPRINGBORN SMITHERS LABORATORIES

Christian Picard
Study Director

8/27/09
Date

QUALITY ASSURANCE STATEMENT

The study conduct, raw data and report for **"D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225"** were inspected by the Quality Assurance Unit (QAU) at Springborn Smithers Laboratories to determine adherence with the study protocol and laboratory standard operating procedures. This report is an accurate representation of the raw data. Dates of study inspections, inspection types, and dates reported to the Study Director and to Management are listed below.

Inspection Date	Inspection Type	Reported to Study Director/Management
4/20/2009	Data	4/20/2009
5/14/2009	Protocol Review	5/14/2009
5/14/2009	In-Life-Water Quality Measurements	5/14/2009
6/3/2009	Method Appendix	6/3/2009
7/13/2009	Data	7/13/2009
7/23 to 24/2009	Draft Report	7/24/2009
7/29/2009	Revised Draft	7/29/2009
8/27/2009	Final report	8/27/2009

SPRINGBORN SMITHERS LABORATORIES

Dorothy A. Antonioli

Dorothy A. Antonioli
Quality Assurance Auditor

27 August 2009
Date

KEY STUDY PERSONNEL

The following Springborn Smithers personnel were responsible for the conduct of the work and reporting of the study results.

Christian Picard	Study Director
Michael J. Bradley	Biologist I
Kristen Follett	Assistant Biologist
Andrew Moore	Assistant Biologist
Alicia Hurd	Chemistry Technician I
Rebecca J. Smith	Chemist I
Barbara E. Smith	Chemist I
Marjorie E. Dix	Senior Research Chemist
Joshua D. True	Technical Report Writer
Susan P. Shepherd	Director, Ecotoxicology

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SUMMARY

D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225

STUDY SPONSOR: CES (Centre European des Silicones, European Chemical Industry Council (Cefic))

PROTOCOL TITLE: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225, Springborn Smithers Laboratories Protocol No.: 062608/OECD/Oligochaete/Spiked Sediment

SPRINGBORN SMITHERS STUDY NUMBER: 13937.6103

TEST SUBSTANCE: D4, Batch No. LL084732, CAS No. 556-67-2, reported to have a purity of 99.77%, was received from Dow Corning Corporation on 8 July 2008.

DEFINITIVE TEST DATES: 14 May to 11 June 2009

TEST ORGANISM: *Lumbriculus variegatus*
Source: Springborn Smithers culture

TEST CONDITIONS: 28-day duration, static conditions, 19 to 23 °C, illumination of 16 hours light: 8 hours darkness at 530 to 540 lux

OVERLYING WATER: Fortified laboratory well water
pH: 8.0
Specific conductance: 600 µmhos/cm
Total hardness as $CaCO_3$: 170 mg/L
Total alkalinity as $CaCO_3$: 100 mg/L

SEDIMENT: Natural Sediment
percent organic carbon: 2.2%
particle size distribution: 93% sand, 6% silt, and 1% clay
pH: 6.5
percent moisture (1/3 bar): 18.0%

NOMINAL SEDIMENT CONCENTRATIONS: 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg

MEAN MEASURED SEDIMENT CONCENTRATIONS: 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg

RESULTS: Based on measured concentrations of D4 in the sediment, the No-Observed-Effect Concentration (NOEC) for this exposure was determined to be 13 mg a.i./kg. The Lowest-Observed-Effect Concentration (LOEC) for this exposure was determined to be 19 mg a.i./kg. Since no concentration tested resulted in ≥ 50% reduction in survival or biomass, the EC50 value was empirically estimated to be > 32 mg a.i./kg, the highest mean measured concentration tested.

1.0 INTRODUCTION

This study was designed to determine the effects of D4, applied to sediment on the sediment-dwelling oligochaete *(Lumbriculus variegatus)*, following a 28-day exposure period. The study was performed under static conditions in artificial sediment for a period of 28 days. Exposure concentrations of D4 were measured on day 0 (test initiation), day 7 and day 28 (test termination) in the sediment. Results of this study were used to estimate the highest concentration which had no significant ($p \leq 0.05$) effect on the test organism performance (No-Observed-Effect Concentration, NOEC), and the lowest concentration that had a significant effect on the exposed organisms (Lowest-Observed-Effect Concentration, LOEC). If possible, the concentration which caused a 50% reduction (EC50) in either oligochaete reproduction or biomass was also estimated.

The study was initiated on 7 August 2008, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental phase of the study was conducted from 14 May to 11 June 2009 at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. All original raw data, the protocol and the original final report produced during this study are archived at Springborn Smithers Laboratories at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

The toxicity test was performed according to the Springborn Smithers Laboratories protocol entitled "Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225" Springborn Smithers Laboratories Protocol No.: 062608/OECD/Oligochaete/Spike Sediment (Appendix 1). This protocol meets the general requirements specified in the OECD Guideline for the Testing of Chemicals, Guideline 225 entitled "Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment" (OECD, 2007).

2.2 Test Substance and Internal Standard

2.2.1 Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance, and archival of a sample of the test substance are the responsibility of the Study Sponsor.

2.2.2 Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane (M_4Q)
Synonym:	Trimethylsiloxysilicate
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

2.3 Test Organism

The oligochaete (*Lumbriculus variegatus*) was selected as a test organism since it is the recommended species for use in OECD Guideline 225 (OECD, 2007) for sediment toxicity testing. They are tolerant to a wide range of sediment types and are widely used for sediment toxicity and bioaccumulation testing. The OECD Guideline 218 for testing of chemicals in spiked sediment relies on the use of the *Chironomus* species in testing. The larvae of *Chironomus* sp. live at the sediment water interface and exposure is to the top layer of the sediment only. The oligochaete, *Lumbriculus variegates*, contacts the sediment and pore water at greater depths and theoretically provides a complementary assessment of sediment toxicity to *Chironomus* sp. testing. The oligochaetes used during this study were obtained from the main laboratory cultures maintained at Springborn Smithers. The oligochaetes from this culture were maintained in a 57-L aquarium containing approximately 40 L of culture water under flow-through conditions. The water used in the main culture is unadulterated well water from a 100-meter bedrock well and has been characterized as soft water with typical ranges as total hardness of 30 to 60 mg/L as $CaCO_3$, alkalinity of 25 to 45 mg/L as $CaCO_3$, pH of 6.9 to 7.7, and a specific conductance of 140 to 200 micromhos/cm. Oligochaetes were fed 15 mL of finely ground suspension (100 mg/mL) of flaked fish food weekly.

Fourteen days prior to test initiation, worms from the culture were removed and artificially fragmented to synchronize the population, using a scalpel to remove the anterior ends. Synchronized oligochaetes were then acclimated to test conditions (i.e., test water and test sediment) for a period of thirteen days to regenerate new heads. The synchronization of worms is performed to avoid "uncontrolled" regeneration and reproduction that may contribute high variation in the test results. Once during the acclimation period, the synchronized oligochaetes were fed 6.4 mL of a finely ground suspension (100 mg/mL) of flaked fish food. During the

holding period, dissolved oxygen ranged from 7.9 to 8.4 mg/L, pH ranged from 7.2 to 8.2 and the temperature ranged from 20 to 21 °C. No mortality was observed in the test population 48 hours prior to test initiation.

2.4 Overlying Water

Overlying water used during this study was fortified well water. The dilution water was prepared by fortifying well water based on the formula for hard water (U.S. EPA, 1975) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. The water used during the definitive exposure was characterized as having a total hardness and total alkalinity as calcium carbonate ($CaCO_3$) of 170 and 100 mg/L, respectively, a pH of 8.0, and a specific conductivity of 600 micromhos per centimeter (µmhos/cm).

Representative samples of the overlying water source were analyzed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM (2002) standard practice.

2.5 Sediment

Natural freshwater sediment was the substrate used in the exposure. The natural sediment (Springborn Smithers Laboratories Batch No. 031809) was collected from a single source, Glen Charlie Pond, Wareham, Massachusetts. Prior to use and characterization, the sediment was wet pressed through a 2.0-mm sieve to remove large particles and benthic organisms and homogenized thoroughly. The natural sediment used in the study was characterized by Agvise Laboratories, Northwood, North Dakota, as having an organic carbon content of 2.2%, a pH of 6.5 and a percent moisture (at 1/3 Bar) of 18.0. The particle size distribution conducted by Agvise Laboratories resulted in average particle size distribution of 93% sand, 6% silt and 1% clay. A percent solids value of 58.46% was determined by Springborn Smithers Laboratories. A sample of the sediment pore water was generated from the sediment batch prior to testing and

yielded a measured concentration of 4.5 mg/L as nitrogen (ammonia). This concentration is well below levels of concern for this organism. A representative sample of the sediment source was analyzed for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected at concentrations that would be considered to have an adverse impact on the results of the test.

2.6 Test Concentrations

Selection of nominal D4 concentrations for the 28-day chronic toxicity test with oligochaetes was based on toxicity information developed at Springborn Smithers Laboratories through preliminary testing. Based on the results of preliminary testing (see Section 3.1) and consultation with the Study Sponsor, the nominal D4 concentrations of 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg were selected for the definitive exposure.

2.7 Test Procedures

2.7.1 Stock Solution Preparation

A 20 mg a.i./mL primary stock was prepared by placing 0.5012 g (0.5000 g as active ingredient) of D4 in a 25-mL volumetric flask and bringing it to volume with acetone. The resulting stock solution was observed to be clear and colorless with no visible undissolved test substance following stirring. A 2.0 mg a.i./mL secondary stock solution was prepared by placing 2.5 mL of the 20 mg a.i./mL primary stock solution in a 25-mL volumetric flask and bringing it to volume with acetone (CAS No. 67-64-1). Dosing stock solutions were prepared from dilutions of 2.0 mg a.i./mL secondary stock solution and the 20 mg a.i./mL primary stock solution as follows:

Stock Concentration (mg a.i./mL)	Volume of Stock Used (mL)	Volume Diluted to with Acetone (mL)	Final Dosing Stock Concentration (mg a.i./mL)
20	8.80	10	17.6
20	4.40	10	8.80
20	2.20	10	4.40
20	1.15	10	2.29
2.0	5.55	10	1.11
2.0	2.73	10	0.546

All stock solutions were observed to be clear and colorless with no visible undissolved test substance following preparation.

2.7.2 Test Substance Application

The method of test substance application to sediment used during this study was provided by the Study Sponsor. For each dose level, a 2.5-kg aliquot of wet sediment (1.462 kg dry weight based on a percent solids of 58.46%) along with 7.5 L of overlying water (sediment:volume ratio of 3:1) were placed in individual glass jars. Immediately prior to dosing, the contents of each jar were shaken to suspend the sediment. An 8.3-mL volume of each dosing stock solution (prepared as in Section 2.9) was then added to each jar. Each jar was shaken again after dosing to distribute the test substance. After a 16-hour settling period, the water was decanted from the sediment. The sediment was transferred to a Hobart mixer and 3.66 g each of *urtica* powder and alpha cellulose were mixed into the sediment of each test group as a food source for the oligochaetes. This weight of urtica powder and alpha cellulose was equal to 0.25% of the dry weight for each sediment batch. Following mixing in the Hobart mixer, the treated sediments were allocated to the replicate test vessels for each exposure level.

A solvent control sample was prepared in the same manner as the treated sediment by adding 2.5 kg of wet sediment (1.462 kg dry weight based on a percent solids of 58.46%) along with 7.5 L of overlying water (sediment:volume ratio of 3:1) and 8.3 mL of acetone in a glass jar and processed in the same manner as the treated sediments. The negative control sediment group was prepared using only untreated sediment and overlying water (no test substance or solvent).

The negative control and solvent control vessels were maintained under the same conditions as the treatment vessels

2.8 Preparation of Test Vessels

The test vessels used were 600-mL clear glass beakers. The sediment and overlying water were added to the test vessels two days prior to test initiation. A 75-mL (1.5-cm layer) aliquot of sediment was added to each test vessel. The wet weight of the sediment in each jar averaged 148 g (86.5 g dry weight). A turbulence reducer, consisting of a plastic disk, was used to minimize the disruption of the sediment layer during the introduction of 300 mL (6 cm) of overlying water. The total medium volume (sediment/water) was maintained at 375 mL and the ratio of sediment to water was 1:4. The turbulence reducer was composed of a modified plastic disk. The initial water level in each test vessel was marked in order to evaluate evaporation. Each test vessel was covered with a clear plastic plate to minimize evaporation and gentle aeration was provided through a glass pipette fixed approximately 2 to 3 cm above the sediment surface. Aeration was delivered at a rate of 1 to 3 bubbles per second.

Four replicate test vessels (A through D) were established for each treatment level and six vessels (A through F) were established for the control and solvent control. Four additional replicate test vessels (E through H for treatment levels and G through J for the controls) were maintained for the purpose of chemical analysis. Each replicate vessel contained ten oligochaetes, a total of 40 oligochates per concentration and 60 oligochates in the control and solvent control for the biological response replicates. The additional replicates were maintained under the same conditions and contained ten test organisms, but were not used to evaluate the biological response of the test organisms

2.9 Test Conditions

Test vessels were positioned in a water bath containing circulating water designed to maintain the test solutions at a temperature of 20 ± 2 °C. Test solutions were gently aerated (1 to

3 bubbles per second) two days prior to addition of the test organisms and throughout the duration of the exposure period. Test vessels were labeled to identify the nominal test concentration, study number and designated replicate. The test area was illuminated with fluorescent bulbs at an intensity range of 530 to 540 lux and a photoperiod of 16 hours light and 8 hours darkness. Light intensity was measured using a VWR Traceable light meter.

2.9.1 Test Initiation

The spiked sediment and overlying water were added to the test vessels two days prior to addition of the test organisms (day -2). At test initiation, oligochaetes were added impartially to an intermediate vessel by adding no more than two oligochaetes to each vessel until all vessels contained two oligochaetes. This procedure was repeated until each vessel contained ten oligochaetes. The test was initiated when each intermediate vessel of oligochaetes was added to each respective test vessel. The ten oligochaetes were released simultaneously into the overlying water of each test vessel just above the sediment surface. A total of 40 oligochaetes for the treatment levels and 60 oligochaetes for the control and solvent control were exposed to the test conditions.

2.10 Test Monitoring

2.10.1 Biological Observations

Replicate test vessels A through D for treatment levels and A through F for the control and solvent control were examined at test initiation and daily thereafter, until test termination (day 28). Daily observations of mortality and abnormal behavior (e.g., leaving sediment) were made and recorded.

On test day 28 of the exposure, the replicate vessels from each exposure level (four replicates) and controls (six replicates) were terminated to determine the number of surviving oligochaetes and the biomass of the surviving oligochaetes. Instances of dead oligochaetes were also recorded. However, dead oligochaetes often decompose in the vessels and are difficult to

recover. If fewer than ten oligochaetes were observed and no dead oligochaetes were recovered, the missing individuals were assumed to have died. Surviving worms were assigned to one of three groups:

a) Large, complete worms (adults) without regenerated body regions.
b) Complete worms with regenerated, lighter-colored body regions.
c) Incomplete worms (i.e., recently fragmented worms with non-regenerated body regions.

The biomass was determined by placing the entire number of surviving oligochaetes from each replicate vessel in a tared weight tin and then drying in an oven at 100 ± 5 °C overnight (18 to 24 hours). Following drying, the tins were cooled to room temperature in desiccators and then weighted to the nearest 0.01 mg on a calibrated analytical balance.

2.10.2 Water Quality Measurements

Water quality measurements made during the study were performed in the replicate exposure vessels (A through D or A through F). Measurements of dissolved oxygen concentration, temperature and pH were made on the day the test organisms were added (day 0) and at test termination (day 28) in each exposure vessel. In addition, dissolved oxygen concentration, temperature and pH were measured daily in one replicate vessel of each treatment level and the controls during the 28-day exposure. Daily water quality measurements were made on alternating replicates. The temperature was continuously monitored in an auxiliary vessel in the water bath throughout the study. Total hardness, alkalinity, specific conductivity, and total ammonia of the test solutions were determined at test initiation and at test termination in a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F). In addition, total ammonia was determined three times per week in the solvent control and the 100 mg a.i./kg nominal treatment level.

The dissolved oxygen concentration and daily temperature were measured using a Yellow Springs Instrument (YSI) Model No. 550A dissolved oxygen meter/temperature probe. Continuous temperature monitoring was performed using a VWR minimum/maximum

thermometer. A YSI pH100 pH meter was used for pH measurements. Total hardness and alkalinity of the test solutions were determined according to APHA et al., (1995). Specific conductivity was monitored with a YSI Model No. 33 salinity-conductivity-temperature (SCT) meter. Total ammonia was measured with an Orion Ammonium Meter (Model 720A Plus) and an Orion electrode (Model 9512).

2.11 Analytical Measurements

Prior to dosing, a sample of each dosing stock solution was taken and analyzed for D4 concentration. In addition, each treatment level and control sediment was sampled and analyzed for D4 concentration after dosing and prior to division into the replicate test vessels. Results of the pretest analyses were used to judge whether sufficient quantities of D4 had been applied during the mixing process.

During the in-life phase of the definitive study, sediment samples were removed and analyzed for D4 concentration on test days 0 (test initiation), test day 7 and test day 28 (test termination). On test days 0, 7 and 28, samples were removed and analyzed from replicate vessels E, F and G, respectively, for each treatment level and vessels G, H and I for the controls. Overlying water samples were removed from the test vessels by decanting all of the overlying water from each vessel into a graduated cylinder. Following removal of the overlying water, the sediment was then centrifuged at approximately 10,000 *g* for 30 minutes. The pore water generated by centrifugation was then removed by pipet. The sediment samples were then collected from each centrifuge tube with a stainless steel spatula.

In addition, three quality control (QC) samples were prepared at each sampling interval and remained with the set of exposure samples throughout the analytical process. These QC samples were prepared in sediment (based on sediment dry weight) at concentrations of D4 similar to the treatment level range. Results of the analyses of the QC samples were used to judge the precision and quality control maintained during the analysis of exposure solution samples.

All samples were analyzed for D4 using gas chromatography with mass selective detection (GC/MSD) based on methodology validated at Springborn Smithers. The method validations were conducted prior to the initiation of the definitive test and established average recoveries of 82.1 ± 9.02% from artificial sediment (Appendix 2) and 101 ± 18.1% from natural freshwater sediment (Appendix 3), respectively. Results from both methods were comparable which indicates that both of these methods were robust and appropriate for the analysis of test samples. Conditions and procedures used throughout the analysis of the exposure solutions and QC samples during this study were similar to those used in the method validation studies. Defined limits of acceptance of quality control sample performance were set at 70.0 to 120%.

2.12 Statistical Analysis

2.12.1 Determination of LOEC and NOEC Values

At the termination of the study, data obtained on oligochaetes reproduction (the total number of oligochaetes recovered at test termination) and biomass were statistically analyzed to identify significant treatment-related effects. The lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) were determined. All statistical analyses were conducted at the 95% level of certainty except in the case of the Chi-Square Test and Bartlett's Test, in which the 99% level of certainty was applied. The 99% level of certainty is preferred for these qualifying tests. The following procedures were used:

1. Chi-Square Test for normality (Weber et al., 1989) was conducted to compare the observed sample distribution with a normal distribution. The assumption that observations are normally distributed must be validated before subsequent analyses, following parametric procedures, can be performed. If the data are not normally distributed, then a non-parametric procedure is used for subsequent analyses. Analysis of both reproduction and biomass met this assumption of normal distribution.

2. As a check on the assumption of homogeneity of variance implicit in parametric statistics, data for each endpoint were analyzed using Bartlett's Test (Sokal and Rohlf, 1981). Analysis of both reproduction and biomass met the assumption of homogeneity of variance.

3. A t-Test was conducted for oligochaete reproduction and biomass data to compare the performance of the control organisms with that of the solvent control organisms in order to determine if there were any statistically significant positive or negative effects. During this study, the t-Test indicated no significant difference between control and solvent control data for both endpoints (reproduction and biomass). Therefore, data from all treatment levels were compared to the pooled control data to determine treatment level effects.

4. Data for both endpoints measured in this study (reproduction and biomass) passed both the tests for normality and homogeneity. Therefore, Bonferroni's t-Test (Weber, et. al, 1989), a parametric statistical procedure, was used to establish treatment effects for both endpoints.

TOXSTAT® Version 3.5 (West Inc. and Gulley, 1996) was used to perform the computations. The results were used to establish, at the 95% level of certainty, the lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant difference (No-Observed-Effect Concentration, NOEC) from the pooled control data.

2.12.2 EC50 Determination

The EC50 is the estimated measured sediment concentration of the test substance which produces 50% reduction in reproduction or biomass in the test population of oligochaetes at test termination. During this study, no concentration tested resulted in ≥ 50% reduction in

reproduction or biomass, therefore the EC50 values were empirically estimated to be greater than the highest mean measured sediment concentration.

3.0 RESULTS

3.1 Preliminary Testing

Prior to initiating the definitive study, a 28-day preliminary range-finding exposure was conducted at Springborn Smithers at nominal D4 levels of 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg, and a solvent (acetone) control. D4 was applied to the sediment in the same manner and test procedures were followed as outlined in Section 2.9. Three replicates of ten oligochaetes were exposed to each treatment level and control. The following table is a summary of the results of the preliminary range-finding exposure:

Nominal Treatment Level (mg/kg)	Mean Number of Oligochaetes Recovered per Replicate	Mean Biomass per Replicate (mg)
Pooled Control	26	25
0.050	25	24
0.50	23	27
5.0	26	23
50	19[a]	25
500	17[a]	20[a]

[a] Statistically reduced when compared to the pooled control, based on Bonferroni's t-Test.

At test termination, the mean number of surviving oligochaetes observed among oligochaetes exposed to the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels was 25, 23, 26, 19 and 17, respectively. During the same period, the mean number of surviving oligochaetes observed in the negative control and solvent control was 26 and 25, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant difference in survival among oligochaetes exposed to the 50 and 500 mg a.i./kg treatment levels compared to the pooled control (26 oligochaetes).

Mean biomass per replicate in the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels averaged 24, 27, 23, 25 and 20 mg, respectively. During the same period, biomass in the negative control and the solvent control averaged 26 and 23 mg, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant difference in biomass (dry weight) among

oligochaetes exposed to the 500 mg a.i./kg treatment level compared to the pooled control (25 mg).

Based on these results and consultation with the Study Sponsor, nominal D4 concentrations of 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg were selected for the definitive study.

A definitive exposure was initiated on 19 February 2009. The test was terminated on 3 March 2009 due to lower than expected analytical recoveries in the day 0 sediment samples. A second definitive test was initiated on 14 May 2009, the results of which are presented below.

3.2 Definitive Testing

3.2.1 Evaluation of Test Conditions

Results of the water quality measurements made during the definitive exposure are presented in Table 1 and Table 2. The water quality parameters were unaffected by the concentrations of D4 tested and remained within acceptable limits. Daily measurements of the temperature in the exposure solutions, and continuous monitoring in an auxiliary vessel set up in the water bath used to house the test vessels established a temperature range of 19 to 23 °C throughout the definitive study. The dissolved oxygen and pH measurements in the treatment levels and the controls ranged from 7.4 to 9.2 mg/L and 7.2 to 8.1, respectively. On day 17 replicate D of the 6.3 mg a.i./kg nominal treatment level had a measurement of 3.8 mg/L due to a malfunction in the aeration system. Aeration was restored and dissolved oxygen levels returned to the range stated in the protocol.

3.2.2 Analytical Results

Analysis of the stock solution samples used to dose the test sediments ranged from 100 to 110% of the nominal fortified concentrations. These results indicated that the appropriate mass of test substance was introduced to the sediment.

All test concentrations are expressed relative to sediment dry weight. Pretest analysis of the sediment samples, following dosing and prior to allocation into the replicate exposure vessels resulted in measured concentrations ranging from 45 to 88% of the nominal treatment levels. The sediment pretest results demonstrated that a reasonable concentration gradient was achieved from which to base the biological results.

Table 3 summarizes the D4 concentrations measured during the 28-day toxicity test. Measured sediment concentrations at day 0 in the 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg treatment levels were 1.1, 3.7, 13, 18, 24 and 39 mg a.i./kg, respectively. Measured sediment concentrations at day 7 in the 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg treatment levels were 1.6, 3.5, 6.9, 14, 19 and 29 mg a.i./kg, respectively, and 0.92, 2.3, 7.0, 8.3, 15 and 29 mg a.i./kg, respectively, on day 28. Based on mean measured concentrations, treatment levels were defined as 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg.

Analysis of seven of the nine quality control samples resulted in measured concentrations which ranged from 89.9 to 109% of the nominal fortified concentrations (1.50, 25.0 and 100 mg a.i./kg) and were consistent with the expectations of the methodology used (Appendix 2 and Appendix 3). Based on these results, it was determined that the appropriate quality control was maintained during the analyses of sediment samples. One of the nine QC samples was measured at 55.2% of nominal concentration. In addition, one of the nine QC samples was inadvertently fortified below the quantification range. QC samples can be out of the acceptable range due to a number of factors, some of which are spiking, handling or instrument errors.

3.2.3 Biological Results

The number of oligochaetes recovered at test termination and mean biomass for each replicate of each treatment level observed throughout the exposure period are presented in Table 4. Following 28 days of exposure, the number of oligochaetes recovered in the control and solvent control was 30 and 30, respectively. The mean biomass per replicate in the control and solvent control was 46 and 51 mg, respectively. The control data met the minimum performance criteria

of the guideline (i.e., population increase of ≥ 1.8). As demonstrated by the performance of the control organisms, the exposure system provided conditions which were appropriate for promoting survival and growth of the test species. Study data for this exposure are presented in Appendix 4.

At test termination (day 28), the mean number of surviving oligochaetes observed among the oligochaetes exposed to the 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg treatment levels was 31, 31, 28, 29, 26 and 22, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant reduction in the mean number of surviving oligochaetes in the 19 and 32 mg a.i./kg treatment levels compared to the pooled control data (30 oligochaetes).

Mean biomass per replicate in the 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg treatment levels was 55, 64, 50, 47, 44 and 44 mg, respectively. Statistical analysis (Bonferroni's t-Test) determined no significant difference in mean biomass compared to the control (49 mg).

Based on mean measured concentrations of D4 in the sediment, the No-Observed-Effect Concentration (NOEC) for this exposure was determined to be 13 mg a.i./kg. The Lowest-Observed-Effect Concentration (LOEC) for this exposure was determined to be 19 mg a.i./kg. Since no concentration tested resulted in ≥ 50% reduction in reproduction or biomass, the EC50 value was empirically estimated to be > 32 mg a.i./kg, the highest mean measured concentration tested.

PROTOCOL DEVIATIONS

1. The protocol states that the temperature of the test solutions will be maintained at 20 ± 2 °C. During this study, temperature readings recorded on test day 17 were 23 °C. In addition, the minimum-maximum thermometer recorded temperatures which exceeded the stated range on test days 17, 18 and 22. The maximum recorded temperature was 23 °C.

2. The protocol states that the test will be illuminated to an intensity of 100 to 500 lux. During this exposure, the light intensity was measured to be 530 to 540 lux.

These deviations did not have a negative impact on the results or interpretation of the study.

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Table 1. Water quality parameters (dissolved oxygen, temperature and pH) measured in the overlying water during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Concentration (mg a.i./kg)	Range		
	Dissolved Oxygen Concentration[ab] (mg/L)	Temperature[ac] (°C)	pH[a]
Control	7.9 – 9.0	19 – 23	7.2 – 8.0
Solvent Control	7.9 – 9.1	19 – 23	7.5 – 8.0
3.1	7.4 – 9.1	19 – 23	7.6 – 8.1
6.3	7.5[d] – 9.2	19 – 23	7.7 – 8.1
13	8.0 – 9.2	19 – 23	7.5 – 8.1
25	8.0 – 9.1	19 – 23	7.2 – 8.1
50	8.0 – 9.2	19 – 23	7.2 – 8.0
100	7.7 – 9.1	19 – 23	7.7 – 8.1

[a] N= 39 for the control and solvent control; N = 35 for all remaining treatment levels.
[b] 30% of the air saturation value at 19 °C = 2.8 mg/L.
30% of the air saturation value at 23 °C = 2.6 mg/L.
[c] Continuous temperature monitoring in an auxiliary vessel set up in the water bath used to house the test vessels established a temperature range of 19 to 23 °C throughout the exposure period.
[d] On test day 17 a dissolved oxygen concentration of 3.8 mg/L was measured in replicate D due to a brief malfunction in the aeration system.

NOTE: Values have been rounded to two significant figures.

Table 2. Water quality parameters (total hardness, total alkalinity, specific conductivity and ammonia) measured in the overlying water during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Concentration (mg a.i./kg)	Total Hardness[a] (mg/L as $CaCO_3$)		Total Alkalinity[a] (mg/L as $CaCO_3$)		Specific Conductance[a] (µmhos/cm)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Solvent Control	140	60	72	32	600	500
100	150	56	74	44	600	500

Nominal Concentration (mg a.i./kg)	Ammonia Analysis (mg/L as N)[b]											
	Day 0	Day 4	Day 6	Day 8	Day 11	Day 13	Day 15	Day 18	Day 20	Day 22	Day 25	Day 28
Solvent Control	0.46	0.34	≤0.10	0.25	0.16	0.34	1.5	1.2	2.3	3.0	2.5	1.8
100	0.55	0.33	0.13	0.17	≤0.10	0.29	1.1	1.2	2.4	2.7	3.7	3.3

[a] Based on measurement of a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F) at test initiation and termination.

[b] Based on measurement of a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F) at test initiation, termination and three times per week.

NOTE: Values have been rounded to two significant figures.

Table 3. Concentrations of D4 (expressed relative to sediment dry weight) measured in sediment samples during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Sediment Concentration (mg a.i./kg)	Measured Sediment Concentration (mg a.i./kg)				Mean Percent Recovery[d]
	Day 0[a]	Day 7[b]	Day 28[c]	Mean[d]	
Control	< 0.60[e]	< 0.60	< 0.60	NA[f]	NA
Solvent Control	< 0.60	< 0.60	< 0.60	NA	NA
3.1	1.1	1.6	0.92	1.2	39
6.3	3.7	3.5	2.3	3.2	50
13	13	6.9	7.0	8.8	68
25	18	14	8.3	13	54
50	24	19	15	19	39
100	39	29	29	32	32
QC[g] #1 1.50	0.827 (55.2)[h]	< 0.00060 (NA)[i]	1.64 (109)		
QC#2 25.0	22.5 (89.9)	24.6 (98.2)	26.4 (106)		
QC#3 100	101 (101)	100 (100)	104 (104)		

[a] Analytical samples were removed from replicate E, with the exception of the control and solvent control which were removed from replicate G.
[b] Analytical samples were removed from replicate F, with the exception of the control and solvent control which were removed from replicate H.
[c] Analytical samples were removed from replicate G, with the exception of the control and solvent control which were removed from replicate I.
[d] Mean measured and percent recovery values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[e] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for the analysis was set at the lowest validated sample concentration.
[f] NA = Not Applicable.
[g] QC = Quality Control sample. Percent of nominal is presented in parentheses below the measured results.
[h] Percent recovery for this QC sample is outside of the acceptable range (i.e., 70.0 to 120%, Appendix 2).
[i] This QC sample was inadvertently fortified below the limit of quantification.

Table 4. Mean measured sediment concentrations, number of oligochaetes and biomass of oligochaetes at test termination of the 28-day exposure with D4 and oligochaetes (*Lumbriculus variegatus*).

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes	Population Increase Factor	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
Control	A	17	12	1	30	3.0	42.38	30 (2)	46 (6)
	B	18	11	1	30	3.0	44.24		
	C	12	12	2	26	2.6	38.83		
	D	16	12	0	28	2.8	53.88		
	E	9	24	0	33	3.3	52.66		
	F	14	13	4	31	3.1	46.88		
Solvent Control	A	13	12	3	28	2.8	41.79	30 (3)	51 (9)
	B	16	10	0	26	2.6	46.15		
	C	23	6	2	31	3.1	48.96		
	D	21	10	2	33	3.3	48.22		
	E	20	11	1	32	3.2	67.07		
	F	16	9	3	28	2.8	55.00		
Pooled Control					356	3.0		30 (2)	49 (8)
1.2	A	19	12	0	31	3.1	49.65	31 (1)	55 (6)
	B	17	11	2	30	3.0	61.45		
	C	19	12	0	31	3.1	59.22		
	D	24	8	1	33	3.3	50.84		
3.2	A	10	14	6	30	3.0	55.52	31 (3)	64 (6)
	B	12	15	3	30	3.0	63.67		
	C	9	19	0	28	2.8	67.13		
	D	9	22	3	34	3.4	70.49		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation
[c] Statistically different ($p \leq 0.05$) compared to the pooled control data, based on Bonferroni's t-Test.

Table 4. (Continued) Mean measured sediment concentrations, number of oligochaetes and biomass of oligochaetes at test termination of the 28-day exposure with D4 and oligochaetes (*Lumbriculus variegatus*).

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes	Population Increase Factor	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
8.8	A	16	10	2	28	2.8	44.78	28 (1)	50 (8)
	B	14	15	0	29	2.9	42.72		
	C	16	12	1	29	2.9	50.07		
	D	17	9	0	26	2.6	61.14		
13	A	10	16	0	26	2.6	44.35	29 (3)	47 (3)
	B	11	18	1	30	3.0	51.40		
	C	21	11	0	32	3.2	47.43		
	D	7	21	1	29	2.9	45.46		
19	A	11	10	4	25	2.5	37.64	26 (3)[c]	44 (5)
	B	15	11	1	27	2.7	46.63		
	C	15	7	0	22	2.2	48.07		
	D	15	13	0	28	2.8	44.77		
32	A	11	11	0	22	2.2	49.88	22 (1)[c]	44 (6)
	B	12	11	0	23	2.3	37.21		
	C	12	7	2	21	2.1	48.01		
	D	10	10	3	23	2.3	42.62		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation
[c] Statistically different ($p \leq 0.05$) compared to the pooled control data, based on Bonferroni's t-Test.

APPENDIX 1 - STUDY PROTOCOL

SPRINGBORN SMITHERS
LABORATORIES
Massachusetts Research Center
790 Main Street • Wareham, MA • 02571-1037 • Phone· (508) 295-2550 • Fax (508) 295-8107

TEST PROTOCOL

PROTOCOL TITLE: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: CES (Centre European des Silicones, European Chemical Industry Council (Cefic)

Address: Avenue E. van Nieuwenhuyse 4, B-1160 Brussels, Belgium

Phone: 989-496-8226

Study Monitor: Kurt Drottar **E-mail:** k.drottar@dowcorning.com

Sponsor Protocol/Project No.:

Test Substance Name: D4

Purity: 99.75% **Lot #:** LL084732

Analytical Standard:

Purity: **Lot #:**

Additional Comments and Modifications:

Sponsor Approval: **Date:** 8/6/08

TO BE COMPLETED BY SPRINGBORN SMITHERS LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility Springborn Smithers Laboratories 790 Main Street, Wareham, MA 02571-1037

Study Director: Christian Picard Study No.. 13937 - 6103

Test Concentrations: *

Proposed Experimental Dates: (Start) * (Termination) *

Study Director Signature 8/7/08 **Study Initiation Date**

* To be provided by protocol amendment, if applicable.

Other Locations: 2900 Quakenbush Road, P O Box 620 • Snow Camp, North Carolina 27349 • Phone· (336) 376-0141 • Fax· (336) 376-0145
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Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

1.0 OBJECTIVE

The purpose of this test will be to determine the impact of a test substance to the sediment-dwelling oligochaete (*Lumbriculus variegatus*), following a 28-day exposure period. The study will assess the impact of the test substance on the reproduction and biomass of the oligochaetes. The methods described in the protocol meet the requirements specified in the OECD Guideline for the Testing of Chemicals, Guideline 225 entitled "Sediment Water Lumbriculus Toxicity Test Using Spiked Sediment" (OECD, 2007).

2.0 MATERIALS AND METHODS

2.1 Chemical System

2.1.1 Test Substance

Upon arrival at Springborn Smithers Laboratories, all test substances and reference substances will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date

2.1.2 Test Substance Concentrations

Test substance concentrations will be based on the results of a 28-day static range-finding test. Concentrations of 500, 50, 5.0, 0.5, 0.05 mg/kg dry weight plus controls will be selected for the static range-finding test. A negative control consists of overlying water and sediment without test substance.

For the definitive test, each test substance concentration will be 50% of the next higher concentration of the test substance.

2.1.3 Solvent Control

An organic solvent (N,N-dimethylformamide) will be used as a carrier to solubilize the test substance. The solvent volume utilized will remain constant across the test concentration series. A solvent control will be included in each test and will consist of

sediment plus the equivalent volume of solvent used during the application of the test solutions to the sediment. The solvent volume will be kept as low as possible.

2.1.4 Application of Test Substance to Sediment

The appropriate amount of test substance will be removed for dosing the exposure system (e.g., weighed on an analytical balance or volumetrically measured with a graduated pipette). A Chemical Usage Log will be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test substance is used. The amount of test substance will be applied to the sediment according to the following formula:

$$\text{Sediment Concentration (e.g., mg a.i./kg)} = \frac{\text{T.S.}}{\text{S.A.} \times \text{D.W}}$$

where:

T.S.	=	test substance (mg a.i.)
S.A.	=	wet sediment amount (kg)
D.W.	=	(percent dry weight of sediment) ÷ 100

The test substance will be applied by the following method:

For each dose level, a glass jar will be filled with natural sediment followed by approximately 3 times the volume of overlying water, to give a water-volume to sediment-volume ratio of 3:1. The jar contents will be shaken to suspend the sediment immediately prior to dosing individually with a solution containing octamethylcyclotetrasiloxane, D_4, in N,N-dimethylformamide (DMF). The jar will be shaken again after dosing with D_4 to distribute the test substance. After settling for 4 hours, the water will be decanted from the sediments. The sediment will be stirred to assure homogeneity and then treated sediment will be allocated to individual test vessels.

2.2 Test System

2.2.1 Species

The oligochaete, *Lumbriculus variegatus*, will be the species used in this reproduction test. Prior to test initiation, synchronized oligochaetes of the same size are removed from the culture aquaria and added to the exposure systems. The synchronization of worms is performed to avoid "uncontrolled" regeneration and reproduction that may contribute high variation in the test results.

2.2.2 Justification of Test System

The oligochaete *Lumbriculus variegatus* is recommended for use in OECD guidance (OECD 2007) for sediment toxicity testing. This species will be used because it is tolerant to a wide range of sediment types and widely used for sediment toxicity and bioaccumulation testing. The OECD guideline 225 for testing of chemicals in spiked

sediment relies on use of *Chironomus* species in testing. The larvae of *Chironomus sp.* live at the sediment water-interface and exposure is to the top layer of sediment only. The oligochaete *Lumbriculus variegatus* will contact sediment and pore water at greater depths and theoretically provides a complimentary assessment of sediment toxicity to *Chironomus sp.* testing.

2.2.3 Synchronization of Oligochaetes

Approximately two weeks prior to test initiation, the worms will be artificially fragmented and the posterior ends will be left to regenerate new heads. Worms are artificially fragmented using a scalpel to remove the anterior portion of each worm. The worms will then be held under test conditions (i.e., water and test sediment) for a period of 10 to 14 days to regenerate new heads. This provides organisms that are "synchronized" worms and are equally exposed to the treated sediment.

2.2.4 Origin

Lumbriculus variegatus cultures will be maintained at Springborn Smithers Laboratories. The oligochaetes will be cultured in aquaria under flow-through conditions. The water used in the main culture is unadulterated well water from a 100-meter bedrock well supplemented on demand with untreated Town of Wareham well water, and will be characterized as soft water with typical ranges as total hardness of 30 to 60 mg/L as $CaCO_3$, alkalinity of 25 - 45 mg/L as $CaCO_3$, pH of 6.9 to 7.7, and a specific conductance of 140 to 200 microohms/cm. Culture water will be maintained at 23 ± 1 °C. An appropriate number of oligochaetes from the main culture population will be artificially fragmented and placed in a small aquarium (e.g., 10 liter aquarium) with test sediment and unadulterated well water under flow-through conditions. The synchronized population will be acclimated to the test conditions by renewing the overlying water in the small aquarium with test dilution water at the rate of approximately one volume replacement per day. The test dilution water will consist of hard fortified well water with a total hardness of 160-180 mg/L as $CaCO_3$, alkalinity of 110-130 mg/L as $CaCO_3$, pH of 7.9-8.3 and a specific conductance of 400-600 micromhos/cm.

The origin of the culture and the background of the test culture will be described in the final report. The oligochaetes will be held in test sediment for a period of at least 10 to 14 days (synchronization period) to acclimate the organisms to the test sediment.

2.2.5 Feeding

The main culture of oligochaetes will be fed 15 mL of a flaked fish food suspension (100 mg/mL), weekly. The synchronized population will be fed a flaked fish food suspension after seven days of being acclimated to test conditions. The feeding rate of the synchronized oligochaetes will be modified based on the size of this population. Oligochaetes will be fed once during the study (see section 2.3.4).

2.2.6 Handling

Fine mesh nets and wide-bore pipettes will be used to remove and transfer the oligochaetes from the cultures to the test system, taking care to minimize possible stress due to handling. Oligochaetes that are damaged or dropped during transfer will not be used.

2.2.7 Loading

Prior to test initiation, a subsample of oligochaetes will be taken to determine dry weight of the organisms. The ratio of total organic carbon (TOC) of the sediment to organisms dry weight in each vessel will be calculated.

2.3 Physical System

2.3.1 Test Containers

The test chambers to be used in the 28-day static toxicity test will be 600-mL glass beakers. The test chambers will be chemically clean before the test is started. The test chambers will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with water. Each test chamber contains 1.5 cm (75 mL) of sediment and 6 cm (300 mL) of overlaying water. The total medium volume (sediment/water) is maintained at 375 mL and the ratio of sediment to water is 1:4. Each test chamber will be covered with a plexiglass plate to minimize evaporation. The test chambers will be labeled to identify the treatment/control and the replicate designation.

2.3.2 Replication and Control of Bias

Four replicates (A through D) will be included with each test concentration. The control and solvent control will have six replicates (A through F). Each replicate vessel will contain ten individuals, a total of either 40 or 60 oligochaetes per concentration or control. Oligochaetes will be added impartially to an intermediate beaker by adding no more than two oligochaetes to each vessel until all beakers contain two oligochaetes. This procedure will be repeated until each intermediate beaker contains ten oligochaetes. The test will be initiated when each intermediate beaker of 10 oligochaetes is added to each respective test vessel. Four additional replicate test vessels (E through H for each test concentration and G through J for the controls) will be established for analytical measurements during the test. These vessels will be maintained under the same conditions as the exposure vessels and will contain test organisms. These additional replicates will not be included in the biological observations for the study.

2.3.3 Overlying Water

Dilution water will consist of hard fortified well water with a total hardness of 160 to 180 mg/L as $CaCO_3$. The well water (total hardness about 30 mg/L as $CaCO_3$) will be fortified according to the formulation for hard water presented in "*Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians*" (U.S. EPA, 1975). Dilution water will be filtered through an amberlite XAD-7 resin column prior to delivery to the diluter. The column is 30 cm long and 1.6 cm wide. This filtration will effectively remove any potential organic contaminants from the water. Total hardness, total alkalinity, pH and specific conductance of the diluent water will be monitored on each batch prior to use to assure that these parameters are within the normal acceptable ranges. Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1995). Ranges for these parameters will generally be: total hardness, 160 to 180 mg/L as $CaCO_3$; alkalinity, 110 to 130 mg/L as $CaCO_3$; specific conductance, 400 to 600 µmhos/cm; and pH, 7.9 to 8.3.

New batches of diluent water will be prepared when either the previous batch is exhausted, when a water quality parameter (total hardness, alkalinity, etc.) differs from the normal ranges, or after two weeks of holding. The diluent water will be aerated with an air pump and air stones to bring the pH and dissolved gases into equilibrium with the atmosphere. Fiberglass containers will be used to hold the diluent water, and water will be pumped from this holding tank to the diluter. Periodic analysis of representative samples of the dilution water source will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP 7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to oligochaetes.

2.3.4 Sediment

The test will be conducted using natural freshwater sediment of medium organic carbon content (e.g., 2 – 5% OC). The freshwater sediment used in this study will be collected from a site known to be free from contamination. The natural sediment will be wet pressed through a 2 mm sieve to remove large particles and will be characterized for total organic carbon content, pH, percent sand, silt, clay (particle size distribution) and percent water holding capacity by Agvise Laboratories, Northwood, North Dakota. The ammonia concentration in the sediment pore water will be analyzed prior to the use of the sediment. Periodic analyses of representative samples of the sediment will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP 7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations that may be harmful to oligochaetes.

In addition, *Urtica* sp. powder will be added to the sediment at a rate of 0.25% of the sediment dry weight as a food source for the oligochaetes during the test, along with alpha-cellulose at 0.25% of the sediment dry weight.

2.4 Test Conditions

2.4.1 Temperature

Water temperature of the test solutions will be maintained at 20 ± 2 °C by conducting the test in a water bath maintained at the appropriate test temperature. The temperature between replicates vessels at any one measurement interval should be ± 1 °C.

2.4.2 Lighting

The test will be conducted in a light controlled laboratory. The test will be illuminated to a light intensity of 100 to 500 lux using fluorescent bulbs. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer. Light intensity will be measured once during the study.

2.4.3 Dissolved Oxygen

Total dissolved oxygen will not be allowed to drop below 30% of the air saturation value (ASV). Aeration (with oil free air) will be initiated prior to test initiation to maintain the dissolved oxygen concentration at or above 30% of the ASV.

2.4.4 Preparation of Test Vessels

The sediment and overlying water will be added to the test vessels two days prior to the addition of the test organisms. During this period the test vessels will be held under test conditions. The amount of sediment is added to each test vessel to a layer of 1.5 cm deep (75 mL). To avoid separation of the sediment ingredients, a turbulence reducer is used to add the 6 cm of overlying water (300 mL of water). The turbulence reducer consists of a plastic disk that floats as the water is poured in the test vessel, taking care not to disturb the sediment. The initial water level in each test vessel will be marked so as to evaluate evaporation.

Each test vessel is covered with a plastic plate and gentle aeration is provided through a glass pipette fixed approximately 2 to 3 cm above the sediment surface. Aeration is delivered at a rate of 1 to 3 bubbles per second. If necessary, the water level in each test vessel will be topped off with deionized water (i.e., twice weekly) to prevent a change of more than 10% (i.e., 30 mL) of the total overlying water volume

2.4.5 Initiation of Test Organisms

Two days before exposure initiation (day -2) the treated and control sediments will be added to the replicate test vessels and the overlying water will be added (section 2.4.4). This allows the sediment and water to equilibrate prior to addition of the test organisms.

The exposure will be initiated (Day 0) when all vessels contain ten synchronized oligochaetes and the exposure will terminate 28 days thereafter.

2.5 Sampling and Observations

2.5.1 Sampling

Prior to test initiation and before the sediment is allocated to the replicate test vessels, a sample of sediment from each treatment level and controls will be removed and analyzed. In addition, a sample of the stock solution used to apply the test substance will be sampled and analyzed.

During the in-life phase, samples of the sediment from each treatment level and controls will be removed on test day 0, test day 7 and at test termination (test day 28) for determination of test substance concentration The sample volume of sediment will be dependent upon the analytical methodology employed for determination of test substance concentrations. Three quality control (QC) samples will be prepared at each sampling interval and stored and analyzed with the set of study samples. The QC samples will be prepared in sediment at test substance concentrations similar to the treatment level range. Results of these analyses indicate the accuracy of the analytical method for measuring test substance concentration at each sampling period. The analytical method used to measure test substance concentrations in the exposure solutions will be validated by Springborn Smithers Laboratories at the expected nominal concentration range prior to test initiation.

2.5.2 Sampling Devices

The entire volume of overlying water will be removed from each test vessel carefully by decanting or pipetting. After removal of the overlying water the sediment will be mixed thoroughly. An appropriate size sample of the mixed bulk sediment will then be removed with a stainless steel spatula for determination of sediment test substance concentrations.

2.5.3 Water Quality Measurements

On the day of addition of the test organisms (day 0) and test termination (day 28), temperature, dissolved oxygen (DO) concentration and pH will be measured and recorded in each test vessel established for biological observations (replicates A through D for treatment levels and A through F for controls). In addition, temperature, dissolved oxygen (DO) concentration and pH will be measured and recorded daily in a replicate test vessel established for biological observations of each concentration and control(s). Daily measurements will be alternated among replicate vessels. Total hardness, alkalinity, and specific conductance will be determined on day 0 and at test termination in a sample from the highest concentration tested and the solvent control. Total ammonia will be measured in the highest concentration and the solvent control at test initiation and three times weekly throughout the duration of the test. Overlying water removed for water quality determination on day 0 will be taken from additional replicates set-up for chemical analysis. Temperature will be monitored continuously in an auxiliary vessel, maintained in the water bath with all test vessels, by using a minimum-maximum thermometer. Readings of temperature extremes will be recorded daily.

2.5.4 Biological Observations

Following addition of the oligochaetes and daily thereafter, until termination, observations of oligochaete mortality and abnormal behavior (e.g., leaving sediment) will be made and recorded.

2.5.5 Test Termination

On test day 28 of the exposure, the replicate vessels from each exposure level (four replicates) and controls (six replicates) will be terminated to determine number of surviving oligochaetes and biomass of surviving oligochaetes.

Surviving worms will be assigned to one of three groups:

a) large complete worms (adults) without regenerated body regions

b) complete worms with regenerated, lighter colored body regions

c) incomplete worms (i.e., recently fragmented worms with non-regenerated body regions)

The biomass is determined by placing the entire number of surviving oligochaetes in each replicate vessel in a tared weigh tin and then drying in an oven at 100 ± 5°C overnight (18 to 24 hours). Following drying, the tins are cooled to room temperature in desiccators and then weighed to the nearest 0.01 mg on a calibrated analytical balance.

2.5.6 Acceptability Criteria

For the test, the following criteria have to occur in the control or the test will be considered unacceptable:

- Based on the results of a ring-test, the average number of living worms per replicate vessel in the control should have increased by a factor of 1.8 at the end of the exposure compared to the number of worms per replicate at the start of the exposure.
- The dissolved oxygen concentration should not be < 30% of the air saturation value (ASV) at the end of the test.
- The pH range in overlying water at the beginning and end of the test should be between 6 and 9.

3.0 STATISTICAL ANALYSES

3.1 Endpoints

The endpoints used for determination of significant effects by statistical evaluation include number of surviving oligochaetes per replicate vessel and biomass of oligochaetes per replicate vessel. All concentration-effect relationships are based on the nominal concentrations of test substance in the sediment.

3.2 Statistical Methods (Determination of LOEC/NOEC)

If a solvent is used as a carrier for the test substance, a t-Test ($p \leq 0.05$) will be used to compare the results (survival) of the solvent control to the control data. If the data are similar, they will be pooled for further analyses. If the data are not similar, the remaining statistical analyses will be performed comparing the treatment data to the solvent control data.

The data will be tested for normality and homogeneity of variance using Shapiro-Wilks' Test or Chi-Square Test (depending on the number of replicates in the analysis) and Bartlett's Test or Cochran's Test. If the data passes these two tests, then a parametric method will be used to evaluate the results of test, e.g., William's Test or Dunnett's Test. If the data fails the test for normality and homogeneity of variance, then a non-parametric method will be used to evaluate the results of the test, e.g., Dunn's (Sokal and Rohlf, 1981) or Steel's One-Many Rank Test (Weber et al, 1989). If necessary, mean values will be transformed using square root, arcsine square root, or log conversion procedures. The Lowest-Observed-Effect-Concentration (LOEC) is defined as the lowest test concentration that shows a statistically significant effect and the No-Observed-Effect-Concentration (NOEC) is the highest test concentration that shows no statistically significant difference from the control.

3.3 Transformations

Transformation of data is performed with data representing endpoint estimates obtained as a proportion (e.g., survival). Prior to analyzing data of this type, the observed proportion in each vessel is transformed by using the arcsine square-root transformation.

4.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

5.0 REPORTING

The raw data and final draft of the report will be reviewed by the Quality Assurance Unit and the Study Director. Chemical and water quality measurements will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The draft report will be initially submitted to the Study Sponsor for review. Upon acceptance by the Sponsor, the final report will be submitted. The final report will meet the formatting requirements of EPA's PR Notice 86-5. All reports will include, but will not be limited to, the following information:

- The report and project numbers from Springborn Smithers Laboratories and Sponsor study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Study Monitor, Study Director and Principal Investigator.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).
- Characterization and origin of the dilution water.
- Characterization and origin of the sediment.
- Scientific name of the test organisms, source and culturing information.
- Test container volume, sediment and water volume, number of replicates used per concentration, and mass of oligochaetes used per treatment/control.
- Description of exposure system and application of test substance to sediment.
- Test temperatures, dissolved oxygen concentration, and pH; and photoperiod and light intensity used, as well as specific conductance, total ammonia, total alkalinity and total hardness measured.
- Description or reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- The nominal test substance concentration in sediment.

- Number of surviving worms and biomass per replicate vessels for each treatment level and control.
- Data evaluation and conclusions.
- Deviations from the protocol not addressed in protocol amendments will be listed, together with a discussion of the impact on the study.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of raw data and final report.

6.0 PROTOCOL CHANGES

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any. If necessary, amendments other than the one providing the information required by page one of this protocol, may initially be verbally authorized, followed by Springborn Smithers' written documentation. In such cases, the effective date of the amendment will be the date of verbal authorization.

7.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records, and reports will comply with the Organization for Economic Co-operation and Development (OECD) Good Laboratory Practices

8.0 REFERENCES

APHA, AWWA, WPCF. 1995. Standard Methods for the Examination of Water and Wastewater. 19th Edition, Washington, DC

OECD, 1997. Good Laboratory Practice in the Testing of Chemicals. Paris, France.

OECD. 2007. OECD Guideline 225. Sediment Water Lumbriculus Toxicity Test Using Spiked Sediment. 16 October 2007. Paris, France.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. 2nd Edition. W H. Freeman and Company, New York. 859 pp.

U.S. EPA. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Ecological Research Series (EPA-660/3-75-009). 61 pp.

U.S. EPA. 40 CFR, Part 160. Federal Insecticide, Fungicide, and Rodenticide Act. Good Laboratory Practices Standards; Final Rule. Office of the Federal Register, National Archives and Records Administration. U.S. Government Printing Office, Washington, D.C.

Weber, C.I. et al. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics, 27: 103-117.

Williams, D.A. 1972. A comparison of several dose levels with a zero dose control. Biometrics, 28: 519-531.

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PROTOCOL AMENDMENT

Amendment No.: 1

Effective Date: 17 December 2008

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937.6103

Amendment:

1. Protocol Cover Page, Study Specific Information
The following information is being provided as requested on the protocol cover page·

Internal Standard. Tetrakis (Trimethylsiloxy) Silane Lot Number· 8D-12648 Purity· 99.8%

Test Concentrations: 100, 50, 25, 13 and 6.3 mg/kg plus controls

Proposed Experimental Dates (Start) January 2009 (Termination) February 2009

2. Page 2, Section 2.1.3 Solvent Control
The protocol states that an organic solvent (DMF) will be used as a carrier to solubilize the test substance. In this study, acetone will be the organic solvent used to solubilize the test substance

3. Page 3, Section 2.1.4 Application of Test Substance to the Sediment
The protocol states that the sediment will be dosed with a solution containing D4 dissolved in DMF. In this study, the sediment will be dosed with a solution containing D4 dissolved in acetone.

4. Page 9, Section 3.1 Endpoints
The protocol states that all concentration-effect relationships will be based on nominal concentrations of the test substance in the sediment In this study, all concentration-effect relationships will be based on measured concentrations of the test substance in the sediment

Other Locations: 2900 Quakenbush Road, P.O Box 620 • Snow Camp, North Carolina 27349 • Phone· (336) 376-0141 •Fax (336) 376-0145
Seestrasse 21 • Horn, CH-9326, Switzerland • Phone (41) 71 844-6970 • Fax· (41) 71 841-8630

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13797.6103
Page 2 of 2

None of the above changes will have a negative impact on the study.

Approval Signatures.

Christian R. Picard
Springborn Smithers Study Director

12/22/08
Date

Study Sponsor Representative

12/22/08
Date

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PROTOCOL AMENDMENT

Amendment No.: 2

Effective Date: 13 February 2009

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937 6103

Amendment:

1. Page 3, Section 2.1.4 – Application of the Test Substance
The protocol states that the dosed sediment/water mixture will be allowed to settle for four hours and the water will then be decanted from the sediment. In this study, the dosed sediment/water mixture will be allowed to settle overnight (approximately 16 hours) before decanting the water from the sediment.

2. Page 8, Section 2.5.2 – Sampling Devices
The protocol states that the sediment in the test vessel will be mixed thoroughly and an appropriate sized sample of mixed bulk sediment will be removed with a stainless steel spatula for analysis. In this study, an appropriate sized sediment sample will be removed from the test vessels on days 0, 7 and 28 and centrifuged at approximately 1200 *g* for 15 to 30 minutes. After centrifuging, the pore water is decanted or removed by pipette. An appropriate size sample of sediment will then be removed from the centrifuge tube with a stainless steel spatula for determination of sediment concentrations.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R. Picard
Springborn Smithers Study Director — Date: 2/19/09

Study Sponsor Representative — Date: 2/19/09

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SMITHERS
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PROTOCOL AMENDMENT

Amendment No.: 3

Effective Date: 8 May 2009

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937 6103

Amendment:

1. Protocol Cover Page Information and Amendment 1

An additional test concentration of 3 1 mg/kg is being added to the range of concentrations listed in Amendment 1.

2. Amendment 2

Amendment 2 states that sediment samples will be centrifuged at approximately 1200 *g* for 15 to 30 minutes. In this study, sediment samples will be centrifuged at approximately 10,000 *g* for 15 to 30 minutes.

None of the above changes will have a negative impact on the study.

Approval Signatures.

Christian R. Picard
Springborn Smithers Study Director

5/11/09
Date

Study Sponsor Representative

5/11/09
Date

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APPENDIX 2 – ANALYTICAL METHODOLOGY (ARTIFICIAL SEDIMENT)

SUMMARY

Methodology was validated (25 March 2009) to quantify the amount of D4 present in artificial sediment. Recovery samples containing the test substance were extracted twice with acetonitrile and hexane. The hexane extracts were combined and diluted with hexane prior to analysis. An aliquot of the final extracts were analyzed by automated injection on a gas chromatographic system equipped with mass selective detection (GC/MSD).

This method was validated by fortification of artificial sediment with D4 at nominal concentrations of 0.600, 10.0 and 100 µg a.i./g. Recoveries of D4 averaged 82.1 ± 9.02%. Due to instrumental carryover, the LOQ is set to the lowest validated concentration, i.e., 0.600 µg/g. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

EXPERIMENTAL

Equipment

1. Instrument:	Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector series 5973, Hewlett Packard autosampler, Hewlett Packard Model 7683 injector and Hewlett Packard ChemStation Version C.00.000 for data acquisition.
2. Balances:	Sartorius BL210S, Orion 0960, Sartorius MA45C moisture balance
3. Shaker table:	Lab-Line orbit shaker
4. Centrifuge:	Beckman GS-6R
5. Laboratory equipment:	syringes, volumetric flasks, volumetric pipets, Pasteur pipets, autosampler vials, 45-mL glass vials with PTFE-lined caps, and amber glass bottles with Teflon®-lined caps

Reagents

1. Acetone:	Burdick & Jackson, reagent grade
2. Hexane:	Burdick & Jackson, reagent grade
3. Acetonitrile:	Burdick & Jackson, reagent grade
4. Nitric acid:	JT Baker, reagent grade
5. Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Synonym:	Trimethylsiloxysilicate (M_4Q)
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

PROCEDURES

Preparation of Stock Solutions

A 10.0 mg a.i./mL D4 stock solution was prepared by placing 0.2514 g of D4 (0.2508 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with acetone. The 100 mg a.i./L secondary stock solution was used to fortify the low-level recovery samples. The 1000 mg a.i./L secondary stock solution was used to fortify the mid- and high-level recovery samples.

A 1.00 mg a.i./mL D4 stock solution was prepared by placing 0.1005 g of the test substance (0.1003 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with hexane. Two secondary stock solutions (10.0 and 100 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 1.00 mg a.i./L primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with hexane. The 10.0 and 100 mg a.i./L secondary stock solutions were used to prepare calibration standards.

A 10.0 mg/mL tetrakis(trimethylsiloxy)-silane internal standard stock solution was prepared by placing 0.2504 g of the internal standard in a 25.0-mL volumetric flask and bringing it to volume with acetone. A 1000 mg/L secondary stock solution was prepared by placing 5.00 mL of the 10.0 mg/mL primary stock solution in a 50.0 mL volumetric flask and bringing it to volume with acetone. The 1000 mg/L secondary internal standard stock solution was added to the mid- and high-level recovery samples.

A second 100 mg/L secondary internal standard stock solution was prepared by placing 0.500 mL of the 10.0 mg/mL primary internal standard stock solution in a 50.0-mL volumetric flask and bringing it to volume with hexane. The 100 mg/L secondary internal standard stock solution was added to the low-level recovery samples, the control samples and the calibration standards.

All stock solutions were stored in a refrigerator in glass amber bottles fitted with Teflon®-lined caps.

Preparation of Calibration Standards

Calibration standards were prepared in hexane by fortifying with the 10.0 and 100 mg a.i./L secondary stock solutions to yield concentrations of 0.0200, 0.0500, 0.100, 0.200, 0.300 and 0.500 mg a.i./L. Each calibration standard was fortified with 150 µL of the 100 mg/L secondary stock solution using the scheme outlined in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Calibration Standard Concentration (mg a.i./L)
NA[a]	NA	100	0.150	50.0	0.00
10.0	0.100	100	0.150	50.0	0.0200
10.0	0.250	100	0.150	50.0	0.0500
10.0	0.500	100	0.150	50.0	0.100
100	0.100	100	0.150	50.0	0.200
100	0.150	100	0.150	50.0	0.300
100	0.250	100	0.150	50.0	0.500

[a] NA = Not Applicable.

Sample Fortification and Preparation

All glassware used for the preparation and extraction of recovery samples was subjected to rigorous cleaning before use. The glassware was soaked and rinsed well with 20% nitric acid in water. This was followed by a water rinse to remove the nitric acid, and then a rinse with acetone to remove residual organics. Glassware was rinsed with copious amounts of hexane prior to use.

The recovery samples were prepared by fortifying artificial sediment (5.00 g, dry weight) with D4 at concentrations of 0.600, 10.0 and 100 µg a.i./g. The low-level recovery samples were prepared by fortifying 5.00 g (dry weight) of artificial sediment using the 100 mg a.i./L secondary stock solution. The mid- and high-level recovery samples were prepared by fortifying 5.00 g (dry weight) of artificial sediment using the 1000 mg a.i./L primary stock solution. Each

concentration level was produced in triplicate. In addition, three recovery samples 5.00 g (dry weight) were left unfortified to serve as controls.

A 0.600-mL aliquot of the 1000 mg/L secondary internal standard stock solution was added to the high-level recovery samples. A 0.0600-mL aliquot of the 1000 mg/L secondary internal standard stock solution was added to the mid-level recovery samples. A 0.150-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low-level recovery samples.

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Sample Mass (g)	Sample Concentration (µg a.i./g)
NA	NA	100	0.150	5.00	0.00
100	0.0300	100	0.150	5.00	0.600
1000	0.0500	1000	0.0600	5.00	10.0
1000	0.500	1000	0.600	5.00	100

[a] NA = Not Applicable.

Extraction

All recovery samples (5.00 g, dry weight) were prepared in 45-mL glass vials with PTFE-lined caps. An aliquot (6.00-mL) of acetonitrile was added to each sample. An aliquot of hexane (12.0 mL) was added to the vials that contained both sediment and acetonitrile. The samples were placed on a shaker table at 150 rpm for 30 minutes and centrifuged at 1500 rpm for 15 minutes to separate the layers. At this point the samples contained three layers: the top layer was hexane, the middle was acetonitrile and water and the bottom was the sediment. The upper hexane layer was transferred to 50.0-mL volumetric flasks. An addition aliquot of hexane (12.0-mL) was added to the acetonitrile and sediment in the vials. The samples were placed on a shaker table at 150 rpm for an additional 30 minutes and centrifuged at 1500 rpm for 15 minutes. The extracts were combined and taken to volume with hexane. The mid- and high-level samples were further diluted into the calibration standard range with hexane. An aliquot from each recovery sample was collected for analysis by gas chromatographic analysis with mass selective detection and negative chemical ionization (GC-MSD/NCI).

ANALYSIS

GC Parameters:

Column: DB-624 30m x 0.32mm x 1.8 µm
Temperature: 40 °C (initial) and held for 2.00 minutes
Ramps:

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
10.0	250	0.00
30.0	280	2.33

Run Time: 26.33 min
Injection Volume: 2.0 µL
Gas Flows: Carrier gas: Helium, at a constant flow of 65.2 mL/min
Inlet Mode: Splitless
Purge Time: on at 1.00 min at 60 mL/min
Inlet Temperature: 260 °C
Retention Time: approximately 11.3 minutes

MSD parameters

Solvent delay: 8.0 minutes
Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
281	100	quantitation D4
369	100	quantitation internal standard

Temperatures: MSD Transfer Line - 280 °C
MS Quad – 150 °C
MS Source – 230 °C

Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the system was performed by programmed automated injection.

CALCULATIONS

A calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (mg a.i./L) in the calibration standards against the natural logarithm (ln) of the peak area of the calibration standards. The equation of the line (equation 1) was algebraically

manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the natural logarithm (ln) of the peak area of the recovery sample and the dilution factor. Equations 2, 3 and 4 were then used to calculate measured concentrations and analytical results.

(1) $$\ln y = a(\ln x)^2 + b(\ln x) + c$$

(2) $$\ln x = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

(3) $$DC\,(x) = \text{inverse}(\ln x)$$

(4) $$A = DC \text{ x } DF$$

where:

y	=	detector response (peak area) for analyte
a, b, c	=	regression constants
x	=	detected concentration (µg a.i./L) in the sample
C	=	constant c minus the natural log of the peak area; C = (c - lny)
DC (x)	=	detected concentration in the sample
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

(5) $\ln(Area_{MIN}) = \ln(0.5 \times A_{LS})$

(6) $\ln(LOQ_{INST}) = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(7) $LOQ_{INST} = \text{inverse}\,(\ln LOQ_{INST})$

(8) $LOQ = LOQ_{INST} \times DF_{CTRL}$

where:

$Area_{MIN}$	=	one half of the mean detector response (peak area) of the low concentration calibration standard (two injections)
A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
a, b, c	=	regression constants
C	=	regression constant c – $Area_{MIN}$; $C = (c - \ln(Area_{MIN}))$
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

RESULTS AND DISCUSSION

The mean recovery was 82.1% ± 9.02%. Due to instrumental carryover the LOQ was set at 0.600 µg a.i./g, the lowest validated concentration. Measured test sample concentrations < 0.600 µg/g are reported as < 0.600 µg/g. The low concentration recoveries are reported even if they fall below 0.600 µg/g, because that is their nominal concentration. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%.

Analytical results for the recovery of D4 from artificial sediment are presented in Table 2A. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample are presented in Figure 2A, Figure 2B and Figure 2C, respectively. A typical regression analysis for D4 is presented in Figure 2D.

Table 2A. Analytical results for the recovery of D4 from artificial sediment during the method validation study.

Sample ID	Fortified Concentration (µg a.i./g)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
Ctrl A	0.00	10.0	< 0.600	NA
Ctrl B	0.00	10.0	< 0.600	NA
Ctrl C	0.00	10.0	< 0.600	NA
Low A	0.600	10.0	0.512	85.4
Low B	0.600	10.0	0.452	75.4
Low C	0.600	10.0	0.366	61.0
Mid A	10.0	40.0	8.07	80.7
Mid B	10.0	40.0	8.92	89.2
Mid C	10.0	40.0	8.35	83.5
High A	100	400	88.1	88.1
High B	100	400	87.8	87.8
High C	100	400	87.5	87.5
MEAN				**82.1**
STD DEV				**9.02**
% CV				**11.0**

[a] Concentrations expressed as less than values were below the limit of quantitation (LOQ). Due to instrumental carryover the LOQ for this analysis is set at 0.600 µg/g, the lowest validated concentration. Measured test samples concentrations <0.600 µg/g are reported as < 0.600 µg/g.

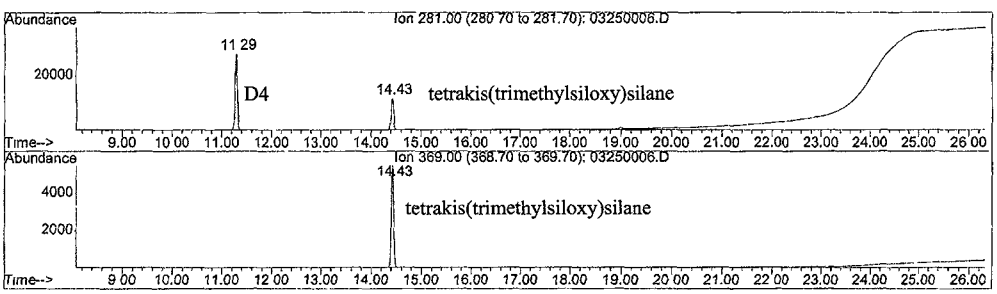
[b] NA = Not Applicable.

Note: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Figure 2A. Representative chromatogram of a 0.2 mg a.i./L calibration standard used for quantification during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250006.D
Operator    : MED
Acquired    : 25 Mar 2009  19:17     using AcqMethod D4SIM
Sample Name: std 0.2mg/l
Misc Info   :
Vial Number: 6
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```

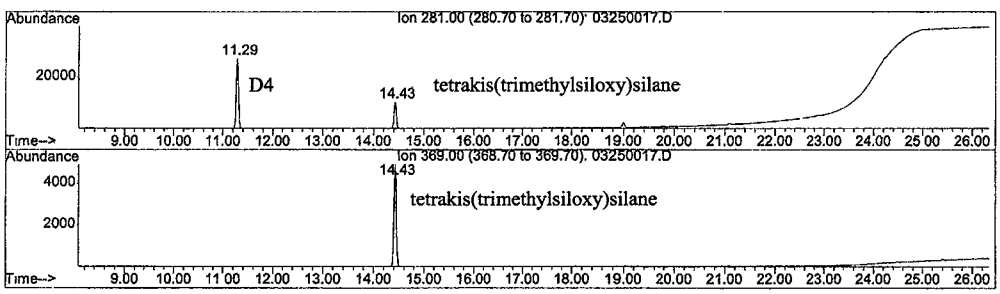


Retention Time	Area	Area %	Ratio %
281.00 amu			
11.290	905398	70.974	100.000
14.428	370270	29.026	40.896
369.00 amu			
14.429	173970	100.000	100.000

Figure 2B. Representative chromatogram of a 10.0 µg a.i./g recovery sample used during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250017.D
Operator    : MED
Acquired    : 26 Mar 2009   1:55     using AcqMethod D4SIM
Sample Name: MID B
Misc Info   :
Vial Number: 17
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```

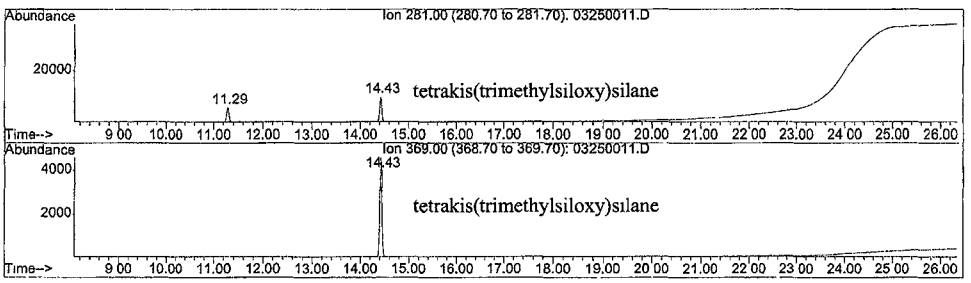


Retention Time	Area	Area %	Ratio %
281.00 amu			
11.289	866427	72.749	100.000
14.429	324553	27.251	37.459
369.00 amu			
14.432	153533	100.000	100.000

Figure 2C. Representative chromatogram of a control sample during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250011.D
Operator    : MED
Acquired    : 25 Mar 2009  22:22     using AcqMethod D4SIM
Sample Name: CTRL B
Misc Info   :
Vial Number: 11
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```



Retention Time	Area	Area %	Ratio %
281.00 amu			
11.293	183597	37.211	59.262
14.429	309804	62.789	100.000
369.00 amu			
14.430	145441	100.000	100.000

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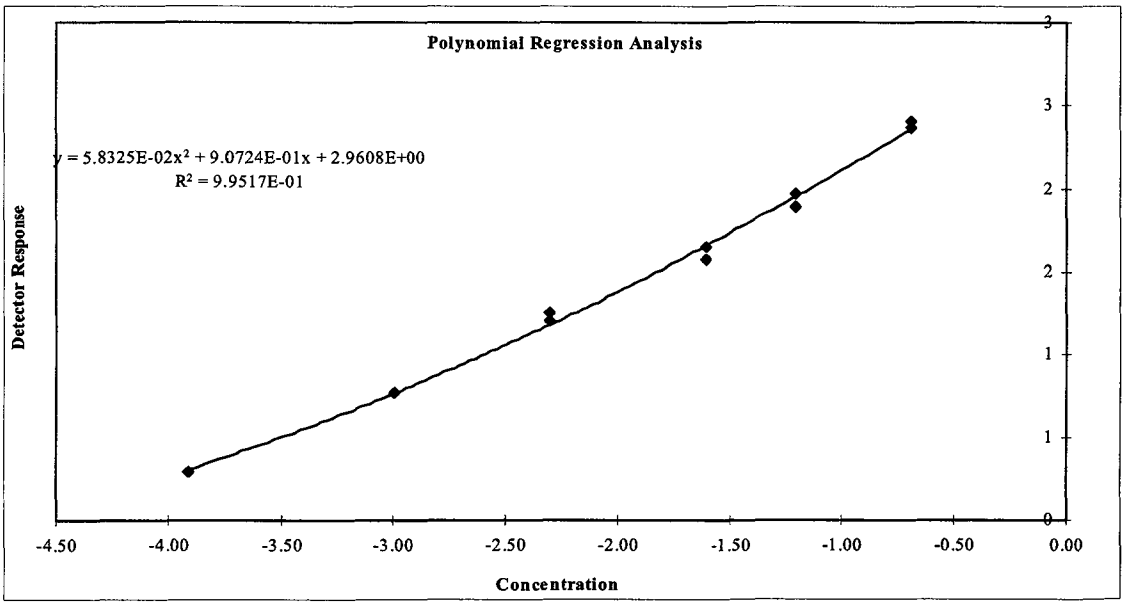
Note: The small peak observed at 11.3 minutes has an area that is below that of the lowest standard and is below the LOQ for this analysis. It is likely due to instrumental carryover from residual silicones in the system.

Figure 2D. A typical regression analysis for the calibration standards used to quantitate D4 during the method validation study.



Regression Analysis

$r^2 = 0.99517$
$y = 0.058325x^2 + 0.90724 + 2.9608$

ln Standard Concentration mg a.i./L	Standard Concentration mg a.i./L	ln Response Area	Standard Response Area	Internal Standard Response Area
-3.912	0.0200	--[a]	368592	167565
-2.996	0.0500	--[a]	444997	166994
-2.303	0.100	1.2528	599001	171133
-1.609	0.200	1.6495	905398	173970
-1.204	0.300	1.9713	1242092	172997
-0.693	0.500	2.4071	1942742	174992
-3.912	0.0200	0.2891	253442	189805
-2.996	0.0500	0.7647	412187	191858
-2.303	0.100	1.2058	601713	180176
-1.609	0.200	1.5649	963981	201573
-1.204	0.300	1.8918	1334634	201269
-0.693	0.500	2.3646	2063702	193960

[a] Standard not used due to extreme variance.

APPENDIX 3 – METHOD VALIDATION (NATURAL SEDIMENT)

SUMMARY

Methodology was validated (11 December 2008) to quantify the amount of D4 present in freshwater sediment. Recovery samples containing the test substance were extracted twice with acetonitrile and hexane. The hexane extracts were combined and diluted with hexane prior to analysis. An aliquot of the final extracts were analyzed by gas chromatography with mass selective detection and negative chemical ionization (GC-MSD/NCI).

This method was validated by fortification of freshwater sediment with D4 at nominal concentrations of 0.600, 10.0 and 100 µg a.i./g. Recoveries of D4 averaged 101 ± 18.1%. Due to instrumental carryover, the limit of quantitation (LOQ) was set at 0.600 µg a.i./g, lowest fortified sample concentration. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

EXPERIMENTAL

Equipment

1. Instrument:	Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector series 5973, Hewlett Packard autosampler, Hewlett Packard Model 7683 injector and Hewlett Packard ChemStation Version A.01.00 for data acquisition.
2. Balances:	Orion 0960, American Scientific Products SP182, Mettler PJ3000, Sartorius MA45C moisture balance
3. Shaker table:	Lab-Line orbit shaker
4. Centrifuge:	Beckman GPR
5. Laboratory equipment:	syringes, volumetric flasks, volumetric pipets, Pasteur pipets, autosampler vials, 45-mL glass vials with PTFE-lined caps, and amber glass bottles with Teflon®-lined caps

Reagents

1. Acetone:	Burdick & Jackson, reagent grade
2. Hexane:	Burdick & Jackson, reagent grade
3. Acetonitrile:	Burdick & Jackson, reagent grade
4. Nitric acid:	JT Baker, reagent grade
5. Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Synonym:	Trimethylsiloxysilicate (M_4Q)
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

PROCEDURES

Preparation of Stock Solutions

A 10.0 mg a.i./mL primary stock solution was prepared by placing 0.2516 g of D4 (0.2510 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with acetone. The 100 mg a.i./L secondary stock solution was used to fortify the low-level recovery samples. The 1000 mg a.i./L secondary stock solution was used to fortify the mid- and high-level recovery samples.

A 1.00 mg a.i./mL primary stock solution was prepared by placing 0.1007 g of D4 (0.1004 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with hexane. Two secondary stock solutions (10.0 and 100 mg a.i./mL) were prepared by placing 0.500 and 5.00 mL, respectively, of the 1.00 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with hexane. The 10.0 and 100 mg a.i./L secondary stock solutions were used to prepare calibration standards.

A 10.0 mg/mL tetrakis(trimethylsiloxy) silane internal standard stock solution was prepared by placing 0.2506 g of the internal standard in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg/L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg/mL primary internal standard stock solution in separate 50.0-mL volumetric flasks and bringing each volume with hexane and acetone, respectively. A 0.0500-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low recovery samples prior to analysis. Aliquots of 0.0200 and 0.200 mL of the 1000 mg/L secondary internal standard stock solution were added to the mid- and high-level recovery samples, respectively, prior to analysis.

All stock solutions were stored in a refrigerator in glass amber bottles fitted with Teflon®-lined caps.

Preparation of Calibration Standards

Calibration standards were prepared in hexane by fortifying with the 10.0 and 100 mg a.i./L secondary stock solutions to yield concentrations of 0.0200, 0.0500, 0.100, 0.200, 0.300 and 0.500 mg a.i./L. A 50-µL aliquot of the 100 mg/L secondary internal standard stock solution was added to the calibration standards prior to analysis. The calibration standard fortification procedure is presented in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Calibration Standard Concentration (mg a.i./L)
NA[a]	NA	100	0.0500	50.0	0.00
10.0	0.100	100	0.0500	50.0	0.0200
10.0	0.250	100	0.0500	50.0	0.0500
10.0	0.500	100	0.0500	50.0	0.100
100	0.100	100	0.0500	50.0	0.200
100	0.150	100	0.0500	50.0	0.300
100	0.250	100	0.0500	50.0	0.500

[a] NA = Not Applicable.

Sample Fortification and Preparation

All glassware used for the preparation and extraction of recovery samples was subjected to rigorous cleaning before use. The glassware was soaked and rinsed well with 20% nitric acid in water. This was followed by a water rinse to remove the nitric acid, and then a rinse with acetone to remove residual organics.

The recovery samples were prepared by fortifying freshwater sediment (5.00 g, dry weight) with D4 at concentrations of 0.600, 10.0 and 100 µg a.i./g. The low-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 100 mg a.i./L secondary stock solution. The mid- and high-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 1000 mg a.i./L secondary stock solution. Each concentration level was produced in triplicate. In addition, three recovery samples 5.00 g (dry weight) were left unfortified to serve as controls.

A 0.0500-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low recovery samples prior to analysis. Aliquots of 0.0200 and 0.200 mL of the 1000 mg/L secondary internal standard stock solution were added to the mid- and high-level recovery samples, respectively, prior to analysis. The sample fortification procedure is presented in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Sample Mass (g)	Sample Concentration (µg a.i./g)
NA	NA	100	0.0500	5.00	0.00
100	0.0300	100	0.0500	5.00	0.600
1000	0.0500	1000	0.0200	5.00	10.0
1000	0.500	1000	0.200	5.00	100

[a] NA = Not Applicable.

Extraction

All recovery samples were prepared in 45-mL glass vials with PTFE-lined caps. An aliquot (6.00-mL) of acetonitrile was added to each sample. An aliquot of hexane (12.0 mL) was added to the vials that contained both sediment and acetonitrile. The samples were placed on a shaker table at 150 rpm for 30 minutes and centrifuged at 1500 rpm for 15 minutes to separate the layers. At this point, the samples contained three layers: the top layer was hexane, the middle was acetonitrile and water and the bottom was the sediment. The upper hexane layer was transferred to 50.0-mL volumetric flasks. An additional aliquot of hexane (12.0-mL) was added to the acetonitrile and sediment in the vials. The samples were placed on a shaker table at 150 rpm for an additional 30 minutes and centrifuged at 1500 rpm for 15 minutes. The hexane extracts were combined, taken to volume with hexane and mixed well. The mid- and high-level samples were further diluted into the calibration standard range with hexane. An aliquot from each recovery sample was collected for analysis by gas chromatographic analysis with mass selective detection (GC/MSD).

ANALYSIS

GC Parameters:

Column: Varian WCOT Fused Silica, CP-SIL 8 CB, 30m x 0.25 mm x 0.25 µm

Temperature: 40 °C (initial) and held for 2.00 minutes

Ramps:

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
10.0	250	0.00
30.0	300	2.33

Run Time: 27.0 min
Injection Volume: 2.0 µL
Gas Flows: Carrier gas: Helium, at a constant flow of 1.0 mL/min
Inlet Mode: Splitless
Purge Time: on at 1.00 min at 60 mL/min
Inlet Temperature: 260 °C
Retention Time: approximately 8.0 minutes

MSD parameters

Solvent delay: 6.0 minutes

Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
297	100	quantitation D4
311	100	quantitation internal standard

Temperatures: MSD Transfer Line - 280 °C
MS Quad – 106 °C
MS Source – 150 °C

Chemical Ionization gas: Methane

Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the system was performed by programmed automated injection.

CALCULATIONS

A calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (mg a.i./L) in the calibration standards against the natural logarithm (ln) of the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the natural logarithm (ln) of the peak area of the recovery sample and the dilution factor. Equations 2, 3 and 4 were then used to calculate measured concentrations and analytical results.

(1) $\ln y = a(\ln x)^2 + b(\ln x) + c$

(2) $\ln x = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(3) $DC(x) = \text{inverse}(\ln x)$

(4) $A = DC \times DF$

where:

y	=	detector response (peak area) for analyte
a, b, c	=	regression constants
x	=	detected concentration (µg a.i./L) in the sample
C	=	constant c minus the natural log of the peak area; C = (c - lny)
DC (x)	=	detected concentration in the sample
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

(5) $\ln(Area_{MIN}) = \ln(0.5 \times A_{LS})$

(6) $\ln(LOQ_{INST}) = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(7) $LOQ_{INST} = \text{inverse}(\ln LOQ_{INST})$

(8) $LOQ = LOQ_{INST} \times DF_{CTRL}$

where:

$Area_{MIN}$	=	one half of the mean detector response (peak area) of the low concentration calibration standard (two injections)
A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
a, b, c	=	regression constants
C	=	regression constant c – $Area_{MIN}$; C = (c - ln($Area_{MIN}$))
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

RESULTS AND DISCUSSION

The mean recovery was 101% ± 18.1%, with a limit of quantitation of 0.600 µg a.i./g. Due to instrumental carryover, the LOQ was set at lowest fortified sample concentration. These parameters, while relatively constant, do vary somewhat among runs and produce small variations in the LOQ. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

Analytical results for the recovery of D4 from freshwater sediment are presented in Table 3A. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample are presented in Figure 3A, Figure 3B and Figure 3C, respectively. A typical regression analysis for D4 is presented in Figure 3D.

Table 3A. Analytical results for the recovery of D4 from freshwater sediment during the method validation study.

Sample ID	Fortified Concentration (µg a.i./g)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
Ctrl A	0.00	10.0	< 0.600	NA
Ctrl B	0.00	10.0	< 0.600	NA
Ctrl C	0.00	10.0	< 0.600	NA
Low A	0.600	10.0	0.448	74.7
Low B	0.600	10.0	0.838	140
Low C	0.600	10.0	0.687	114
Mid A	10.0	40.0	10.5	105
Mid B	10.0	40.0	9.15	91.5
Mid C	10.0	40.0	9.21	92.1
High A	100	400	93.9	93.9
High B	100	400	97.9	97.9
High C	100	400	97.4	97.4
			MEAN	**101**
			STD DEV	**18.1**
			% CV	**18.0**

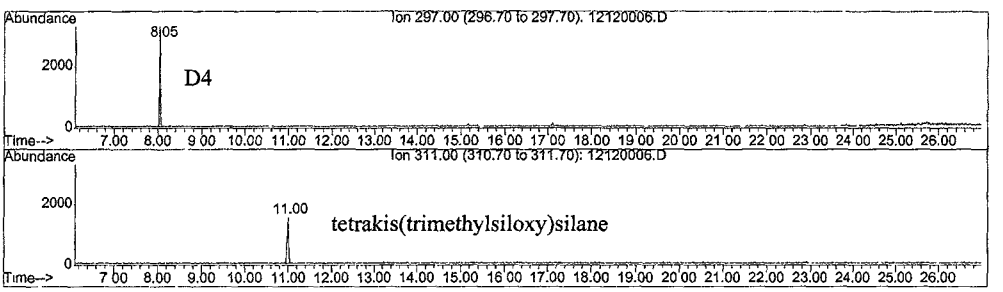
[a] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for the analysis was set at the lowest fortified sample concentration, 0.600 µg a.i./g, due to instrumental carryover.
[b] NA = Not Applicable.

Note: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Figure 3A. Representative chromatogram of a 0.200 mg a.i./L calibration standard used for quantification during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120006.D
Operator    : MED
Acquired    : 12 Dec 2008  14:46     using AcqMethod D4CISIM
Sample Name: std 0.2mg/l
Misc Info   :
Vial Number: 6
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

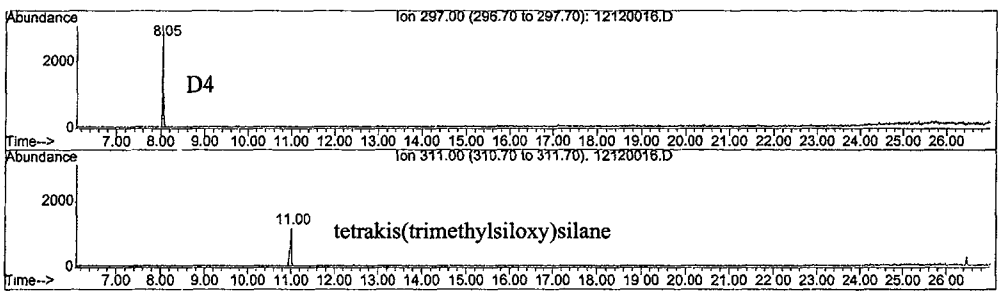


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.049	45339	100.000	100.000
311.00 amu			
10.999	37584	100.000	100.000

Figure 3B. Representative chromatogram of a 10 µg a.i./g recovery sample used during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120016.D
Operator    : MED
Acquired    : 12 Dec 2008  20:50     using AcqMethod D4CISIM
Sample Name: MID A
Misc Info   :
Vial Number: 16
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

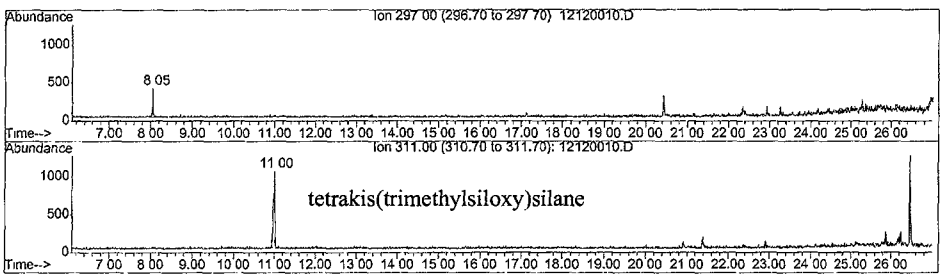


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.045	42937	100.000	100.000
311.00 amu			
11.000	28351	100.000	100.000

Figure 3C. Representative chromatogram of a control sample during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120010.D
Operator    : MED
Acquired    : 12 Dec 2008  17:12     using AcqMethod D4CISIM
Sample Name: CTRL A
Misc Info   :
Vial Number: 10
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

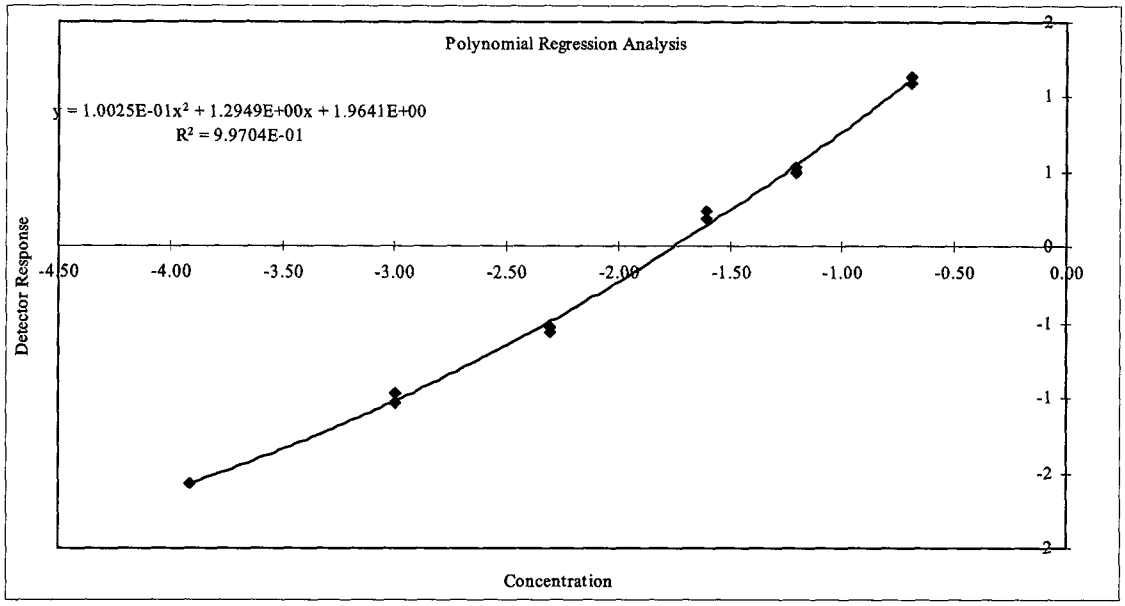


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.047	6030	100.000	100.000
311.00 amu			
11.000	27235	100.000	100.000

12120010.D Tue Dec 16 15:10:33 2008 Page 1

Note: The peak at a retention time for D4, approximately 8.0 minutes, has an area below the lowest standard and is below the LOQ for the analysis. It is likely from instrumental carryover due to residual silicones in the system.

Figure 3D. A typical regression analysis for the calibration standards used to quantitate D4 during the method validation study.



Regression Analysis

$r^2 = 0.99704$

$y = 1.0025E\text{-}01x^2 + 1.2949E+00x + 1.9641E+00$

ln Standard Concentration mg a.i./L	Standard Concentration mg a.i./L	ln Response Area	Standard Response Area	IS Response Area
-3.912	0.0200		20941	44180
-2.996	0.0500	-0.9694	15814	41692
-2.303	0.100	-0.5545	23227	40441
-1.609	0.200	0.1876	45339	37584
-1.204	0.300	0.5018	63772	38610
-0.693	0.500	1.1380	96855	31037
-3.912	0.0200	-1.5684	6585	31600
-2.996	0.0500	-1.0258	11387	31762
-2.303	0.100	-0.5259	19046	32224
-1.609	0.200	0.2356	36943	29188
-1.204	0.300	0.5285	53895	31769
-0.693	0.500	1.0920	90338	30312

APPENDIX 4 – STUDY DATA

13937.6103 28 Day Oligochaete Results

Nominal Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes Recovered	Population Increase Factor	Tare Weight (g)	Tare Weight with Oligochaetes (g)	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
Control	A	17	12	1	30	3.0	1.23856	1.28094	42.38	30(2)	46(6)
	B	18	11	1	30	3.0	1.22723	1.27147	44.24		
	C	12	12	2	26	2.6	1.22901	1.26784	38.83		
	D	16	12	0	28	2.8	1.23434	1.28822	53.88		
	E	9	24	0	33	3.3	1.22692	1.27958	52.66		
	F	14	13	4	31	3.1	1.23580	1.28268	46.88		
Solvent Control	A	13	12	3	28	2.8	1.23938	1.28117	41.79	30(3)	51(9)
	B	16	10	0	26	2.6	1.22611	1.27226	46.15		
	C	23	6	2	31	3.1	1.24618	1.29514	48.96		
	D	21	10	2	33	3.3	1.23065	1.27887	48.22		
	E	20	11	1	32	3.2	1.22551	1.29258	67.07		
	F	16	9	3	28	2.8	1.23023	1.28523	55.00		
Pooled Control					356	3.0				30(2)	49(8)
3.1	A	19	12	0	31	3.1	1.22510	1.27475	49.65	31(1)	55(6)
	B	17	11	2	30	3.0	1.22090	1.28235	61.45		
	C	19	12	0	31	3.1	1.24622	1.30544	59.22		
	D	24	8	1	33	3.3	1.23232	1.28316	50.84		
6.3	A	10	14	6	30	3.0	1.21536	1.27088	55.52	31(3)	64(6)
	B	12	15	3	30	3.0	1.22850	1.29217	63.67		
	C	9	19	0	28	2.8	1.23838	1.30551	67.13		
	D	9	22	3	34	3.4	1.23233	1.30282	70.49		
13	A	16	10	2	28	2.8	1.23063	1.27541	44.78	28(1)	50(8)
	B	14	15	0	29	2.9	1.22938	1.2721	42.72		
	C	16	12	1	29	2.9	1.2229	1.27297	50.07		
	D	17	9	0	26	2.6	1.22521	1.28635	61.14		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation

13937.6103 28 Day Oligochaete Results (Continued)

Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes Recovered	Population Increase Factor	Tare Weight (g)	Tare Weight with Oligochaetes (g)	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
25	A	10	16	0	26	2.6	1.23198	1.27633	44.35	29(3)	47(3)
	B	11	18	1	30	3.0	1.24069	1.29209	51.40		
	C	21	11	0	32	3.2	1.22556	1.27299	47.43		
	D	7	21	1	29	2.9	1.23199	1.27745	45.46		
50	A	11	10	4	25	2.5	1.22294	1.26058	37.64	26(3)	44(5)
	B	15	11	1	27	2.7	1.22783	1.27446	46.63		
	C	15	7	0	22	2.2	1.24010	1.28817	48.07		
	D	15	13	0	28	2.8	1.23125	1.27602	44.77		
100	A	11	11	0	22	2.2	1.22367	1.27355	49.88	22(1)	44(6)
	B	12	11	0	23	2.3	1.22541	1.26262	37.21		
	C	12	7	2	21	2.1	1.22857	1.27658	48.01		
	D	10	10	3	23	2.3	1.24487	1.28749	42.62		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation

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Study Title

28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to D5 Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

Data Requirement

OPPTS Draft Guideline 850.1735

Author

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Study Completed On

20 April 2009

Submitted To

CES (Centre European des Silicones, European Chemical Industry Council (Cefic))
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Springborn Smithers Laboratories
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Laboratory Project ID

Springborn Smithers Study No. 13937.6101

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The data and report prepared for **"28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to D5 Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735"** were produced and compiled in accordance with all pertinent U.S. EPA Good Laboratory Practice Regulations (40 CFR, Part 160) with the following exceptions: routine water, sediment and food contaminant screening analyses were conducted using standard U.S. EPA procedures by GeoLabs, Inc., Braintree, Massachusetts and are considered facility records under Springborn Smithers Laboratories' SOP 7.92. Since the analyses were conducted following standard validated methods, these exceptions have no impact on the study results.

SPRINGBORN SMITHERS LABORATORIES

Christian R. Picard
Study Director

4/20/09
Date

QUALITY ASSURANCE UNIT STATEMENT

The study conduct, raw data and report for **"28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to D5 Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735"** were inspected by the Quality Assurance Unit at Springborn Smithers Laboratories to determine adherence with the study protocol and laboratory standard operating procedures. This report is an accurate representation of the raw data. Dates of study inspections, study inspection types, and dates reported to the Study Director and to Management are provided below.

Inspection Date	Inspection Type	Reported to Study Director/Management
12/22/08	Protocol review	12/22/08
12/22/08	Water quality measurements	12/22/08
1/8/09	Data	1/8/09
2/6 & 2/10/09	Data	2/10/09
2/10/09	Draft report	2/10/09
2/24/09	Data & revised draft report	2/24/09
4/20/09	Final report	4/20/09

SPRINGBORN SMITHERS LABORATORIES

Kathleen T Terrell for DAA — 20 April 2009

Dorothy A. Antonioli — Date

Quality Assurance Auditor

KEY STUDY PERSONNEL

The following Springborn Smithers personnel were responsible for the conduct of the work and reporting of the study results.

Christian R. Picard	Study Director
Michael J. Bradley	Biologist I
Jennifer Donovan	Biology Technician II
Kristen Follett	Assistant Biologist
Andrew Moore	Assistant Biologist
Robyne M. Zuendel	Chemist I
Marjorie E. Dix	Senior Research Chemist
Andrea M. Soares	Technical Report Writer
Susan P. Shepherd	Director, Ecotoxicology

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SUMMARY

28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to D5 Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

SPONSOR:	CES (Centre European des Silicones, European Chemical Industry Council (Cefic))
PROTOCOL TITLE:	Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735, Springborn Smithers Laboratories Protocol No.: 062608/28-Day/Hyalella
SPRINGBORN SMITHERS STUDY NUMBER:	13937.6101
TEST SUBSTANCE:	D5, Batch No. 0000341832, CAS No. 541-02-6, reported to have a purity of 99.16%[1], was received from Dow Corning Corporation on 8 July 2008.
DEFINITIVE TEST DATES:	2 to 30 December 2008
TEST ORGANISM:	*Hyalella azteca*, 8 days old Source: Springborn Smithers culture
TEST CONDITIONS:	28-day duration, renewal of overlying water, 21 to 25 °C, illumination of 16 hours light: 8 hours darkness at 840 to 870 lux
OVERLYING WATER:	Well water pH: 7.1 Specific conductivity: 230 µmhos/cm Total hardness (as $CaCO_3$): 54 mg/L Total alkalinity (as $CaCO_3$): 20 mg/L

[1] Original characterization of the test substance yielded a purity of 99.19%. All calculations for this study are based on the original purity.

SEDIMENT: Natural freshwater sediment, collected from Glen Charlie Pond, Wareham, Massachusetts
percent organic carbon - 4.8%
particle size distribution - 83% sand, 12% silt, and 5% clay
pH - 6.0
percent moisture (1/3 bar) - 28.6%

NOMINAL SEDIMENT CONCENTRATIONS: 16, 31, 63, 125, 250 and 500 mg a.i./kg (expressed relative to sediment dry weight)

MEAN MEASURED SEDIMENT CONCENTRATIONS: 18, 28, 62, 130, 230 and 460 mg a.i./kg (expressed relative to sediment dry weight)

RESULTS: Based on mean measured sediment concentrations and amphipod survival and growth (dry weight) data, the following results were generated from this study:

Amphipod Survival (28-Day Exposure):

Lowest-Observed-Effect Concentration (LOEC)	230 mg a.i./kg
No-Observed-Effect Concentration (NOEC)	130 mg a.i./kg
28-Day LC50 (95% confidence intervals)	310 mg a.i./kg (210 to 360 mg a.i./kg)

Amphipod Growth (28-Day Exposure):

Lowest-Observed-Effect Concentration (LOEC)	> 130 mg a.i./kg
No-Observed-Effect Concentration (NOEC)	130 mg a.i./kg
28-Day EC50 (95% confidence intervals)	> 130 mg a.i./kg (NA[a])

[a] NA = Not Applicable. EC50 value was empirically estimated, therefore, corresponding 95% confidence intervals could not be calculated.

1.0 INTRODUCTION

The purpose of this study was to determine the effects of D5, applied to sediment, on the survival and growth of the sediment-dwelling freshwater amphipods (*Hyalella azteca*). The study was performed under static-renewal conditions for a period of 28 days. Exposure concentrations of D5 in sediment were measured on days 0 (test initiation), 7 and 28 (test termination). The LC50 for amphipod survival and the EC50 for amphipod growth were determined. Results of this study were used to estimate the highest concentration that had no significant ($p \leq 0.05$) effect on the test organism performance (No-Observed-Effect Concentration, NOEC), and the lowest concentration that had a significant effect on the exposed organisms (Lowest-Observed-Effect Concentration, LOEC).

The study was initiated on 7 August 2008, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental phase of the 28-day definitive test was conducted from 2 to 30 December 2008 at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. All original raw data, the protocol and the original final report produced during this study are stored in Springborn Smithers' archives at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Springborn Smithers Laboratories protocol entitled "Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735", Springborn Smithers Laboratories Protocol No.: 062608/28-Day/Hyalella (Appendix 1). The methods described in this protocol meet the testing requirements of the U.S. Environmental Protection Agency's Ecological Effects

Test Guideline (Draft) OPPTS 850.1735 Whole Sediment Acute Toxicity Invertebrates, Freshwater (U.S. EPA, 1996).

2.2 Test Substance and Internal Standard

2.2.1 Test Substance

The test substance, D5, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D5
Synonyms:	decamethylcyclopentasiloxane, Dow Corning® 1693 Fluid
Batch No.:	0000341832
CAS No.:	541-02-6
Purity:	99.19%[1]
Expiration Date:	31 December 2010

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-23) was stored at room temperature in the original container in a dark, ventilated cabinet and was used to prepare test solutions and quality control samples during testing. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance, and archival of a sample of the test substance are the responsibility of the Study Sponsor.

[1] Recharacterization of the test substance during the study resulted in a purity of 99.16%. All calculations for this study are based on the original purity.

2.2.2 Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8% (tested as 100%)
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. The internal standard was used in the quantitation of D5 in the exposure solutions and quality control samples during testing.

2.3 Test Organism

The freshwater amphipod, *Hyalella azteca,* was selected as the test organism because of their sensitivity to a variety of chemical substances in addition to being one of the guideline recommended species for freshwater benthic invertebrate tests. The amphipod is easily cultured, making it suitable for toxicity testing. The amphipods used during this study were obtained from laboratory cultures maintained at Springborn Smithers. Prior to test initiation, amphipods were maintained in 20-L glass aquaria containing approximately 15 L of culture water under flow-through conditions. The culture water was from the same source as water used as overlying water during the test.

Amphipods (8 days old) used in the exposure were collected from reproducing adult amphipods removed from the main culture tanks 9 days prior to test initiation. The adult amphipods were placed in 9.5-L aquaria (isolation tanks) containing approximately 8 L of water. Juvenile amphipods (< 24 hours old) produced by these isolated adults were then removed from the isolation tanks and pipetted into 1-L beakers containing approximately 0.80 L of laboratory dilution water. The juvenile amphipods were reared under static conditions for 8 days with

gentle oil-free aeration. During the holding period, dissolved oxygen ranged from 8.3 to 8.6 mg/L and temperature was maintained at 22 °C. No mortality was observed in the test population 48 hours prior to test initiation.

During holding and acclimation, the freshwater amphipods were fed 2.5 mL of a combination of yeast, cereal leaves and flaked fish food suspension (YCT) and 2.5 mL of *Ankistrodesmus falcatus*, a unicellular green algae, every other day. During the 28-day exposure, each replicate test vessel received 1.5 mL of YCT daily. Representative samples of the food source were analyzed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations considered toxic in any of the samples analyzed. Based on these analyses, food sources were considered to be of acceptable quality since analyte concentrations were below levels of concern.

2.4 Overlying Water

Amphipod cultures are maintained in water from the same source as the dilution water utilized during this study and have successfully survived and reproduced over multiple generations. The water used during the definitive exposure was characterized as having a total hardness and total alkalinity as calcium carbonate ($CaCO_3$) of 54 mg/L and 20 mg/L, respectively, a pH of 7.1 and a specific conductivity of 230 µmhos/cm. Representative samples of the dilution water source were analyzed for the presence of pesticides, PCBs and metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected in any of the water samples analyzed in agreement with ASTM guidelines (2002). In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration of the dilution water source was 0.69 mg/L for December 2008. The above mentioned information confirmed the acceptability of this dilution water for bioassays.

2.5 Sediment

The sediment (Springborn Smithers Batch No. 091908) used during this study was collected from Glen Charlie Pond, Wareham, Massachusetts. Prior to use and characterization, the sediment was wet-pressed through a 2.0-mm sieve to remove large particles. The sediment used in this study was characterized by Agvise Laboratories, Northwood, North Dakota, as having a percent organic carbon of 4.8%, a particle size distribution of 83% sand, 12% silt, and 5% clay, a pH of 6.0 and a percent moisture at 1/3 bar (water holding capacity) of 28.6%. The percent solids was measured by Springborn Smithers Laboratories to be 54.30%. A sample of the sediment pore water was generated from the sediment batch prior to testing and yielded a measured concentration of 2.4 mg/L as nitrogen (ammonia). This concentration is well below levels of concern for this organism. A representative sample of the sediment source was analyzed for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected at concentrations that would be considered to have an adverse impact on the results of the test.

2.6 Test Conditions

Test vessels were positioned in a water bath containing circulating water designed to maintain the test solutions at a temperature of 23 ± 1 °C. The test area was illuminated with fluorescent bulbs at an intensity range of 840 to 870 lux and a photoperiod of 16 hours light and 8 hours darkness. Light intensity was measured using a VWR Traceable light meter.

2.7 Test Concentrations

Selection of nominal D5 concentrations for the 28-day definitive toxicity test with freshwater amphipods was based on toxicity information developed at Springborn Smithers through preliminary testing. Based on the results of preliminary testing (see Section 3.1) and consultation with the Study Sponsor, the nominal D5 concentrations chosen for the definitive

study were 16, 31, 63, 125, 250 and 500 mg a.i./kg. Test concentrations are expressed relative to sediment dry weight.

2.8 Test Procedures

2.8.1 Stock Solution Preparation

A 50 mg a.i./mL primary stock solution was prepared by placing 1.2599 g of D5 (1.2497 g as active ingredient) into a 25-mL volumetric flask and bringing to volume with dimethylformamide (DMF, CAS No. 68-12-2). The resultant solution was observed to be clear and colorless with no visible undissolved test substance following preparation.

A 5.0 mg a.i./mL secondary stock solution was prepared by placing 2.5 mL of the 50 mg a.i./mL primary stock solution in a 25-mL volumetric flask and bringing it to volume with DMF. This stock solution was observed to be clear and colorless with no visible undissolved test substance following preparation.

2.8.2 Sediment Dosing Stock Solutions

Six individual dosing stock solutions were prepared in DMF for application of the test substance to the sediment. The following preparation scheme was used:

Stock Solution Used (mL)	Volume of Stock Solution Used (mL)	Volume Diluted to (mL) with DMF	Dosing Stock Concentration (mg a.i./mL)
50	9.87	10	49.4
50	4.94	10	24.7
50	2.47	10	12.4
50	1.24	10	6.20
5.0	6.12	10	3.06
5.0	3.16	10	1.58

All dosing stock solutions were observed to be clear and colorless with no visible undissolved test substance following preparation.

2.8.3 Application of Test Substance to Sediment

The method of test substance application to sediment used during this study was provided by the Study Sponsor. For each dose level, a 1.5-kg aliquot of wet sediment (0.8145 kg dry weight based on a percent solids of 54.30%) along with 4.5 L of overlying water (sediment:volume ratio of 3:1) were placed in individual glass jars. Immediately prior to dosing, the contents of each jar were shaken to suspend the sediment. An 8.25-mL volume of each dosing stock solution (prepared as in Section 2.8.2) was then added to each jar. Each jar was shaken again after dosing to distribute the test substance. After a four-hour settling period, the water was decanted from the sediment and the treated sediment was allocated to the individual test vessels.

Dosing Stock Solution Used (mg a.i./mL)	Volume Stock Used (mL)	Wet Weight Sediment Used (kg)	Dry Weight Sediment Used (kg)	Nominal Concentration (mg a.i./kg dry weight)
49.4	8.25	1.5	0.8145	500
24.7	8.25	1.5	0.8145	250
12.4	8.25	1.5	0.8145	125
6.20	8.25	1.5	0.8145	63
3.06	8.25	1.5	0.8145	31
1.58	8.25	1.5	0.8145	16

A solvent control sample was prepared in the same manner as the treated sediment by adding 1.5 kg of wet sediment (0.8145 kg dry weight based on a percent solids of 54.30%) along with 4.5 L of overlying water (sediment:volume ratio of 3:1) and 8.25 mL of DMF in a glass jar and processed in the same manner as the treated sediments. The negative control sediment group was prepared using only untreated sediment and overlying water (no test substance or solvent). The negative control and solvent control vessels were maintained under the same conditions as the treatment vessels.

2.8.4 Preparation of Test Vessels

The test chambers used in the static-renewal test were 300-mL glass vessels which were chemically cleaned prior to use and rinsed several times using dilution water. Each test vessel

had a hole cut on the top edge of the beaker which was covered with 40-mesh Nitex® screen for drainage. Each vessel contained 100 mL (approximately 4-cm layer) of sediment (equivalent to 127 g wet weight per vessel or 69.2 g dry weight per vessel) and 175 mL of overlying water. The total overlying/sediment volume was maintained at approximately 275 mL. The test chambers were labeled to identify the nominal test concentration, study number and the replicate designation.

2.8.5 Renewal of Overlying Water

During the 28-day study, the overlying water was renewed by adding two volume additions (i.e., 350 mL) per test vessel per day using an intermittent delivery system in combination with a calibrated water-distribution system (Zumwalt et al., 1994). The intermittent delivery system was calibrated to provide 1 L of water per cycle to the water-distribution system (Figure 1), which subsequently provided 50 mL of water per cycle to each replicate test chamber. The water delivery system cycled approximately 7 times per day, providing approximately 350 mL per vessel every 24 hours (i.e., approximately two overlying volume replacements per vessel per day).

The calibration of the overlying water renewal system was checked prior to test initiation and confirmed at test termination. If there was any indication during the test that the renewal system calibration had changed (e.g., system malfunction or unexplained differences in dissolved oxygen concentration or temperature in the test vessels), the calibration of the necessary renewal system components were checked. During the test, the renewal system was visually inspected at least twice daily. A complete check of intermittent delivery system function was made once daily.

2.8.6 Allocation of Sediment to Exposure Vessels

One day prior to test initiation (day -1), the treated and control sediments were allocated to the twelve replicate vessels per treatment or control (100 mL per vessel). Overlying water was gently added to each vessel and then each vessel was placed under the renewal system.

Twelve replicates were maintained for each test concentration and the controls. Eight replicates (A through H) were used to evaluate the biological response of the test organisms. The remaining four replicates (I through L) were maintained for the purpose of chemical analysis. Each replicate vessel contained ten amphipods, a total of 80 amphipods per concentration or control. The additional replicates were maintained under the same conditions and contained test organisms, but were not used to evaluate the biological response of the test organisms.

2.8.7 Test Initiation

Amphipods were added impartially to an intermediate test beaker by adding no more than two amphipods to each vessel until all beakers contained two amphipods. This procedure was repeated until each beaker contained ten amphipods. The test was initiated when each intermediate beaker of ten amphipods was added to each respective test vessel.

2.9 Test Monitoring

2.9.1 Water Quality Measurements

At test initiation and termination, temperature, dissolved oxygen concentration and pH were measured in the overlying water of each replicate vessel of each treatment level and control used for biological monitoring (A through H) during the 28-day exposure. On test days 1 through 27, temperature and dissolved oxygen were measured in one alternating replicate each day. In addition, the temperature was continuously monitored in an auxiliary vessel in the temperature controlled water bath used to house the test vessels throughout the study using a VWR minimum/maximum thermometer. Total ammonia concentration of the overlying water was

monitored at test initiation and test termination in each treatment level and control solution from a composite sample (replicates A through H). The dissolved oxygen concentration and daily temperature were measured using a Yellow Springs Instrument (YSI) Model No. 550A dissolved oxygen meter/temperature probe. The pH was measured using a YSI Model pH100 pH meter. The concentration of total ammonia was determined using an Orion (Model 720A plus) meter in combination with an Orion Ammonia Electrode Model 9512.

2.9.2 Biological Observations

All vessels were examined at test initiation and at 24-hour intervals thereafter, until test termination (day 28). Observations of mortality and abnormal behavior were made and the physical characteristics of the test samples were recorded. At test termination (day 28), the total number and growth of surviving amphipods was determined in each test vessel. The remaining sediment was sieved to remove all surviving amphipods. Growth of the surviving amphipods were determined by pooling all surviving amphipods from each replicate in a tared weighing tin and drying at 60 ± 5 °C for approximately 24 hours. The pooled amphipods were then weighed on a calibrated analytical balance to the nearest 0.01 mg. Growth as dry weight per amphipod for each replicate was calculated using the following equation:

$$G = TW/NS$$

Where:

G	=	Growth as dry weight per amphipod
TW	=	Total weight of surviving amphipods
NS	=	Number of surviving amphipods

2.10 Analytical Measurements

Prior to test initiation and before division into the replicate exposure chambers, a sample of sediment from each treatment level and control was removed and analyzed. In addition,

subsamples of the dosing stock solutions used to dose the sediments were also analyzed for test substance concentration. Results of the pretest analyses were used to judge whether sufficient quantities of D5 had been applied to the sediment.

During the in-life phase of the definitive study, sediment samples were removed and analyzed for D5 concentration on test days 0, 7 and 28. On day 0, samples were removed and analyzed from replicate I of all treatment levels and controls. On day 7, samples were removed and analyzed from replicate J of all treatment levels and controls, while on day 28, samples were removed and analyzed from replicate K of all treatment levels and controls. Overlying water samples were removed from the test vessels by decanting all of the overlying water from each vessel into a graduated cylinder. Following removal of the overlying water, the sediment was then centrifuged at approximately 1200 *g* for 30 minutes. The pore water generated by centrifugation was then removed by pipet. The sediment samples were then collected from each centrifuge tube with a stainless steel spatula.

In addition, three quality control (QC) samples were prepared at each sampling interval and remained with the set of exposure samples throughout the analytical process. These QC samples were prepared in sediment (based on sediment dry weight) at concentrations of D5 similar to the treatment level range. Results of the analyses of the QC samples were used to judge the precision and quality control maintained during the analysis of exposure solution samples.

All samples were analyzed for D5 using gas chromatography with mass selective detection and negative chemical ionization (GC-MSD/NCI) based on methodology validated at Springborn Smithers. The method validation was conducted prior to the initiation of the definitive test and established an average recovery of 109% ± 7.41% from freshwater sediment (Appendix 2). Conditions and procedures used throughout the analysis of the exposure solutions and QC samples during this study were similar to those used in the method validation studies. Defined limits of acceptance of quality control sample performance were set at 70.0 to 120%.

2.11 Statistical Analyses

2.11.1 Determination of LOEC and NOEC

At the termination of the study, data obtained on amphipod survival and growth (dry weight) were statistically analyzed to identify significant treatment-related effects. The lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) were determined. Analyses were performed using the mean replicate organism response in each treatment group rather than individual response values. All statistical analyses were conducted at the 95% level of certainty except in the case of Shapiro-Wilks' (Weber et al., 1989), Chi-Square Test (Weber et al., 1989) and Bartlett's Test (Weber et al., 1989), in which the 99% level of certainty was applied. The 99% level of certainty is preferred for these qualifying tests. The following procedures were used:

1. Determination of adverse effects on the percent survival was determined after transformation (e.g., arcsine square-root percentage) of the data.

2. A t-Test was conducted for the survival and growth data to compare the performance of the negative control organisms with that of the solvent control organisms in order to determine if there were any statistically significant positive or negative effects. During this study, the t-Test indicated no significant difference between negative control and solvent control survival and growth data. Therefore, data from all dose levels was compared to the negative control data to determine treatment level effects (U.S. EPA, 2008).

3. Chi-Square Test for normality (Weber et al., 1989) and Shapiro-Wilks' Test for normality (Weber et al., 1989) was conducted for survival data and growth data, respectively, to compare the observed sample distribution with a normal distribution. The assumption that observations are normally distributed must be

validated before subsequent analyses, following parametric procedures, can be performed. If the data are not normally distributed, then a non-parametric procedure is used for subsequent analyses. Analysis of growth data met this assumption of normal distribution.

4. As a check on the assumption of homogeneity of variance implicit in parametric statistics, data were analyzed using Bartlett's Test (Sokal and Rohlf, 1981). Growth data met the assumption for homogeneity of variance.

5. Survival data was not normally distributed, therefore, Steel's Many-One Rank Test (Steel, 1959), a non-parametric statistical procedure, was used to establish treatment effects for this endpoint. Growth data met the assumptions for both normality and homogeneity of variance, therefore, Dunnett's Test (Dunnett, 1955, 1964), a parametric statistical procedure, was used to establish treatment effects for this endpoint.

TOXSTAT® Version 3.5 (Gulley et al., 1996) was used to perform the computations. The results were used to establish, at the 95% level of certainty, the lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant difference (No-Observed-Effect Concentration, NOEC) from the negative control data.

2.11.2 LC50 Determination

The LC50 is the estimated measured sediment concentration of the test substance which produces 50% mortality in the test population of amphipods at test termination. The linear interpolation method within TOXSTAT® Version 3.5 (Gulley et al., 1996) was used to determine the LC50 value for survival.

2.11.3 EC50 Determination

The EC50 is the estimated measured sediment concentration of test substance which produces a 50% reduction in growth (measured as dry weight) in the test population of amphipods at test termination. During this study, no concentration tested resulted in ≥ 50% reduction in growth, therefore the EC50 value was empirically estimated to be greater than the highest mean measured sediment concentration that was statistically analyzed.

3.0 RESULTS

3.1 Preliminary Testing

Prior to initiation of the definitive study, amphipods were exposed to nominal D5 concentrations of 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg. The D5 was applied in the manner described and test procedures were followed as outlined in Section 2.8. In addition, a solvent control and negative control sediment were maintained. One day after allocation of the spiked sediments to the exposure vessels, amphipods were added to each exposure vessel and maintained under test conditions for a period of 28 days. Three replicate vessels, each containing 10 amphipods, were established for each treatment level and the controls.

At test termination, 97, 97, 97, 100 and 43% survival was observed among amphipods exposed to the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels, respectively. During the same period, survival of amphipods in the negative control and solvent control averaged 93 and 97%, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant difference in survival among amphipods exposed to the 500 mg a.i./kg treatment level compared to the pooled control (95% survival).

Following 28 days of exposure, dry weight among surviving amphipods exposed to the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels averaged 0.60, 0.78, 0.82, 1.09 and 1.46 mg, respectively. During the same period, dry weight among surviving amphipods in the negative

control and the solvent control averaged 0.49 and 0.72 mg, respectively. Statistical analysis (Bonferroni's t-Test) determined no significant difference in growth (dry weight) among amphipods exposed to any of the treatment levels tested compared to the pooled control (0.61 mg).

Based on these results and consultation with the Study Sponsor, nominal D5 concentrations of 16, 31, 63, 125, 250 and 500 mg a.i./kg were selected for the definitive study.

3.2 Definitive Test

3.2.1 Evaluation of Test Conditions

Results of the water quality measurements made during the definitive sediment exposure are presented in Table 1 and Table 2. Throughout the exposure period, all water quality parameters were unaffected by the sediment concentrations of D5 tested and remained within acceptable limits. Dissolved oxygen was maintained between 40 and 100% saturation (i.e., between 3.4 and 8.4 mg/L at 24 °C). Daily measurements of the temperature in the exposure solutions ranged from 22 to 24 °C. Continuous monitoring in an auxiliary vessel in the water bath used to house the test vessels established a temperature range of 21 to 25 °C throughout the definitive study.

3.2.2 Analytical Results

Analysis of the stock solution samples used to dose the test sediments ranged from 91 to 99% of the nominal fortified concentrations. These results indicated that the appropriate mass of test substance was introduced to the sediment.

All test concentrations are expressed relative to sediment dry weight. Pretest analysis of the sediment samples, following dosing and prior to allocation into the replicate exposure vessels resulted in measured concentrations ranging from 26 to 42% of the nominal treatment levels. The lower than expected pretest sample recoveries are most likely due to improper sample

mixing during sample processing and are not indicative of actual test concentrations based on the day 0 analytical recoveries.

Table 3 summarizes the D5 concentrations measured during the 28-day toxicity test. Measured sediment concentrations at day 0 in the 16, 31, 63, 125, 250 and 500 mg a.i./kg treatment levels were 14, 33, 66, 120, 170 and 390 mg a.i./kg, respectively. Measured sediment concentrations at day 7 in the 16, 31, 63, 125, 250 and 500 mg a.i./kg treatment levels were 22, 23, 63, 150, 220 and 500 mg a.i./kg, respectively, and 19, 29, 57, 140, 300 and 490 mg a.i./kg, respectively, on day 28. Based on mean measured concentrations, treatment levels were defined as 18, 28, 62, 130, 230 and 460 mg a.i./kg.

Analysis of seven of the nine quality control samples resulted in measured concentrations which ranged from 76.4 to 117% of the nominal fortified concentrations (2.00, 50.0 and 500 mg a.i./kg) and were consistent with the expectations of the methodology used (Appendix 2). Based on these results, it was determined that the appropriate quality control was maintained during the analyses of sediment samples. Two of the nine QC samples were measured at 150 and 126% recovery. QC samples can be out of the acceptable range due to a number of factors, some of which are spiking, handling or instrument errors.

3.2.3 Biological Results

A summary of survival and growth data at termination of the sediment exposure of *Hyalella azteca* to D5 is presented in Table 4 and Table 5 and is illustrated in Figure 2 and Figure 3, respectively. Following 28 days of exposure, amphipod survival in the negative control and solvent control averaged 98 and 84%, respectively. During the same period, amphipod growth in the negative control and solvent control averaged 0.49 and 0.54 mg per amphipod, respectively. Amphipod survival and growth observed in both the negative control and solvent control during this period met the minimum standard criteria established by the OPPTS Guideline (i.e., 80% survival and 0.15 mg per amphipod). As demonstrated by the negative control and solvent

control organism performance, the exposure system provided test conditions that were appropriate for promoting acceptable survival and growth of *Hyalella azteca*.

At test termination (test day 28), survival observed among amphipods in the 18, 28, 62, 130, 230 and 460 mg a.i./kg treatment levels averaged 94, 83, 98, 93, 64 and 19%, respectively. Statistical analysis (Steel's Many-One Rank Test) determined a significant difference in survival among amphipods exposed to the 230 and 460 mg a.i./kg treatment levels compared to the negative control organisms (98%). Due to the survival effect observed, the 230 and 460 mg a.i./kg treatment levels were excluded from further statistical analysis (i.e., growth).

Four replicates in this study (solvent control replicate E, 28 mg a.i./kg replicate A, 230 mg a.i./kg replicates C and E) had low percent survival that was not believed to be caused by exposure to the test substance or solvent. The low survival in these replicate vessels may have been due to human error during organism addition/recovery or poor water quality that was not captured during the daily monitoring of the test vessels throughout the exposure. The high percent survival in all other replicates within these respective groups confirms that the low survival was not toxicant or solvent related. However, data from all replicates in the exposure were included in the statistical analysis of survival and growth endpoints.

At test termination (test day 28), growth observed among amphipods in the 18, 28, 62 and 130 mg a.i./kg treatment levels was 0.51, 0.36, 0.42 and 0.46 mg, respectively. Statistical analysis (Dunnett's Test) determined a significant difference in growth among amphipods exposed to the 28 mg a.i./kg treatment level compared to the negative control organisms (0.49 mg). However, due to the lack of statistical significance at the higher treatment levels tested (i.e., 62 and 130 mg a.i./kg), this effect was not considered to be biologically relevant.

Table 6 summarizes the established endpoints for this study (LOEC, NOEC, LC50 and EC50 values) based on mean measured sediment concentrations. Based on mean measured sediment concentrations and amphipod survival, the Lowest-Observed-Effect Concentration (LOEC) and

No-Observed-Effect Concentration (NOEC) were determined to be 230 and 130 mg a.i./kg, respectively. The 28-day LC50 value for amphipod survival was determined to be 310 mg a.i./kg, with 95% confidence intervals of 210 to 360 mg a.i./kg.

Based on mean measured sediment concentrations and amphipod growth, the LOEC and NOEC were determined to be > 130 and 130 mg a.i./kg, respectively. Since no concentration tested resulted in ≥ 50% reduction, the 28-day EC50 value for amphipod growth was empirically estimated to be > 130 mg a.i./kg, the highest mean measured sediment concentration statistically analyzed.

PROTOCOL DEVIATIONS

1. The protocol states that the temperature of the test solutions will be maintained at 23 ± 1 °C by conducting the study in a water bath maintained at the appropriate test temperature. On four separate study days, the minimum temperature measured by the minimum/maximum thermometer monitoring the water bath was 21 °C. On five separate study days, the maximum temperature measured by the minimum/maximum thermometer monitoring the water bath was 25 °C. These temperatures are within the tolerance level of the test organism and did not have a negative impact on the results or interpretation of the study.

2. The protocol states that the alkalinity and specific conductance of the overlying water will range from 24 to 45 mg/L as $CaCO_3$ and from 110 to 160 µmhos/cm, respectively. During this study, the alkalinity and specific conductance of the overlying water was 20 mg/L as $CaCO_3$ and 230 µmhos/cm, respectively. The differences observed in the overlying water parameters are due to seasonal fluctuations and did not have a negative impact on the results or interpretation of the study.

3. The protocol states that the sediment will be mixed thoroughly after removal of the overlying water and an appropriate size sample of the mixed bulk sediment will then be removed with a stainless steel spatula for analysis. In this study, an appropriate size sediment sample was removed from the test vessels on days 0, 7 and 28 and centrifuged at approximately 1200 *g* for 30 minutes. After centrifuging, the pore water was decanted and removed by pipet. An appropriate size sample of sediment was then removed from the centrifuge tube with a stainless steel spatula for determination of sediment concentrations. Based on the chemical characteristics of the test substance and the study focus on sediment concentrations, this deviation did not significantly impact the outcome of the exposure.

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Table 1. Results of the water quality parameters (dissolved oxygen, temperature and pH) measured in the overlying water during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Nominal Sediment Concentration (mg a.i./kg)	Ranges		
	Dissolved Oxygen[a] (mg/L)	Temperature[ab] (°C)	pH[a]
Control	6.0 - 8.3	22 - 24	6.7 - 7.0
Solvent Control	5.1 - 8.0	22 - 24	6.8 - 7.0
16	5.2 - 7.9	22 - 24	6.8 - 7.0
31	4.8 - 8.4	22 - 24	6.8 - 7.0
63	5.0 - 7.6	22 - 24	6.9 - 7.0
125	5.0 - 7.9	22 - 24	6.7 - 7.1
250	4.8 - 7.7	22 - 24	6.7 - 7.1
500	5.0 - 7.8	22 - 24	6.8 - 7.1

[a] N = 37, based on daily measurement of each treatment level and control. Daily measurements were alternated among the eight replicate test vessels (A through H).

[b] Continuous measurement in an auxiliary vessel maintained in the water bath used to house the test vessels established a temperature range of 21 to 25 °C.

NOTE: Measurements have been rounded to two significant figures.

Table 2. Results of the water quality parameters (total hardness, total alkalinity, specific conductance and ammonia) measured in the overlying water during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Nominal Sediment Concentration (mg a.i./kg)	Total Hardness as $CaCO_3$ (mg/L)		Total Alkalinity as $CaCO_3$ (mg/L)		Specific Conductivity (µmhos/cm)		Ammonia as Nitrogen (mg/L)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Control	48	48	12	16	290	300	0.45	≤ 0.10
Solvent Control	52	52	12	20	290	300	0.44	≤ 0.10
16	52	52	12	20	290	300	0.46	≤ 0.10
31	52	52	12	18	280	300	0.81	≤ 0.10
63	48	48	12	18	290	300	0.53	≤ 0.10
125	48	52	10	18	290	300	0.49	≤ 0.10
250	48	52	10	18	290	300	0.80	≤ 0.10
500	44	52	10	18	290	300	0.53	≤ 0.10

NOTE: Values have been rounded to two significant figures.

Table 3. Concentrations of D5 (expressed relative to sediment dry weight) measured in sediment samples during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Nominal Sediment Concentration (mg a.i./kg)	Measured Sediment Concentration (mg a.i./kg)				Mean Percent Recovery[d]
	Day 0[a]	Day 7[b]	Day 28[c]	Mean[d]	
Control	< 2.0[e]	< 2.0	< 2.0	NA[f]	NA
Solvent Control	< 2.0	< 2.0	< 2.0	NA	NA
16	14	22	19	18	110
31	33	23	29	28	91
63	66	63	57	62	99
125	120	150	140	130	110
250	170	220	300	230	92
500	390	500	490	460	92
QC[g] #1 2.00	3.00 (150)[h]	2.34 (117)	1.53 (76.4)		
QC#2 50.0	51.4 (103)	63.0 (126)[h]	49.0 (98.0)		
QC#3 500	535 (107)	520 (104)	550 (110)		

[a] Analytical samples were removed from replicate I.
[b] Analytical samples were removed from replicate J.
[c] Analytical samples were removed from replicate K.
[d] Mean measured and percent recovery values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[e] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for this analysis was set at 2.0 mg a.i./kg, the lowest validated concentration.
[f] NA = Not Applicable.
[g] QC = Quality Control sample. Percent of nominal is presented in parentheses below the measured results.
[h] Percent recovery for this QC sample is outside of the acceptable range (i.e., 70.0 to 120%, Appendix 2).

Table 4. Mean measured sediment concentrations tested and amphipod survival and growth (dry weight) in each replicate vessel at test termination of the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	Test Day 28	
		Mean Percent Survival	Mean Dry Weight Per Amphipod Larvae (mg)
Control	A	100	0.55
	B	100	0.62
	C	90	0.48
	D	100	0.50
	E	90	0.41
	F	100	0.51
	G	100	0.46
	H	100	0.41
Solvent Control	A	100	0.47
	B	80	0.46
	C	90	0.57
	D	100	0.58
	E	10	0.81
	F	90	0.50
	G	100	0.44
	H	100	0.48
18	A	90	0.50
	B	90	0.54
	C	90	0.60
	D	100	0.47
	E	100	0.52
	F	90	0.43
	G	100	0.45
	H	90	0.54
28	A	20	0.29
	B	80	0.44
	C	80	0.33
	D	100	0.35
	E	100	0.34
	F	90	0.25
	G	100	0.49
	H	90	0.38

[a] NA = Not Applicable. No surviving organisms were observed in this replicate.

Table 4. (Continued). Mean measured sediment concentrations tested and amphipod survival and growth (dry weight) in each replicate vessel at test termination of the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	Test Day 28	
		Mean Percent Survival	Mean Dry Weight Per Amphipod Larvae (mg)
62	A	100	0.36
	B	100	0.51
	C	100	0.43
	D	100	0.40
	E	80	0.31
	F	100	0.47
	G	100	0.41
	H	100	0.50
130	A	90	0.54
	B	90	0.42
	C	90	0.45
	D	100	0.42
	E	80	0.47
	F	100	0.48
	G	90	0.48
	H	100	0.47
230	A	80	0.32
	B	70	0.45
	C	10	0.31
	D	80	0.36
	E	0	NA[a]
	F	100	0.32
	G	90	0.48
	H	80	0.49
460	A	30	0.49
	B	40	0.45
	C	0	NA
	D	10	0.06
	E	0	NA
	F	40	0.58
	G	20	0.38
	H	10	0.43

[a] NA = Not Applicable. No surviving organisms were observed in this replicate.

Table 5. Mean percent survival and mean dry weight during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Mean Measured Sediment Concentration (mg a.i./kg)	Test Day 28	
	Mean Percent Survival (SD[a])	Mean Dry Weight Per Larvae in mg (SD)
Control	98 (5)	0.49 (0.07)
Solvent Control	84 (31)	0.54 (0.12)
18	94 (5)	0.51 (0.06)
28	83 (27)	0.36 (0.08)[b]
62	98 (7)	0.42 (0.07)
130	93 (7)	0.46 (0.04)
230	64 (37)[c]	0.39 (0.08)[d]
460	19 (16)[c]	0.40 (0.18)[d]

[a] SD = Standard Deviation.
[b] Significantly reduced compared to the negative control, based on Dunnett's Test. However, due to the lack of statistical significance at the higher treatment levels tested (i.e., 62 and 130 mg a.i./kg), this effect was not considered to be biologically relevant.
[c] Significantly reduced compared to the negative control, based on Steel's Many-One Rank Test.
[d] This treatment level was excluded from statistical analysis for growth due to the survival effect observed.

Table 6. Established endpoints for the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.

Based on Mean Measured Sediment Concentrations (mg a.i./kg)		
Endpoint	**Amphipod Survival**	**Amphipod Growth**
Lowest-Observed-Effect Concentration (LOEC)	230	> 130
No-Observed-Effect Concentration (NOEC)	130	130
28-Day LC50/EC50 (95% confidence intervals)	310 (210 to 360)	> 130 (NA[a])

[a] NA = Not Applicable. EC50 value was empirically estimated; therefore, corresponding 95% confidence intervals could not be calculated.

Figure 1. Diagram of water-distribution system (Zumwalt, 1994).

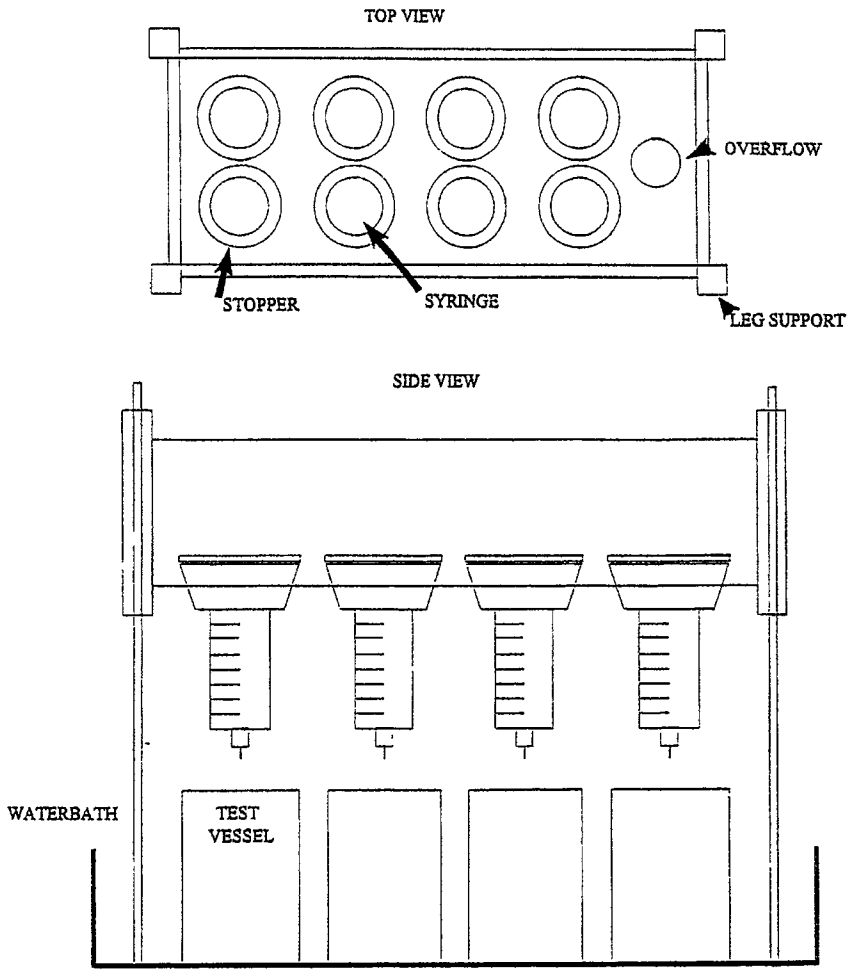
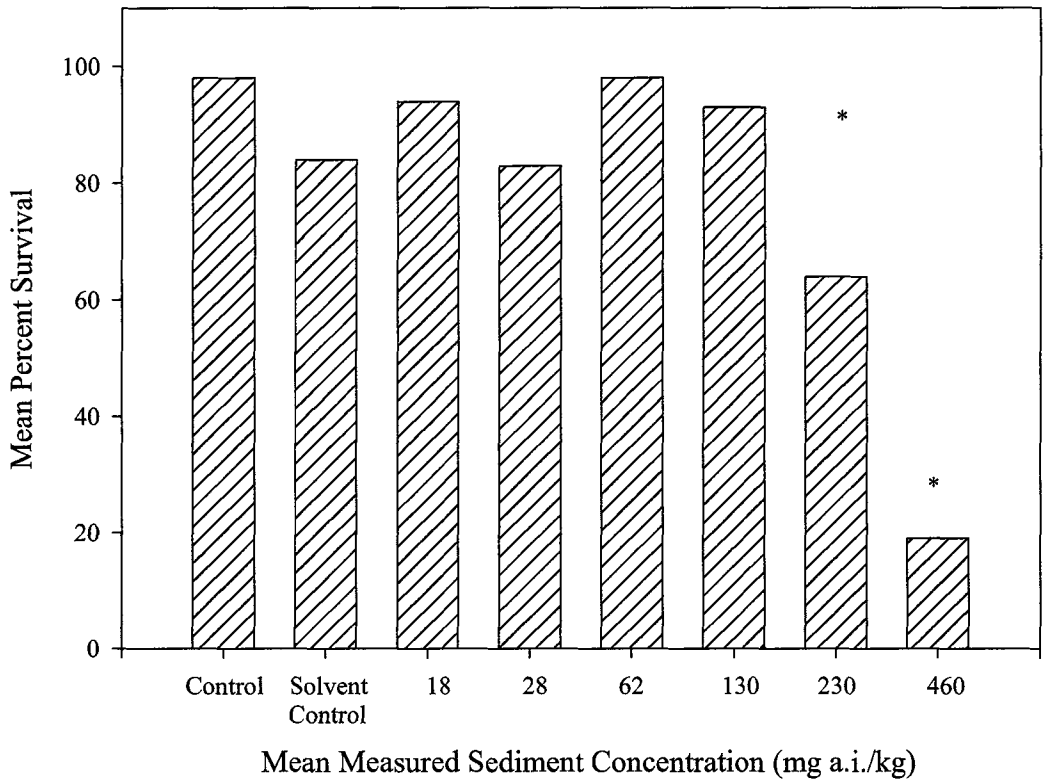
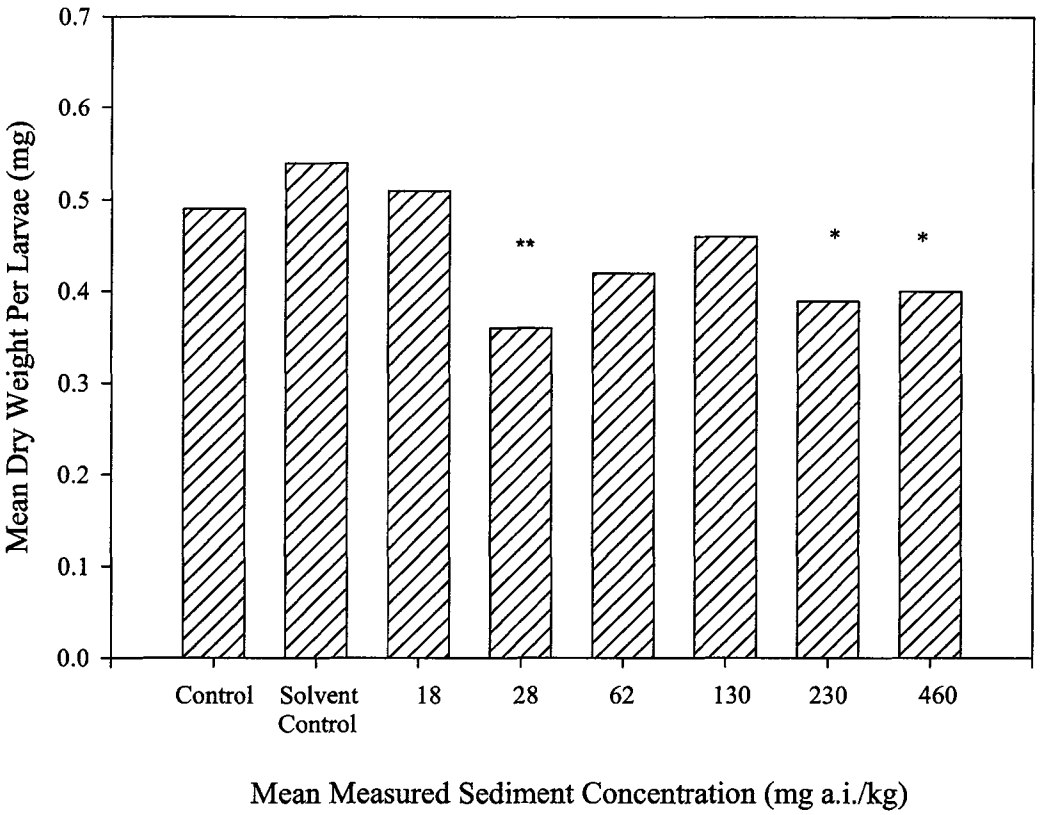


Figure 2. Mean percent survival during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.



*Statistically different compared to the control, based on Steel's Many-One Rank Test. Treatment level was excluded from statistical analysis of growth due to the survival effect observed.

Figure 3. Mean amphipod growth (dry weight) during the 28-day static-renewal exposure of freshwater amphipods (*Hyalella azteca*) to D5 applied to sediment.



*This treatment level was excluded from statistical analysis of growth due to the survival effect observed.
**Statistically different compared to the control, based on Dunnett's Test. However, due to the lack of statistical significance in the higher treatment levels tested, this effect was not considered to be biologically relevant.

APPENDIX 1 - STUDY PROTOCOL

SPRINGBORN SMITHERS
LABORATORIES
Massachusetts Research Center
790 Main Street • Wareham, MA • 02571-1037 • Phone: (508) 295-2550 • Fax (508) 295-8107

TEST PROTOCOL

PROTOCOL TITLE: Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

TO BE COMPLETED BY THE STUDY SPONSOR:		
Study Sponsor:	CES (Centre European des Silicones, European Chemical Industry Council (Cefic)	
Address:	Avenue E. van Nieuwenhuyse 4, B-1160 Brussels, Belgium	
		Phone: 989-496-8226
Study Monitor:	Kurt Drottar	**E-mail:** k.drottar@dowcorning.con
Sponsor Protocol/Project No.:		
Test Substance Name: D5		
Purity: 99.19%	**Lot #:** 0000341832	
Analytical Standard:		
Purity:	**Lot #:**	
Additional Comments and Modifications:		
Sponsor Approval:		**Date:** 8/6/08

TO BE COMPLETED BY SPRINGBORN SMITHERS LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Springborn Smithers Laboratories 790 Main Street, Wareham, MA 02571-1037

Study Director: Christian Picard Study No.: 13937-6101

Test Concentrations: *

Proposed Experimental Dates: (Start) * (Termination) *

Study Director Signature **Study Initiation Date** 8/7/08

* To be provided by protocol amendment, if applicable.

Other Locations: 2900 Quakenbush Road, P.O. Box 620 • Snow Camp, North Carolina 27349 • Phone: (336) 376-0141 • Fax. (336) 376-0145
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Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

1.0 OBJECTIVE

The purpose of this test will be to determine the impact of a test substance to the freshwater, sediment-dwelling amphipod (*Hyalella azteca*), under static-renewal conditions for 28 days. The study will assess the impact of the test substance on the survival and growth of the amphipods. The methods described in this protocol are designed to meet the testing requirements of the U.S. Environmental Protection Agency's Ecological Effects Test Guideline (Draft) OPPTS 850.1735 Whole Sediment Acute Toxicity Invertebrates, Freshwater (U.S. EPA, 1996).

2.0 MATERIALS AND METHODS

2.1 Chemical System

2.1.1 Test Substance

Upon arrival at Springborn Smithers Laboratories, the test and reference substance(s) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

2.1.2 Test Substance Concentration Selection

Test substance concentrations will be based on the results of a 28-day preliminary range-finding test. Concentrations of 500, 50, 5.0, 0.5, 0.05 mg/kg dry weight plus controls will be selected for the static renewal range-finding test. A negative control consists of overlying water and sediment without test substance.

For the definitive test, five test concentrations and a negative control will be used. Each test substance concentration will be 50% of the next higher concentration of the test substance. The range of concentrations will be selected to determine a No-Observed-Effect Concentration (NOEC) and a Lowest-Observed-Effect Concentration (LOEC) based on the survival and growth of the amphipods. If possible, based on the concentrations selected, an LC50 and EC50 will also be calculated.

2.1.3 Solvent Control

An organic solvent (N,N-dimethylformamide) will be used as a carrier to solubilize the test substance. The solvent volume utilized will remain constant across the test concentration series. A solvent control will be included in each test and will consist of sediment plus the equivalent volume of solvent used during the application of the test solutions to the sediment. The solvent volume will be kept as low as possible.

2.1.4 Application of Test Substance to Sediment

The test substance will be weighed on an analytical balance for which a calibration log is maintained. A Chemical Usage Log will be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test substance is used. The amount of test substance will be applied to the sediment according to the following formula:

$$Sediment\ Concentration\ (e.g., mg\ a.i./kg) = \frac{T.S.}{S.A. \times D.W.}$$

where:

T.S. = test substance (mg a.i.)
S.A. = sediment amount (kg
D.W. = (percent dry weight of sediment) ÷ 100

The test substance will be applied by the following method:

For each dose level, a glass jar will be filled with natural of sediment followed by approximately 3 times the volume of overlying water, to give a water-volume to sediment-volume ratio of 3:1. The jar contents will be shaken to suspend the sediment immediately prior to dosing individually with a solution containing decamethylcyclopentasiloxane, D_5, in N,N-dimethylformamide (DMF). The jar will be shaken again after dosing with D_5 to distribute the test substance. After settling for 4 hours, the water will be decanted from the sediments. The sediment will be stirred to assure homogeneity and then treated sediment will be allocated to individual test vessels.

2.2 Test System

2.2.1 Species

The freshwater invertebrate, *Hyalella azteca,* is the species used in this test. Test organisms will be 7 to 10 days old at initiation of the test. Amphipods used in the exposure will be the young amphipods produced by adult amphipods removed from

isolation tanks 7 to 10 days prior to test initiation. The adult amphipods are placed in 9.5-L aquaria (isolation tank) with approximately 8 L of water. Young produced by these isolated adults will then be removed from the isolation tanks and pipetted into 1-L beakers containing approximately 0.80 liters of water until test initiation. Amphipods will not be used if > 10% mortality is observed during the 48 hours prior to test initiation.

2.2.2 Justification of Test System

Characteristics which make this test organism suitable for this acute toxicity test are their ease of handling and their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater invertebrate species.

2.2.3 Origin

Hyalella azteca cultures will be maintained at Springborn Smithers Laboratories. Amphipods will be cultured in 20-L glass aquaria (containing approximately 15 L of culture water) under flow-through conditions. Water used to culture the amphipods is similar to the overlying water used during the 28-day test. Culture water will be maintained at 23 ± 1 °C.

2.2.4 Feeding

While being maintained in the culture prior to the test, adult and juvenile amphipods will be fed once daily. They will be fed a combination of Yeast, Cerophyl® and flaked fish food suspension (YCT) and a unicellular green algae *Ankistrodesmus falcatus*. During testing, 1.5 mL of YCT Suspension will be added daily to each test vessel. A sample of the food source will be periodically analyzed using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP 7.92, for the presence of pesticides, PCBs and selected toxic metals. If food collects on the sediment surface during testing, feeding may be suspended for one or more days.

2.2.5 Handling

Wide-bore pipets will be used to transfer the amphipods, taking care to minimize possible stress due to handling. Amphipods that are damaged or dropped during transfer will not be used.

2.3 Physical System

2.3.1 Sediment

The test will be conducted using natural sediment of medium organic carbon (OC) content, between 2 and 5% OC. The freshwater sediment used in the study will be collected from a site known to be free of contamination. The sediment will be wet pressed through a 2 mm sieve to remove large particles, and will be characterized for total organic carbon content, pH, percent sand, silt, clay (particle size distribution) and percent water holding capacity by AGVISE Laboratories, Northwood, North Dakota. In addition, the ammonia concentration in the sediment pore water will be analyzed prior to the use of the sediment. Periodic analysis of representative samples of the sediment source will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree,

Massachusetts, in accordance with Springborn Smithers' SOP 7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the test organisms.

2.3.2 Test Containers

The test chambers used in the static-renewal test will be 300-mL beakers. Each test vessel will have a notch cut on the top edge of the beaker and will be covered with 40-mesh Nitex® screen for drainage. Each vessel will contain 100 mL (an approximate 4-cm layer) of sediment and 175 mL of overlying water. The overlying/sediment volume will thus be maintained at approximately 275 mL. The average wet weight of sediment added to all test vessels will be determined by randomly selecting three replicate test vessels from each treatment level and control and weighing the mass of sediment added to each vessel on test day –1. The test chambers will be labeled to identify the treatment/control, study number, and the replicate designation.

2.3.3 Replication and Control of Bias

Twelve replicates will be included with each test concentration and control. Eight replicates (A through H) will be used to evaluate the biological response of the test organisms. The other four replicate vessels (I through L) will be maintained for the purpose of chemical analysis. Each replicate vessel for monitoring the biological response will contain ten individuals, a total of 80 amphipods per concentration or control. The additional replicates will be maintained under the same conditions and contain test organisms, but will not be used to evaluate the biological response of the test organisms. Amphipods will be added impartially to an intermediate test beaker by adding no more than two amphipods to each vessel until all beakers contain two amphipods. This procedure will be repeated until each beaker contains ten amphipods. The test will be initiated when each intermediate beaker of amphipods is added to each respective test vessel.

2.3.4 Overlying Water

Overlying water will consist of unadulterated water from a 100-meter bedrock well, supplemented on demand with untreated Town of Wareham well water, and will be characterized as soft water with the following typical parameter ranges:

- total hardness of 30 - 60 mg/L as $CaCO_3$
- total alkalinity of 25 - 45 mg/L as $CaCO_3$
- pH of 6.9 to 7.7
- specific conductance of 110 to 160 micromhos/cm

Total hardness, total alkalinity, pH and specific conductance of the overlying water source will be monitored weekly at a central location in the laboratory to assure that these parameters are within the normal acceptable ranges. Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1995). Periodic analysis of representative samples of the dilution water source will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP

7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the test organisms.

2.3.5 Renewal of Overlying Water

During the 28-day study, the overlying water will be renewed by adding two volume additions (i.e., 350 mL) per day using an intermittent delivery system in combination with a calibrated water-distribution system (Zumwalt et al., 1994). The intermittent delivery system will be calibrated to provide 1 liter of water per cycle to the water-distribution system, which subsequently provides 50 mL of water per cycle to each replicate test chamber. The water delivery system cycles 7 times per day, providing 2 volume additions every 24 hours. Delivery of two volume replacements per day is sufficient to provide consistent and acceptable water quality characteristics throughout the duration of the 28-day exposure.

The calibration of the renewal system will be checked prior to test initiation and at test termination. If there is any indication during the test that the renewal system calibration has changed (e.g., renewal system malfunction or unexplained differences in dissolved oxygen concentration or temperature in the test vessels), calibration of the necessary renewal system components will be checked. During the test, the renewal system will be visually inspected at least twice daily.

2.4 Test Conditions

2.4.1 Temperature

Water temperature of the test solutions will be maintained at 23 ± 1 °C by conducting the test in a temperature-controlled room or water bath maintained at the appropriate test temperature. Temperature will be monitored continuously in one test solution by using a minimum-maximum thermometer. Readings of temperature extremes will be recorded daily.

2.4.2 Lighting

The test will be conducted in a light controlled laboratory. The test will be illuminated to a light intensity of 500 to 1000 lux using fluorescent bulbs. The light intensity will be measured once during the test. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer.

2.4.3 Dissolved Oxygen

Total dissolved oxygen should be between 40% and 100% of saturation for the duration of the test. For contingency purposes, aeration (with oil free air) may be initiated to maintain the dissolved oxygen concentration at or above 40% of saturation.

2.4.4 Test Initiation

The day before test initiation (day -1) the treated sediments and control sediments will be added to the replicate test vessels and the overlying water will be added. The water

will be added gently to prevent resuspension of the sediment layer in the water column. This allows the sediment and water to equilibrate prior to addition of the test organisms.

Amphipods (7-10 days old) will be removed from the cultures. The test will be initiated (day 0) when all vessels contain ten amphipods (eighty per test concentration and controls).

2.5 Sampling And Observations

2.5.1 Measurement of Test Substance Concentration

Prior to test initiation and before the sediment is allocated to the replicate test vessels, a sample of sediment from each treatment level and controls will be removed and analyzed. In addition, a sample of the stock solution used to apply the test substance will be sampled and analyzed.

During the in-life phase, samples of the sediment from each treatment level and controls will be removed on test day 0, test day 7 and at test termination (test day 28) for determination of test substance concentration. The sample volume of sediment will be dependent upon the analytical methodology employed for determination of test substance concentrations. Three quality control (QC) samples will be prepared at each sampling interval and stored and analyzed with the set of study samples. The QC samples will be prepared in sediment at test substance concentrations similar to the treatment level range. Results of these analyses indicate the accuracy of the analytical method for measuring test substance concentration at each sampling period. The analytical method used to measure test substance concentrations in the exposure solutions will be validated by Springborn Smithers Laboratories at the expected nominal concentration range prior to test initiation.

2.5.2 Sampling Procedures

The entire volume of overlying water will be removed from each test vessel carefully by decanting or pipetting. After removal of the overlying water the sediment will be mixed thoroughly. An appropriate size sample of the mixed bulk sediment will then be removed with a stainless steel spatula for determination of sediment test substance concentrations.

2.5.3 Water Quality Measurements

At test initiation and test termination, temperature, dissolved oxygen (DO) concentration and pH will be measured in the overlying water and recorded for each test vessel (replicates A through H). On test days 1 through 27, temperature and dissolved oxygen will be measured and recorded in one alternating replicate each day. Total hardness, alkalinity, specific conductance and total ammonia concentration will be determined in the overlying water at test initiation and test termination in a composite sample from replicates A through H of each treatment level and control.

2.5.4 Biological Observations

Survival and growth (dry weight) of the amphipods will be determined in replicates A through H for each treatment level plus controls on test day 28 by sieving the sediment to remove all surviving amphipods. The growth of surviving amphipods will be recorded at test termination by pooling all surviving amphipods from each replicate vessel and drying at 60 ± 5°C for 24 hours. Pooled amphipods will be weighed on a calibrated analytical balance to the nearest 0.01 mg.

2.5.5 Acceptability Criteria

Mean survival and growth of the amphipods in the laboratory controls must be ≥ 80% and ≥ 0.15 mg per amphipod, respectively, at test day 28.

3.0 STATISTICAL ANALYSES

3.1 Endpoints

The endpoint used for determination of significant effects by statistical evaluation is survival and growth of the amphipods. All concentration-effect relationships will be based on the nominal or measured concentrations of test substance.

3.2 Statistical Methods (Determination of LOEC/NOEC)

If a solvent is used as a carrier for the test substance, a t-Test ($p \leq 0.05$) will be used to compare the survival results of the solvent control to the control data. If the data are similar, they will be pooled for further analyses. If the data are not similar, the remaining statistical analyses will be performed comparing the treatment data to the solvent control data.

The data will be tested for normality and homogeneity of variance using Shapiro-Wilk's Test and Bartlett's Test. If the data passes these two tests, then a parametric method will be used to evaluate the results of test, e.g., Dunnett's Test. If the data fails the test for normality and homogeneity of variance, then a non-parametric method will be used to evaluate the results of the test, e.g., Dunn's (Sokal and Rohlf, 1981) or Steel's One-Many Rank Test (Weber *et al*, 1989). If necessary, mean values will be transformed using square root, arcsine square root, or log conversion procedures. The Lowest-Observed-Effect-Concentration (LOEC) is defined as the lowest test concentration that shows a statistically significant effect and the No-Observed-Effect-Concentration (NOEC) is the highest test concentration that shows no statistically significant difference from the control.

3.3 Transformations

Transformation of data will be performed with data representing endpoint estimates obtained as a proportion (e.g., survival). Prior to analyzing data of this type, the observed proportion in each vessel will be transformed by using the arcsine square-root transformation.

3.4 LC50/EC50 Calculation

The LC50 is the estimated measured concentration of the test substance in sediment that produces 50% mortality in the test population of amphipods at test termination. The EC50 is the estimated measured concentration of the test substance in sediment that produces a 50% reduction in growth of amphipods at test termination. A computer program will be used to estimate the LC50 and EC50. The method selected and reported will be determined by the database (i.e., presence or absence of 100% response, number of partial responses, etc.).

4.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

5.0 REPORTING

The raw data and final draft of the report will be reviewed by the Quality Assurance Unit and the Study Director. Chemical and water quality measurements will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The draft report will be initially submitted to the Study Sponsor for review. Upon acceptance by the Sponsor, the final report will be submitted. The final report will meet the formatting requirements of EPA's PR Notice 86-5. All reports will include, but will not be limited to, the following information:

- The study number from Springborn Smithers Laboratories and Sponsor Study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program Coordinator (if applicable), Study Director and Principal Investigator.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).
- Characterization and origin of the dilution water.
- Characterization and preparation of the sediment.
- Scientific name of the test organisms, source, age and culturing information.
- Test container volume, sediment and water volume, number of replicates used per concentration, and number of amphipods used per treatment.
- Description of exposure system and application of test substance to sediment.

- Test temperatures, dissolved oxygen concentration, and pH; and photoperiod and light intensity used, as well as specific conductance, total ammonia, total alkalinity and total hardness measured.
- Observations of insolubility of the test substance, including the test levels and when observed.
- Definition of criteria used to determine the sublethal effects, and general observations on non-quantifiable effects.
- Number of surviving amphipods in each treatment at each observation period, in tabular form.
- Description or reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- If applicable, means and standard deviations of measured concentrations of the test compound, as well as nominal test concentrations.
- The 28-day LC50 with 95 percent confidence limits for survival of amphipods.
- The Lowest-Observed-Effect Concentration (LOEC) tested based on statistical analyses.
- The No-Observed-Effect Concentration (NOEC) tested based on statistical analyses.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the protocol, raw data and final report.

6.0 PROTOCOL CHANGES

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any. If necessary, amendments other than the one providing the information required by page one of this protocol, may initially be verbally authorized, followed by Springborn Smithers' written documentation. In such cases, the effective date of the amendment will be the date of verbal authorization.

7.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records, and reports will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide, Fungicide, and Rodenticide Act (40 CFR, Part 160).

8.0 REFERENCES

APHA, AWWA, WPCF. 1995. Standard Methods for the Examination of Water and Wastewater. 19th Edition, Washington, DC.

Ditsworth, G. R., D. W. Schults, J. K. P. Jones. 1990. Preparation of Benthic Substrates for Sediment Toxicity Testing. Environmental Toxicology and Chemistry. Vol. 9, pp. 1523-1529.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. 2nd Edition. W.H. Freeman and Company, New York. 859 pp.

Weber, C.I. et al. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

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U.S. EPA. 1996. Office of Prevention, Pesticides and Toxic substances. Ecological Effects Test Guideline, OPPTS 850.1735. Whole Sediment Acute Toxicity Invertebrates, Freshwater. "Public Draft" EPA 712-C-96-354 April 1996. U.S. Environmental Protection Agency. Washington D.C.

Zumwalt, D.C., F.J. Dwyer, I.E. Greer and C.G. Ingersoll. 1994. A water-renewal system that accurately delivers small volumes of water to exposure chambers. Environmental Toxicology and Chemistry. pp. 1311-1314.

SPRINGBORN SMITHERS
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PROTOCOL AMENDMENT

Amendment No.: 1

Effective Date: 14 November 2008

Protocol Title: Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

Protocol Number: 062608/28-Day/Hyalella

Species: *Hyalella azteca*

Study Sponsor: Cefic

Test Substance: D5

Study No.: 13937.6101

Amendment:

1. Protocol Cover Page, Study Specific Information

The following information is being provided as requested on the protocol cover page:

Internal Standard: Tetrakis (Trimethylsiloxy) Silane Lot Number: 8D-12648 Purity: 99.8%

Test Concentrations: 500, 250, 125, 63, 31 and 16 mg/kg plus controls

Proposed Experimental Dates: start: 2 December 2008 termination: 30 December 2008

2. Page 2, Section 2.12 Test Substance Concentration and Selection

The protocol states that five concentrations and controls will be used for the definitive test. In this study, six concentrations and controls will be used for the definitive test.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R Picard — 12/4/08
Springborn Smithers Study Director — Date

Study Sponsor Representative — 12/4/08
Date

Other Locations: 2900 Quakenbush Road, P O. Box 620 • Snow Camp, North Carolina 27349 • Phone (336) 376-0141 •Fax (336) 376-0145
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PROTOCOL AMENDMENT

Amendment No.: 2

Effective Date: 5 January 2009

Protocol Title: Protocol for Conducting a 28-Day Toxicity Test Exposing Freshwater Amphipods (*Hyalella azteca*) to a Test Substance Applied to Sediment Under Static-Renewal Conditions Following OPPTS Draft Guideline 850.1735

Protocol Number: 062608/28-Day/Hyalella

Species: *Hyalella azteca*

Study Sponsor: Cefic

Test Substance: D5

Study No.: 13937.6101

Amendment:

1. Page 8, Section 3.2 – Statistical Methods (Determination of LOEC/NOEC)

The protocol states that if the negative control and solvent control data are statistically similar, this data will be pooled for further statistical comparisons. The protocol also states that if the negative control and solvent control data are statistically different, the remaining statistical analyses will be made comparing the treatment data to the solvent control data. In this study, all statistical comparisons for each biological endpoint will be made against the negative control data regardless of whether the control and solvent control data are statistically similar.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R. Picard
Springborn Smithers Study Director

1/14/09
Date

Study Sponsor Representative

1/14/09
Date

Other Locations: 2900 Quakenbush Road, P.O Box 620 • Snow Camp, North Carolina 27349 • Phone: (336) 376-0141 •Fax: (336) 376-0145
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APPENDIX 2 - ANALYTICAL METHODOLOGY

SUMMARY

Methodology was validated (13 November 2008) to quantify the amount of D5 present in freshwater sediment. Recovery samples containing the test substance were extracted twice with acetone in hexane. The hexane extracts were combined and diluted with hexane prior to analysis. An aliquot of the final extracts were analyzed by gas chromatography with mass selective detection and negative chemical ionization (GC-MSD/NCI).

This method was validated by fortification of freshwater sediment with D5 at nominal concentrations of 2.00, 50.0 and 500 µg a.i./g. Recoveries of D5 averaged 109 ± 7.41% with a limit of quantitation of 0.501 µg a.i./g. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%.

EXPERIMENTAL

Equipment

1. Instrument:	Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector series 5973, Hewlett Packard autosampler, Hewlett Packard Model 7683 injector and Hewlett Packard ChemStation Version A.01.00 for data acquisition.
2. Balance:	Mettler Toledo AB204 and Sartorius MA-45 moisture balance
3. Shaker table:	Lab-Line orbit shaker 0189
4. Centrifuge:	Beckman GPR
5. Laboratory equipment:	syringes, volumetric flasks, volumetric pipets, Pasteur pipets, autosampler vials, 45-mL glass vials with PTFE-lined caps, and amber glass bottles with Teflon®-lined caps

Reagents

1. Acetone:	Burdick & Jackson, reagent grade
2. Hexane:	Burdick & Jackson, reagent grade
3. Acetonitrile:	Burdick & Jackson, reagent grade
4. Methanol:	Burdick & Jackson, reagent grade
5. Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

Test Substance

The test substance, D5, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D5
Synonyms:	decamethylcyclopentasiloxane, Dow Corning® 1693 Fluid
Batch No.:	0000341832
CAS No.:	541-02-6
Purity:	99.16%[1]
Expiration Date:	31 December 2010

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-23) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8% (tested as 100%)
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used to prepare calibration standards during testing.

[1] Original characterization of the test substance yielded a purity of 99.19%. All calculations for this study are based on the original purity.

PROCEDURES

Preparation of Stock Solutions

A 10.0 mg a.i./mL D5 stock solution was prepared by placing 0.2524 g of D5 (0.2504 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetone. This stock solution was used to fortify the mid- and high-level recovery samples. A 100 mg a.i./L secondary stock solution was prepared by placing 0.500 mL of the 10.0 mg a.i./mL primary stock solution in a 50.0-mL volumetric flask and bringing it to volume with acetone. This stock solution was used to fortify the low-level recovery samples.

A 1.00 mg a.i./mL D5 stock solution was prepared by placing 0.1012 g of the test substance (0.1004 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with hexane. Two secondary stock solutions (10.0 and 100 mg a.i./mL) were prepared by placing 0.500 and 5.00 mL, respectively, of the 1.00 mg a.i./L primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with hexane. The 10.0 and 100 mg a.i./L secondary stock solutions were used to prepare calibration standards.

A 10.0 mg/mL tetrakis(trimethylsiloxy)-silane internal standard stock solution was prepared by placing 0.2501 g of the internal standard in a 25.0-mL volumetric flask and bringing it to volume with acetone. The primary internal standard stock solution was added to the high-level recovery samples. Two secondary stock solutions (100 and 1000 mg/L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg/mL primary internal standard stock solution in separate 50.0-mL volumetric flasks and bringing each volume with acetone. The 100 and 1000 mg/L secondary internal standard stock solutions were added to the low- and mid-level recovery samples.

A second 100 mg/L secondary internal standard stock solution was prepared by placing 0.500 mL of the 10.0 mg/mL primary internal standard stock solution in a 50.0-mL volumetric flask and bringing it to volume with hexane. The 100 mg/L secondary internal standard stock solution was added to the calibration standards.

All stock solutions were stored in a refrigerator in glass amber bottles fitted with Teflon®-lined caps.

Preparation of Calibration Standards

Calibration standards were prepared in hexane by fortifying with the 10.0 and 100 mg a.i./L secondary stock solutions to yield concentrations of 0.0200, 0.0500, 0.100, 0.200, 0.300 and 0.500 mg a.i./L. Additionally, the internal standard was fortified in every calibration standard at a concentration of 0.1 mg/L with the 100 mg/L secondary stock solution.

Sample Fortification and Preparation

The recovery samples were prepared by fortifying freshwater sediment (5.00 g, dry weight) with D5 at concentrations of 2.00, 50.0 and 500 µg a.i./g. The low-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 100 mg a.i./L secondary stock solution. The mid- and high-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 10.0 mg a.i./mL primary stock solution. Each concentration level was produced in triplicate. In addition, three recovery samples 5.00 g (dry weight) were left unfortified to serve as controls.

A 0.100-mL aliquot of the 10.0 mg/mL primary internal standard stock solution was added to the high-level recovery samples. A 0.100-mL aliquot of the 1000 mg/L secondary internal standard stock solution was added to the mid-level recovery samples. A 0.0500-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low-level recovery samples.

Extraction

All recovery samples were prepared in 45-mL glass vials with PTFE-lined caps. An aliquot (6.00-mL) of acetonitrile was added to each sample. An aliquot of hexane (12.0 mL) was added to the vials that contained both sediment and acetonitrile. The samples were paced on a shaker table at 150 rpm for 30 minutes and centrifuged at 1500 rpm for 15 minutes to separate the layers. At this point the samples contained three layers: the top layer was hexane, the middle

was acetonitrile and water and the bottom was the sediment. The upper hexane layer was transferred to 50.0-mL volumetric flasks. An addition aliquot of hexane (12.0-mL) was added to the acetonitrile and sediment in the vials. The samples were placed on a shaker table at 150 rpm for an additional 30 minutes and centrifuged at 1500 rpm for 15 minutes. The extracts were combined and taken to volume with hexane. The mid- and high-level samples were further diluted into the calibration standard range with hexane. An aliquot from each recovery sample was collected for analysis by gas chromatographic analysis with mass selective detection and negative chemical ionization (GC-MSD/NCI).

ANALYSIS

GC Parameters:

Column: Varian WCOT Fused Silica, CP-SIL 8 CB, 30m x 0.25mm x 0.25 µm

Temperature: 40 °C (initial) and held for 2.00 minutes

Ramps:

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
10.0	250	0.00
30.0	300	2.33

Run Time: 27.0 min

Injection Volume: 2.0 µL

Gas Flows: Carrier gas: Helium, at a constant flow of 1.0 mL/min

Inlet Mode: Splitless

Purge Time: on at 1.00 min at 60 mL/min

Inlet Temperature: 260 °C

Retention Time: approximately 10.5 minutes

MSD parameters

Solvent delay: 6.0 minutes

Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
355	100	quantitation D5
311	100	quantitation internal standard

Temperatures: MSD Transfer Line - 280 °C
MS Quad – 106 °C
MS Source – 150 °C

Chemical Ionization gas: Methane

Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the system was performed by programmed automated injection.

CALCULATIONS

A calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (mg a.i./L) of the calibration standards against the natural logarithm (ln) of the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept of the regression analysis, and the natural logarithm of the peak area and the dilution factor of the recovery sample. Equations 2, 3 and 4 were then used to calculate measured concentrations and analytical results.

$$(1) \quad \ln y = m(\ln x) + b$$

$$(2) \quad \ln x = \frac{(\ln y - b)}{m}$$

$$(3) \quad DC(x) = \text{inverse}(\ln x)$$

$$(4) \quad A = DC \times DF$$

where:

lnx	=	natural logarithm of sample concentration
lny	=	natural logarithm of detector response
m	=	slope from regression analysis
b	=	y-intercept from regression analysis
DC (x)	=	detected concentration (µg a.i./g) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg a.i./g)

The limit of quantitation (LOQ) was calculated using the following equation:

$$(5) \quad \ln(Area_{MIN}) = \ln(0.5 \times A_{LS})$$

$$(6) \quad \ln(LOQ_{INST}) = \frac{\ln(0.5 \times A_{LS}) - b}{m}$$

$$(7) \quad LOQ_{INST} = \text{inverse}(\ln(LOQ_{INST}))$$

$$(8) \quad LOQ = LOQ_{INST} \times DF_{CNTL}$$

where:

$Area_{MIN}$	=	one half of the mean detector response (peak area) of the low concentration calibration standard (two injections)
A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
b	=	y-intercept from regression analysis
m	=	slope from regression analysis
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

RESULTS AND DISCUSSION

The mean recovery was 109% ± 7.41%, with a limit of quantitation of 0.501 µg a.i./g. The LOQ will vary from one analysis interval to another, since it is dependent upon the regression of the calibration standards, the peak area of the low standards, and dilution factor of the controls. These parameters, while relatively constant, do vary somewhat among runs and produce small variations in the LOQ. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%.

Analytical results for the recovery of D5 from freshwater sediment are presented in Table 1A. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample are presented in Figure 1A, Figure 2A and Figure 3A, respectively. A typical regression analysis for D5 is presented in Figure 4A.

Table 1A. Analytical results for the recovery of D5 from freshwater sediment during the method validation study.

Sample ID	Fortified Concentration (µg a.i./g)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
Ctrl A	0.00	10.0	< 0.501	NA
Ctrl B	0.00	10.0	< 0.501	NA
Ctrl C	0.00	10.0	< 0.501	NA
Low A	2.00	10.0	2.35	117
Low B	2.00	10.0	2.32	116
Low C	2.00	10.0	2.30	115
Mid A	50.0	200	52.9	106
Mid B	50.0	200	56.0	112
Mid C	50.0	200	56.4	113
High A	500	2000	477	95.5
High B	500	2000	539	108
High C	500	2000	505	101
MEAN				**109**
STD DEV				**7.41**
% CV				**6.78**

[a] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for each analysis is dependent upon the linear regression, the area of the low standards and the dilution factor of the controls. The limit of quantitation for the method validation was 0.501 µg a.i./g.

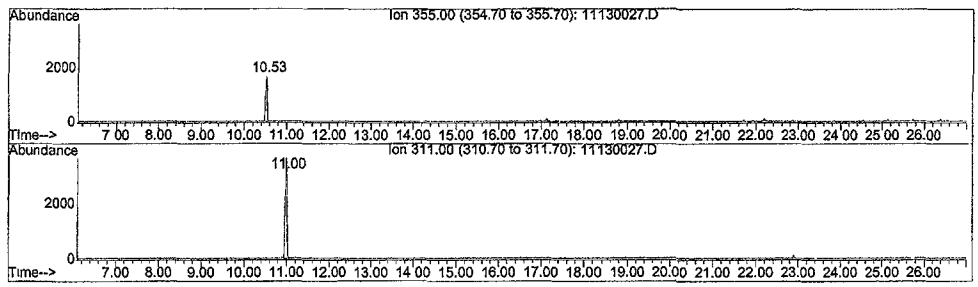
[b] NA = Not Applicable.

Note: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Figure 1A. Representative chromatogram of a 0.200 mg a.i./L calibration standard used for quantification during the method validation study with D5.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\D51113\11130027.D
Operator    : MED
Acquired    : 14 Nov 2008   8:35     using AcqMethod D5CISIM
Sample Name: std 0.2mg/l
Misc Info   :
Vial Number: 27
CurrentMeth: C:\HPCHEM\1\METHODS\D5CISIM.M
```

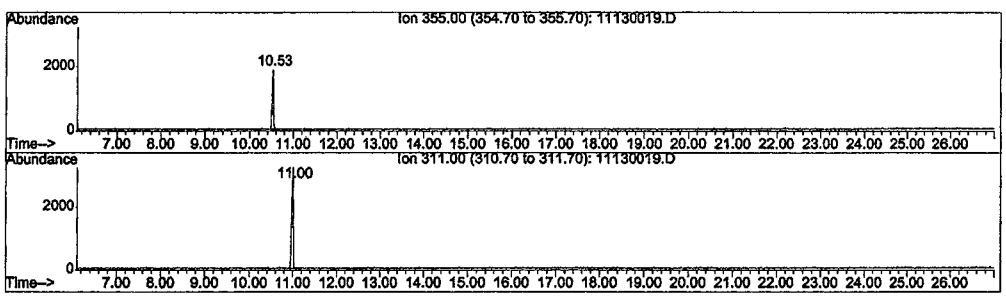


Retention Time	Area	Area %	Ratio %
311.00 amu			
11.003	102696	100.000	100.000
355.00 amu			
10.529	46530	100.000	100.000

Figure 2A. Representative chromatogram of a 500 µg a.i./g recovery sample used during the method validation study with D5.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\D51113\11130019.D
Operator    : MED
Acquired    : 14 Nov 2008   3:44     using AcqMethod D5CISIM
Sample Name: HIGH A
Misc Info   :
Vial Number: 19
CurrentMeth: C:\HPCHEM\1\METHODS\D5CISIM.M
```

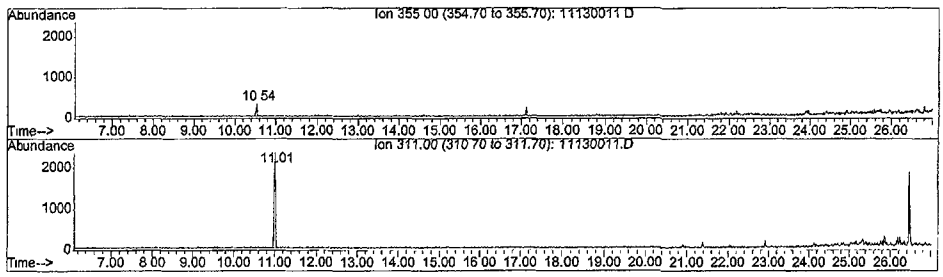


Retention Time	Area	Area %	Ratio %
311.00 amu			
11.005	78925	100.000	100.000
355.00 amu			
10.534	41101	100.000	100.000

Figure 3A. Representative chromatogram of a control sample during the method validation study with D5.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\D51113\11130011.D
Operator    : MED
Acquired    : 13 Nov 2008  22:54     using AcqMethod D5CISIM
Sample Name: CTRL B
Misc Info   :
Vial Number: 11
CurrentMeth: C:\HPCHEM\1\METHODS\D5CISIM.M
```



Retention Time	Area	Area %	Ratio %
311.00 amu			
11.007	56996	100.000	100.000
355.00 amu			
10.537	6665	100.000	100.000

~~For IS only~~ DEN 11/14/08

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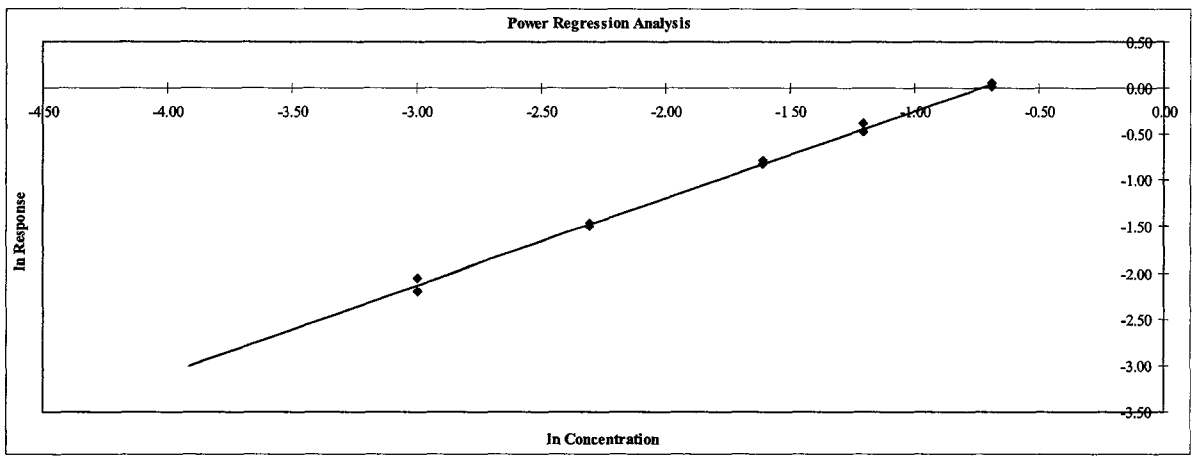
Note: No peak equivalent to retention time for D5. D5 is ordinarily detected at a retention time of approximately 10.5 minutes.

Figure 4A. A typical regression analysis for the calibration standards used to quantitate D5 during the method validation study.



Regression Analysis

$r^2 = 0.99710$
$y = 0.945457x + 0.7018$

ln Standard Concentration mg a.i./L	Standard Concentration mg a.i./L	ln Response Area	Standard Response Area
-3.9120	0.0200	--[a]	11352
-2.9957	0.0500	-2.0630	12439
-2.3026	0.100	-1.5081	20726
-1.6094	0.200	-0.8205	44033
-1.2040	0.300	-0.3796	73017
-0.6931	0.500	0.0104	92785
-3.9120	0.0200	--[a]	7906
-2.9957	0.0500	-2.1986	10517
-2.3026	0.100	-1.4639	21336
-1.6094	0.200	-0.7917	46530
-1.2040	0.300	-0.4722	62493
-0.6931	0.500	0.0561	109079

[a] Standard not used.

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Study Title

D4 – Sediment-Water Lumbriculus Toxicity Test using Spiked Natural Sediment, Following OECD Guideline 225

Data Requirement

OECD Guideline 225

Author

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Study Completed On

27 August 2009

Submitted To

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Laboratory Project ID

Springborn Smithers Study No. 13937.6103

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The data and report presented for **"D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225"** were produced and compiled in accordance with all pertinent OECD (OECD, 1998) Good Laboratory Practice regulations with the following exception: routine water contaminant screening analyses were conducted using standard U.S. EPA procedures by GeoLabs, Inc., Braintree, Massachusetts and are considered facility records under Springborn Smithers Laboratories' SOP 7.92. Since the analyses were conducted following standard validated methods, this exception had no impact on the study results. The internal standard, tetrakis(trimethylsiloxy)silane, was purchased from a commercial supplier and was not characterized under GLPs.

SPRINGBORN SMITHERS LABORATORIES

Christian Picard
Study Director

8/27/09
Date

QUALITY ASSURANCE STATEMENT

The study conduct, raw data and report for **"D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225"** were inspected by the Quality Assurance Unit (QAU) at Springborn Smithers Laboratories to determine adherence with the study protocol and laboratory standard operating procedures. This report is an accurate representation of the raw data. Dates of study inspections, inspection types, and dates reported to the Study Director and to Management are listed below.

Inspection Date	Inspection Type	Reported to Study Director/Management
4/20/2009	Data	4/20/2009
5/14/2009	Protocol Review	5/14/2009
5/14/2009	In-Life-Water Quality Measurements	5/14/2009
6/3/2009	Method Appendix	6/3/2009
7/13/2009	Data	7/13/2009
7/23 to 24/2009	Draft Report	7/24/2009
7/29/2009	Revised Draft	7/29/2009
8/27/2009	Final report	8/27/2009

SPRINGBORN SMITHERS LABORATORIES

Dorothy A. Antonioli

Dorothy A. Antonioli
Quality Assurance Auditor

27 August 2009
Date

KEY STUDY PERSONNEL

The following Springborn Smithers personnel were responsible for the conduct of the work and reporting of the study results.

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SUMMARY

D4 – Sediment-Water Lumbriculus Toxicity Test Using Spiked Natural Sediment, Following OECD Guideline 225

STUDY SPONSOR: CES (Centre European des Silicones, European Chemical Industry Council (Cefic))

PROTOCOL TITLE: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225, Springborn Smithers Laboratories Protocol No.: 062608/OECD/Oligochaete/Spiked Sediment

SPRINGBORN SMITHERS STUDY NUMBER: 13937.6103

TEST SUBSTANCE: D4, Batch No. LL084732, CAS No. 556-67-2, reported to have a purity of 99.77%, was received from Dow Corning Corporation on 8 July 2008.

DEFINITIVE TEST DATES: 14 May to 11 June 2009

TEST ORGANISM: *Lumbriculus variegatus*
Source: Springborn Smithers culture

TEST CONDITIONS: 28-day duration, static conditions, 19 to 23 °C, illumination of 16 hours light: 8 hours darkness at 530 to 540 lux

OVERLYING WATER: Fortified laboratory well water
pH: 8.0
Specific conductance: 600 µmhos/cm
Total hardness as $CaCO_3$: 170 mg/L
Total alkalinity as $CaCO_3$: 100 mg/L

SEDIMENT: Natural Sediment
percent organic carbon: 2.2%
particle size distribution: 93% sand, 6% silt, and 1% clay
pH: 6.5
percent moisture (1/3 bar): 18.0%

NOMINAL SEDIMENT CONCENTRATIONS: 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg

MEAN MEASURED SEDIMENT CONCENTRATIONS: 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg

RESULTS: Based on measured concentrations of D4 in the sediment, the No-Observed-Effect Concentration (NOEC) for this exposure was determined to be 13 mg a.i./kg. The Lowest-Observed-Effect Concentration (LOEC) for this exposure was determined to be 19 mg a.i./kg. Since no concentration tested resulted in ≥ 50% reduction in survival or biomass, the EC50 value was empirically estimated to be > 32 mg a.i./kg, the highest mean measured concentration tested.

1.0 INTRODUCTION

This study was designed to determine the effects of D4, applied to sediment on the sediment-dwelling oligochaete *(Lumbriculus variegatus)*, following a 28-day exposure period. The study was performed under static conditions in artificial sediment for a period of 28 days. Exposure concentrations of D4 were measured on day 0 (test initiation), day 7 and day 28 (test termination) in the sediment. Results of this study were used to estimate the highest concentration which had no significant ($p \leq 0.05$) effect on the test organism performance (No-Observed-Effect Concentration, NOEC), and the lowest concentration that had a significant effect on the exposed organisms (Lowest-Observed-Effect Concentration, LOEC). If possible, the concentration which caused a 50% reduction (EC50) in either oligochaete reproduction or biomass was also estimated.

The study was initiated on 7 August 2008, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental phase of the study was conducted from 14 May to 11 June 2009 at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. All original raw data, the protocol and the original final report produced during this study are archived at Springborn Smithers Laboratories at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

The toxicity test was performed according to the Springborn Smithers Laboratories protocol entitled "Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225" Springborn Smithers Laboratories Protocol No.: 062608/OECD/Oligochaete/Spike Sediment (Appendix 1). This protocol meets the general requirements specified in the OECD Guideline for the Testing of Chemicals, Guideline 225 entitled "Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment" (OECD, 2007).

2.2 Test Substance and Internal Standard

2.2.1 Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance, and archival of a sample of the test substance are the responsibility of the Study Sponsor.

2.2.2 Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane (M_4Q)
Synonym:	Trimethylsiloxysilicate
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

2.3 Test Organism

The oligochaete (*Lumbriculus variegatus*) was selected as a test organism since it is the recommended species for use in OECD Guideline 225 (OECD, 2007) for sediment toxicity testing. They are tolerant to a wide range of sediment types and are widely used for sediment toxicity and bioaccumulation testing. The OECD Guideline 218 for testing of chemicals in spiked sediment relies on the use of the *Chironomus* species in testing. The larvae of *Chironomus* sp. live at the sediment water interface and exposure is to the top layer of the sediment only. The oligochaete, *Lumbriculus variegates*, contacts the sediment and pore water at greater depths and theoretically provides a complementary assessment of sediment toxicity to *Chironomus* sp. testing. The oligochaetes used during this study were obtained from the main laboratory cultures maintained at Springborn Smithers. The oligochaetes from this culture were maintained in a 57-L aquarium containing approximately 40 L of culture water under flow-through conditions. The water used in the main culture is unadulterated well water from a 100-meter bedrock well and has been characterized as soft water with typical ranges as total hardness of 30 to 60 mg/L as $CaCO_3$, alkalinity of 25 to 45 mg/L as $CaCO_3$, pH of 6.9 to 7.7, and a specific conductance of 140 to 200 micromhos/cm. Oligochaetes were fed 15 mL of finely ground suspension (100 mg/mL) of flaked fish food weekly.

Fourteen days prior to test initiation, worms from the culture were removed and artificially fragmented to synchronize the population, using a scalpel to remove the anterior ends. Synchronized oligochaetes were then acclimated to test conditions (i.e., test water and test sediment) for a period of thirteen days to regenerate new heads. The synchronization of worms is performed to avoid "uncontrolled" regeneration and reproduction that may contribute high variation in the test results. Once during the acclimation period, the synchronized oligochaetes were fed 6.4 mL of a finely ground suspension (100 mg/mL) of flaked fish food. During the

holding period, dissolved oxygen ranged from 7.9 to 8.4 mg/L, pH ranged from 7.2 to 8.2 and the temperature ranged from 20 to 21 °C. No mortality was observed in the test population 48 hours prior to test initiation.

2.4 Overlying Water

Overlying water used during this study was fortified well water. The dilution water was prepared by fortifying well water based on the formula for hard water (U.S. EPA, 1975) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. The water used during the definitive exposure was characterized as having a total hardness and total alkalinity as calcium carbonate ($CaCO_3$) of 170 and 100 mg/L, respectively, a pH of 8.0, and a specific conductivity of 600 micromhos per centimeter (µmhos/cm).

Representative samples of the overlying water source were analyzed periodically for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM (2002) standard practice.

2.5 Sediment

Natural freshwater sediment was the substrate used in the exposure. The natural sediment (Springborn Smithers Laboratories Batch No. 031809) was collected from a single source, Glen Charlie Pond, Wareham, Massachusetts. Prior to use and characterization, the sediment was wet pressed through a 2.0-mm sieve to remove large particles and benthic organisms and homogenized thoroughly. The natural sediment used in the study was characterized by Agvise Laboratories, Northwood, North Dakota, as having an organic carbon content of 2.2%, a pH of 6.5 and a percent moisture (at 1/3 Bar) of 18.0. The particle size distribution conducted by Agvise Laboratories resulted in average particle size distribution of 93% sand, 6% silt and 1% clay. A percent solids value of 58.46% was determined by Springborn Smithers Laboratories. A sample of the sediment pore water was generated from the sediment batch prior to testing and

yielded a measured concentration of 4.5 mg/L as nitrogen (ammonia). This concentration is well below levels of concern for this organism. A representative sample of the sediment source was analyzed for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds were detected at concentrations that would be considered to have an adverse impact on the results of the test.

2.6 Test Concentrations

Selection of nominal D4 concentrations for the 28-day chronic toxicity test with oligochaetes was based on toxicity information developed at Springborn Smithers Laboratories through preliminary testing. Based on the results of preliminary testing (see Section 3.1) and consultation with the Study Sponsor, the nominal D4 concentrations of 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg were selected for the definitive exposure.

2.7 Test Procedures

2.7.1 Stock Solution Preparation

A 20 mg a.i./mL primary stock was prepared by placing 0.5012 g (0.5000 g as active ingredient) of D4 in a 25-mL volumetric flask and bringing it to volume with acetone. The resulting stock solution was observed to be clear and colorless with no visible undissolved test substance following stirring. A 2.0 mg a.i./mL secondary stock solution was prepared by placing 2.5 mL of the 20 mg a.i./mL primary stock solution in a 25-mL volumetric flask and bringing it to volume with acetone (CAS No. 67-64-1). Dosing stock solutions were prepared from dilutions of 2.0 mg a.i./mL secondary stock solution and the 20 mg a.i./mL primary stock solution as follows:

Stock Concentration (mg a.i./mL)	Volume of Stock Used (mL)	Volume Diluted to with Acetone (mL)	Final Dosing Stock Concentration (mg a.i./mL)
20	8.80	10	17.6
20	4.40	10	8.80
20	2.20	10	4.40
20	1.15	10	2.29
2.0	5.55	10	1.11
2.0	2.73	10	0.546

All stock solutions were observed to be clear and colorless with no visible undissolved test substance following preparation.

2.7.2 Test Substance Application

The method of test substance application to sediment used during this study was provided by the Study Sponsor. For each dose level, a 2.5-kg aliquot of wet sediment (1.462 kg dry weight based on a percent solids of 58.46%) along with 7.5 L of overlying water (sediment:volume ratio of 3:1) were placed in individual glass jars. Immediately prior to dosing, the contents of each jar were shaken to suspend the sediment. An 8.3-mL volume of each dosing stock solution (prepared as in Section 2.9) was then added to each jar. Each jar was shaken again after dosing to distribute the test substance. After a 16-hour settling period, the water was decanted from the sediment. The sediment was transferred to a Hobart mixer and 3.66 g each of *urtica* powder and alpha cellulose were mixed into the sediment of each test group as a food source for the oligochaetes. This weight of urtica powder and alpha cellulose was equal to 0.25% of the dry weight for each sediment batch. Following mixing in the Hobart mixer, the treated sediments were allocated to the replicate test vessels for each exposure level.

A solvent control sample was prepared in the same manner as the treated sediment by adding 2.5 kg of wet sediment (1.462 kg dry weight based on a percent solids of 58.46%) along with 7.5 L of overlying water (sediment:volume ratio of 3:1) and 8.3 mL of acetone in a glass jar and processed in the same manner as the treated sediments. The negative control sediment group was prepared using only untreated sediment and overlying water (no test substance or solvent).

The negative control and solvent control vessels were maintained under the same conditions as the treatment vessels

2.8 Preparation of Test Vessels

The test vessels used were 600-mL clear glass beakers. The sediment and overlying water were added to the test vessels two days prior to test initiation. A 75-mL (1.5-cm layer) aliquot of sediment was added to each test vessel. The wet weight of the sediment in each jar averaged 148 g (86.5 g dry weight). A turbulence reducer, consisting of a plastic disk, was used to minimize the disruption of the sediment layer during the introduction of 300 mL (6 cm) of overlying water. The total medium volume (sediment/water) was maintained at 375 mL and the ratio of sediment to water was 1:4. The turbulence reducer was composed of a modified plastic disk. The initial water level in each test vessel was marked in order to evaluate evaporation. Each test vessel was covered with a clear plastic plate to minimize evaporation and gentle aeration was provided through a glass pipette fixed approximately 2 to 3 cm above the sediment surface. Aeration was delivered at a rate of 1 to 3 bubbles per second.

Four replicate test vessels (A through D) were established for each treatment level and six vessels (A through F) were established for the control and solvent control. Four additional replicate test vessels (E through H for treatment levels and G through J for the controls) were maintained for the purpose of chemical analysis. Each replicate vessel contained ten oligochaetes, a total of 40 oligochates per concentration and 60 oligochates in the control and solvent control for the biological response replicates. The additional replicates were maintained under the same conditions and contained ten test organisms, but were not used to evaluate the biological response of the test organisms

2.9 Test Conditions

Test vessels were positioned in a water bath containing circulating water designed to maintain the test solutions at a temperature of 20 ± 2 °C. Test solutions were gently aerated (1 to

3 bubbles per second) two days prior to addition of the test organisms and throughout the duration of the exposure period. Test vessels were labeled to identify the nominal test concentration, study number and designated replicate. The test area was illuminated with fluorescent bulbs at an intensity range of 530 to 540 lux and a photoperiod of 16 hours light and 8 hours darkness. Light intensity was measured using a VWR Traceable light meter.

2.9.1 Test Initiation

The spiked sediment and overlying water were added to the test vessels two days prior to addition of the test organisms (day -2). At test initiation, oligochaetes were added impartially to an intermediate vessel by adding no more than two oligochaetes to each vessel until all vessels contained two oligochaetes. This procedure was repeated until each vessel contained ten oligochaetes. The test was initiated when each intermediate vessel of oligochaetes was added to each respective test vessel. The ten oligochaetes were released simultaneously into the overlying water of each test vessel just above the sediment surface. A total of 40 oligochaetes for the treatment levels and 60 oligochaetes for the control and solvent control were exposed to the test conditions.

2.10 Test Monitoring

2.10.1 Biological Observations

Replicate test vessels A through D for treatment levels and A through F for the control and solvent control were examined at test initiation and daily thereafter, until test termination (day 28). Daily observations of mortality and abnormal behavior (e.g., leaving sediment) were made and recorded.

On test day 28 of the exposure, the replicate vessels from each exposure level (four replicates) and controls (six replicates) were terminated to determine the number of surviving oligochaetes and the biomass of the surviving oligochaetes. Instances of dead oligochaetes were also recorded. However, dead oligochaetes often decompose in the vessels and are difficult to

recover. If fewer than ten oligochaetes were observed and no dead oligochaetes were recovered, the missing individuals were assumed to have died. Surviving worms were assigned to one of three groups:

a) Large, complete worms (adults) without regenerated body regions.
b) Complete worms with regenerated, lighter-colored body regions.
c) Incomplete worms (i.e., recently fragmented worms with non-regenerated body regions.

The biomass was determined by placing the entire number of surviving oligochaetes from each replicate vessel in a tared weight tin and then drying in an oven at 100 ± 5 °C overnight (18 to 24 hours). Following drying, the tins were cooled to room temperature in desiccators and then weighted to the nearest 0.01 mg on a calibrated analytical balance.

2.10.2 Water Quality Measurements

Water quality measurements made during the study were performed in the replicate exposure vessels (A through D or A through F). Measurements of dissolved oxygen concentration, temperature and pH were made on the day the test organisms were added (day 0) and at test termination (day 28) in each exposure vessel. In addition, dissolved oxygen concentration, temperature and pH were measured daily in one replicate vessel of each treatment level and the controls during the 28-day exposure. Daily water quality measurements were made on alternating replicates. The temperature was continuously monitored in an auxiliary vessel in the water bath throughout the study. Total hardness, alkalinity, specific conductivity, and total ammonia of the test solutions were determined at test initiation and at test termination in a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F). In addition, total ammonia was determined three times per week in the solvent control and the 100 mg a.i./kg nominal treatment level.

The dissolved oxygen concentration and daily temperature were measured using a Yellow Springs Instrument (YSI) Model No. 550A dissolved oxygen meter/temperature probe. Continuous temperature monitoring was performed using a VWR minimum/maximum

thermometer. A YSI pH100 pH meter was used for pH measurements. Total hardness and alkalinity of the test solutions were determined according to APHA et al., (1995). Specific conductivity was monitored with a YSI Model No. 33 salinity-conductivity-temperature (SCT) meter. Total ammonia was measured with an Orion Ammonium Meter (Model 720A Plus) and an Orion electrode (Model 9512).

2.11 Analytical Measurements

Prior to dosing, a sample of each dosing stock solution was taken and analyzed for D4 concentration. In addition, each treatment level and control sediment was sampled and analyzed for D4 concentration after dosing and prior to division into the replicate test vessels. Results of the pretest analyses were used to judge whether sufficient quantities of D4 had been applied during the mixing process.

During the in-life phase of the definitive study, sediment samples were removed and analyzed for D4 concentration on test days 0 (test initiation), test day 7 and test day 28 (test termination). On test days 0, 7 and 28, samples were removed and analyzed from replicate vessels E, F and G, respectively, for each treatment level and vessels G, H and I for the controls. Overlying water samples were removed from the test vessels by decanting all of the overlying water from each vessel into a graduated cylinder. Following removal of the overlying water, the sediment was then centrifuged at approximately 10,000 *g* for 30 minutes. The pore water generated by centrifugation was then removed by pipet. The sediment samples were then collected from each centrifuge tube with a stainless steel spatula.

In addition, three quality control (QC) samples were prepared at each sampling interval and remained with the set of exposure samples throughout the analytical process. These QC samples were prepared in sediment (based on sediment dry weight) at concentrations of D4 similar to the treatment level range. Results of the analyses of the QC samples were used to judge the precision and quality control maintained during the analysis of exposure solution samples.

All samples were analyzed for D4 using gas chromatography with mass selective detection (GC/MSD) based on methodology validated at Springborn Smithers. The method validations were conducted prior to the initiation of the definitive test and established average recoveries of 82.1 ± 9.02% from artificial sediment (Appendix 2) and 101 ± 18.1% from natural freshwater sediment (Appendix 3), respectively. Results from both methods were comparable which indicates that both of these methods were robust and appropriate for the analysis of test samples. Conditions and procedures used throughout the analysis of the exposure solutions and QC samples during this study were similar to those used in the method validation studies. Defined limits of acceptance of quality control sample performance were set at 70.0 to 120%.

2.12 Statistical Analysis

2.12.1 Determination of LOEC and NOEC Values

At the termination of the study, data obtained on oligochaetes reproduction (the total number of oligochaetes recovered at test termination) and biomass were statistically analyzed to identify significant treatment-related effects. The lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant effect (No-Observed-Effect Concentration, NOEC) were determined. All statistical analyses were conducted at the 95% level of certainty except in the case of the Chi-Square Test and Bartlett's Test, in which the 99% level of certainty was applied. The 99% level of certainty is preferred for these qualifying tests. The following procedures were used:

1. Chi-Square Test for normality (Weber et al., 1989) was conducted to compare the observed sample distribution with a normal distribution. The assumption that observations are normally distributed must be validated before subsequent analyses, following parametric procedures, can be performed. If the data are not normally distributed, then a non-parametric procedure is used for subsequent analyses. Analysis of both reproduction and biomass met this assumption of normal distribution.

2. As a check on the assumption of homogeneity of variance implicit in parametric statistics, data for each endpoint were analyzed using Bartlett's Test (Sokal and Rohlf, 1981). Analysis of both reproduction and biomass met the assumption of homogeneity of variance.

3. A t-Test was conducted for oligochaete reproduction and biomass data to compare the performance of the control organisms with that of the solvent control organisms in order to determine if there were any statistically significant positive or negative effects. During this study, the t-Test indicated no significant difference between control and solvent control data for both endpoints (reproduction and biomass). Therefore, data from all treatment levels were compared to the pooled control data to determine treatment level effects.

4. Data for both endpoints measured in this study (reproduction and biomass) passed both the tests for normality and homogeneity. Therefore, Bonferroni's t-Test (Weber, et. al, 1989), a parametric statistical procedure, was used to establish treatment effects for both endpoints.

TOXSTAT® Version 3.5 (West Inc. and Gulley, 1996) was used to perform the computations. The results were used to establish, at the 95% level of certainty, the lowest test concentration that showed a statistically significant effect (Lowest-Observed-Effect Concentration, LOEC) and the highest test concentration that showed no statistically significant difference (No-Observed-Effect Concentration, NOEC) from the pooled control data.

2.12.2 EC50 Determination

The EC50 is the estimated measured sediment concentration of the test substance which produces 50% reduction in reproduction or biomass in the test population of oligochaetes at test termination. During this study, no concentration tested resulted in ≥ 50% reduction in

reproduction or biomass, therefore the EC50 values were empirically estimated to be greater than the highest mean measured sediment concentration.

3.0 RESULTS

3.1 Preliminary Testing

Prior to initiating the definitive study, a 28-day preliminary range-finding exposure was conducted at Springborn Smithers at nominal D4 levels of 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg, and a solvent (acetone) control. D4 was applied to the sediment in the same manner and test procedures were followed as outlined in Section 2.9. Three replicates of ten oligochaetes were exposed to each treatment level and control. The following table is a summary of the results of the preliminary range-finding exposure:

Nominal Treatment Level (mg/kg)	Mean Number of Oligochaetes Recovered per Replicate	Mean Biomass per Replicate (mg)
Pooled Control	26	25
0.050	25	24
0.50	23	27
5.0	26	23
50	19[a]	25
500	17[a]	20[a]

[a] Statistically reduced when compared to the pooled control, based on Bonferroni's t-Test.

At test termination, the mean number of surviving oligochaetes observed among oligochaetes exposed to the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels was 25, 23, 26, 19 and 17, respectively. During the same period, the mean number of surviving oligochaetes observed in the negative control and solvent control was 26 and 25, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant difference in survival among oligochaetes exposed to the 50 and 500 mg a.i./kg treatment levels compared to the pooled control (26 oligochaetes).

Mean biomass per replicate in the 0.050, 0.50, 5.0, 50 and 500 mg a.i./kg treatment levels averaged 24, 27, 23, 25 and 20 mg, respectively. During the same period, biomass in the negative control and the solvent control averaged 26 and 23 mg, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant difference in biomass (dry weight) among

oligochaetes exposed to the 500 mg a.i./kg treatment level compared to the pooled control (25 mg).

Based on these results and consultation with the Study Sponsor, nominal D4 concentrations of 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg were selected for the definitive study.

A definitive exposure was initiated on 19 February 2009. The test was terminated on 3 March 2009 due to lower than expected analytical recoveries in the day 0 sediment samples. A second definitive test was initiated on 14 May 2009, the results of which are presented below.

3.2 Definitive Testing

3.2.1 Evaluation of Test Conditions

Results of the water quality measurements made during the definitive exposure are presented in Table 1 and Table 2. The water quality parameters were unaffected by the concentrations of D4 tested and remained within acceptable limits. Daily measurements of the temperature in the exposure solutions, and continuous monitoring in an auxiliary vessel set up in the water bath used to house the test vessels established a temperature range of 19 to 23 °C throughout the definitive study. The dissolved oxygen and pH measurements in the treatment levels and the controls ranged from 7.4 to 9.2 mg/L and 7.2 to 8.1, respectively. On day 17 replicate D of the 6.3 mg a.i./kg nominal treatment level had a measurement of 3.8 mg/L due to a malfunction in the aeration system. Aeration was restored and dissolved oxygen levels returned to the range stated in the protocol.

3.2.2 Analytical Results

Analysis of the stock solution samples used to dose the test sediments ranged from 100 to 110% of the nominal fortified concentrations. These results indicated that the appropriate mass of test substance was introduced to the sediment.

All test concentrations are expressed relative to sediment dry weight. Pretest analysis of the sediment samples, following dosing and prior to allocation into the replicate exposure vessels resulted in measured concentrations ranging from 45 to 88% of the nominal treatment levels. The sediment pretest results demonstrated that a reasonable concentration gradient was achieved from which to base the biological results.

Table 3 summarizes the D4 concentrations measured during the 28-day toxicity test. Measured sediment concentrations at day 0 in the 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg treatment levels were 1.1, 3.7, 13, 18, 24 and 39 mg a.i./kg, respectively. Measured sediment concentrations at day 7 in the 3.1, 6.3, 13, 25, 50 and 100 mg a.i./kg treatment levels were 1.6, 3.5, 6.9, 14, 19 and 29 mg a.i./kg, respectively, and 0.92, 2.3, 7.0, 8.3, 15 and 29 mg a.i./kg, respectively, on day 28. Based on mean measured concentrations, treatment levels were defined as 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg.

Analysis of seven of the nine quality control samples resulted in measured concentrations which ranged from 89.9 to 109% of the nominal fortified concentrations (1.50, 25.0 and 100 mg a.i./kg) and were consistent with the expectations of the methodology used (Appendix 2 and Appendix 3). Based on these results, it was determined that the appropriate quality control was maintained during the analyses of sediment samples. One of the nine QC samples was measured at 55.2% of nominal concentration. In addition, one of the nine QC samples was inadvertently fortified below the quantification range. QC samples can be out of the acceptable range due to a number of factors, some of which are spiking, handling or instrument errors.

3.2.3 Biological Results

The number of oligochaetes recovered at test termination and mean biomass for each replicate of each treatment level observed throughout the exposure period are presented in Table 4. Following 28 days of exposure, the number of oligochaetes recovered in the control and solvent control was 30 and 30, respectively. The mean biomass per replicate in the control and solvent control was 46 and 51 mg, respectively. The control data met the minimum performance criteria

of the guideline (i.e., population increase of ≥ 1.8). As demonstrated by the performance of the control organisms, the exposure system provided conditions which were appropriate for promoting survival and growth of the test species. Study data for this exposure are presented in Appendix 4.

At test termination (day 28), the mean number of surviving oligochaetes observed among the oligochaetes exposed to the 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg treatment levels was 31, 31, 28, 29, 26 and 22, respectively. Statistical analysis (Bonferroni's t-Test) determined a significant reduction in the mean number of surviving oligochaetes in the 19 and 32 mg a.i./kg treatment levels compared to the pooled control data (30 oligochaetes).

Mean biomass per replicate in the 1.2, 3.2, 8.8, 13, 19 and 32 mg a.i./kg treatment levels was 55, 64, 50, 47, 44 and 44 mg, respectively. Statistical analysis (Bonferroni's t-Test) determined no significant difference in mean biomass compared to the control (49 mg).

Based on mean measured concentrations of D4 in the sediment, the No-Observed-Effect Concentration (NOEC) for this exposure was determined to be 13 mg a.i./kg. The Lowest-Observed-Effect Concentration (LOEC) for this exposure was determined to be 19 mg a.i./kg. Since no concentration tested resulted in ≥ 50% reduction in reproduction or biomass, the EC50 value was empirically estimated to be > 32 mg a.i./kg, the highest mean measured concentration tested.

PROTOCOL DEVIATIONS

1. The protocol states that the temperature of the test solutions will be maintained at 20 ± 2 °C. During this study, temperature readings recorded on test day 17 were 23 °C. In addition, the minimum-maximum thermometer recorded temperatures which exceeded the stated range on test days 17, 18 and 22. The maximum recorded temperature was 23 °C.

2. The protocol states that the test will be illuminated to an intensity of 100 to 500 lux. During this exposure, the light intensity was measured to be 530 to 540 lux.

These deviations did not have a negative impact on the results or interpretation of the study.

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Table 1. Water quality parameters (dissolved oxygen, temperature and pH) measured in the overlying water during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Concentration (mg a.i./kg)	Range		
	Dissolved Oxygen Concentration[ab] (mg/L)	Temperature[ac] (°C)	pH[a]
Control	7.9 – 9.0	19 – 23	7.2 – 8.0
Solvent Control	7.9 – 9.1	19 – 23	7.5 – 8.0
3.1	7.4 – 9.1	19 – 23	7.6 – 8.1
6.3	7.5[d] – 9.2	19 – 23	7.7 – 8.1
13	8.0 – 9.2	19 – 23	7.5 – 8.1
25	8.0 – 9.1	19 – 23	7.2 – 8.1
50	8.0 – 9.2	19 – 23	7.2 – 8.0
100	7.7 – 9.1	19 – 23	7.7 – 8.1

[a] N= 39 for the control and solvent control; N = 35 for all remaining treatment levels.
[b] 30% of the air saturation value at 19 °C = 2.8 mg/L.
30% of the air saturation value at 23 °C = 2.6 mg/L.
[c] Continuous temperature monitoring in an auxiliary vessel set up in the water bath used to house the test vessels established a temperature range of 19 to 23 °C throughout the exposure period.
[d] On test day 17 a dissolved oxygen concentration of 3.8 mg/L was measured in replicate D due to a brief malfunction in the aeration system.

NOTE: Values have been rounded to two significant figures.

Table 2. Water quality parameters (total hardness, total alkalinity, specific conductivity and ammonia) measured in the overlying water during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Concentration (mg a.i./kg)	Total Hardness[a] (mg/L as $CaCO_3$)		Total Alkalinity[a] (mg/L as $CaCO_3$)		Specific Conductance[a] (µmhos/cm)	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Solvent Control	140	60	72	32	600	500
100	150	56	74	44	600	500

Nominal Concentration (mg a.i./kg)	Ammonia Analysis (mg/L as N)[b]											
	Day 0	Day 4	Day 6	Day 8	Day 11	Day 13	Day 15	Day 18	Day 20	Day 22	Day 25	Day 28
Solvent Control	0.46	0.34	≤0.10	0.25	0.16	0.34	1.5	1.2	2.3	3.0	2.5	1.8
100	0.55	0.33	0.13	0.17	≤0.10	0.29	1.1	1.2	2.4	2.7	3.7	3.3

[a] Based on measurement of a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F) at test initiation and termination.

[b] Based on measurement of a composite sample from the highest treatment level (replicates A through D) and the solvent control (replicates A through F) at test initiation, termination and three times per week.

NOTE: Values have been rounded to two significant figures.

Table 3. Concentrations of D4 (expressed relative to sediment dry weight) measured in sediment samples during the study exposing oligochaetes (*Lumbriculus variegatus*) to D4 applied to sediment.

Nominal Sediment Concentration (mg a.i./kg)	Measured Sediment Concentration (mg a.i./kg)				Mean Percent Recovery[d]
	Day 0[a]	Day 7[b]	Day 28[c]	Mean[d]	
Control	< 0.60[e]	< 0.60	< 0.60	NA[f]	NA
Solvent Control	< 0.60	< 0.60	< 0.60	NA	NA
3.1	1.1	1.6	0.92	1.2	39
6.3	3.7	3.5	2.3	3.2	50
13	13	6.9	7.0	8.8	68
25	18	14	8.3	13	54
50	24	19	15	19	39
100	39	29	29	32	32
QC[g] #1 1.50	0.827 (55.2)[h]	< 0.00060 (NA)[i]	1.64 (109)		
QC#2 25.0	22.5 (89.9)	24.6 (98.2)	26.4 (106)		
QC#3 100	101 (101)	100 (100)	104 (104)		

[a] Analytical samples were removed from replicate E, with the exception of the control and solvent control which were removed from replicate G.
[b] Analytical samples were removed from replicate F, with the exception of the control and solvent control which were removed from replicate H.
[c] Analytical samples were removed from replicate G, with the exception of the control and solvent control which were removed from replicate I.
[d] Mean measured and percent recovery values were calculated using the actual analytical results and not the rounded values (two significant figures) presented in this table.
[e] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for the analysis was set at the lowest validated sample concentration.
[f] NA = Not Applicable.
[g] QC = Quality Control sample. Percent of nominal is presented in parentheses below the measured results.
[h] Percent recovery for this QC sample is outside of the acceptable range (i.e., 70.0 to 120%, Appendix 2).
[i] This QC sample was inadvertently fortified below the limit of quantification.

Table 4. Mean measured sediment concentrations, number of oligochaetes and biomass of oligochaetes at test termination of the 28-day exposure with D4 and oligochaetes (*Lumbriculus variegatus*).

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes	Population Increase Factor	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
Control	A	17	12	1	30	3.0	42.38	30 (2)	46 (6)
	B	18	11	1	30	3.0	44.24		
	C	12	12	2	26	2.6	38.83		
	D	16	12	0	28	2.8	53.88		
	E	9	24	0	33	3.3	52.66		
	F	14	13	4	31	3.1	46.88		
Solvent Control	A	13	12	3	28	2.8	41.79	30 (3)	51 (9)
	B	16	10	0	26	2.6	46.15		
	C	23	6	2	31	3.1	48.96		
	D	21	10	2	33	3.3	48.22		
	E	20	11	1	32	3.2	67.07		
	F	16	9	3	28	2.8	55.00		
Pooled Control					356	3.0		30 (2)	49 (8)
1.2	A	19	12	0	31	3.1	49.65	31 (1)	55 (6)
	B	17	11	2	30	3.0	61.45		
	C	19	12	0	31	3.1	59.22		
	D	24	8	1	33	3.3	50.84		
3.2	A	10	14	6	30	3.0	55.52	31 (3)	64 (6)
	B	12	15	3	30	3.0	63.67		
	C	9	19	0	28	2.8	67.13		
	D	9	22	3	34	3.4	70.49		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation
[c] Statistically different ($p \leq 0.05$) compared to the pooled control data, based on Bonferroni's t-Test.

Table 4. (Continued) Mean measured sediment concentrations, number of oligochaetes and biomass of oligochaetes at test termination of the 28-day exposure with D4 and oligochaetes (*Lumbriculus variegatus*).

Mean Measured Sediment Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes	Population Increase Factor	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
8.8	A	16	10	2	28	2.8	44.78	28 (1)	50 (8)
	B	14	15	0	29	2.9	42.72		
	C	16	12	1	29	2.9	50.07		
	D	17	9	0	26	2.6	61.14		
13	A	10	16	0	26	2.6	44.35	29 (3)	47 (3)
	B	11	18	1	30	3.0	51.40		
	C	21	11	0	32	3.2	47.43		
	D	7	21	1	29	2.9	45.46		
19	A	11	10	4	25	2.5	37.64	26 (3)[c]	44 (5)
	B	15	11	1	27	2.7	46.63		
	C	15	7	0	22	2.2	48.07		
	D	15	13	0	28	2.8	44.77		
32	A	11	11	0	22	2.2	49.88	22 (1)[c]	44 (6)
	B	12	11	0	23	2.3	37.21		
	C	12	7	2	21	2.1	48.01		
	D	10	10	3	23	2.3	42.62		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation
[c] Statistically different ($p \leq 0.05$) compared to the pooled control data, based on Bonferroni's t-Test.

APPENDIX 1 - STUDY PROTOCOL

SPRINGBORN SMITHERS
LABORATORIES
Massachusetts Research Center
790 Main Street • Wareham, MA • 02571-1037 • Phone. (508) 295-2550 • Fax (508) 295-8107

TEST PROTOCOL

PROTOCOL TITLE: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

TO BE COMPLETED BY THE STUDY SPONSOR:		
Study Sponsor:	CES (Centre European des Silicones, European Chemical Industry Council (Cefic)	
Address:	Avenue E. van Nieuwenhuyse 4, B-1160 Brussels, Belgium	
		Phone: 989-496-8226
Study Monitor:	Kurt Drottar	**E-mail:** k.drottar@dowcorning.com
Sponsor Protocol/Project No.:		
Test Substance Name: D4		
Purity: 99.75%	**Lot #:** LL084732	
Analytical Standard:		
Purity:	**Lot #:**	
Additional Comments and Modifications:		
Sponsor Approval:		**Date:** 8/6/08

TO BE COMPLETED BY SPRINGBORN SMITHERS LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Springborn Smithers Laboratories 790 Main Street, Wareham, MA 02571-1037

Study Director: Christian Picard Study No: 13937-6103

Test Concentrations: *

Proposed Experimental Dates: (Start) * (Termination) *

Study Director Signature **Study Initiation Date** 8/7/08

* To be provided by protocol amendment, if applicable

Other Locations: 2900 Quakenbush Road, P O Box 620 • Snow Camp, North Carolina 27349 • Phone: (336) 376-0141 • Fax (336) 376-0145
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Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

1.0 OBJECTIVE

The purpose of this test will be to determine the impact of a test substance to the sediment-dwelling oligochaete (*Lumbriculus variegatus*), following a 28-day exposure period. The study will assess the impact of the test substance on the reproduction and biomass of the oligochaetes. The methods described in the protocol meet the requirements specified in the OECD Guideline for the Testing of Chemicals, Guideline 225 entitled "Sediment Water Lumbriculus Toxicity Test Using Spiked Sediment" (OECD, 2007).

2.0 MATERIALS AND METHODS

2.1 Chemical System

2.1.1 Test Substance

Upon arrival at Springborn Smithers Laboratories, all test substances and reference substances will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer

Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date

2.1.2 Test Substance Concentrations

Test substance concentrations will be based on the results of a 28-day static range-finding test. Concentrations of 500, 50, 5.0, 0.5, 0.05 mg/kg dry weight plus controls will be selected for the static range-finding test. A negative control consists of overlying water and sediment without test substance.

For the definitive test, each test substance concentration will be 50% of the next higher concentration of the test substance.

2.1.3 Solvent Control

An organic solvent (N,N-dimethylformamide) will be used as a carrier to solubilize the test substance. The solvent volume utilized will remain constant across the test concentration series. A solvent control will be included in each test and will consist of

sediment plus the equivalent volume of solvent used during the application of the test solutions to the sediment. The solvent volume will be kept as low as possible.

2.1.4 Application of Test Substance to Sediment

The appropriate amount of test substance will be removed for dosing the exposure system (e.g., weighed on an analytical balance or volumetrically measured with a graduated pipette). A Chemical Usage Log will be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test substance is used. The amount of test substance will be applied to the sediment according to the following formula:

$$\text{Sediment Concentration (e.g., mg a.i./kg)} = \frac{\text{T.S.}}{\text{S.A.} \times \text{D.W}}$$

where:

T.S.	=	test substance (mg a.i.)
S.A.	=	wet sediment amount (kg)
D.W.	=	(percent dry weight of sediment) ÷ 100

The test substance will be applied by the following method:

For each dose level, a glass jar will be filled with natural sediment followed by approximately 3 times the volume of overlying water, to give a water-volume to sediment-volume ratio of 3:1. The jar contents will be shaken to suspend the sediment immediately prior to dosing individually with a solution containing octamethylcyclotetrasiloxane, D_4, in N,N-dimethylformamide (DMF). The jar will be shaken again after dosing with D_4 to distribute the test substance. After settling for 4 hours, the water will be decanted from the sediments. The sediment will be stirred to assure homogeneity and then treated sediment will be allocated to individual test vessels.

2.2 Test System

2.2.1 Species

The oligochaete, *Lumbriculus variegatus*, will be the species used in this reproduction test. Prior to test initiation, synchronized oligochaetes of the same size are removed from the culture aquaria and added to the exposure systems. The synchronization of worms is performed to avoid "uncontrolled" regeneration and reproduction that may contribute high variation in the test results.

2.2.2 Justification of Test System

The oligochaete *Lumbriculus variegatus* is recommended for use in OECD guidance (OECD 2007) for sediment toxicity testing. This species will be used because it is tolerant to a wide range of sediment types and widely used for sediment toxicity and bioaccumulation testing. The OECD guideline 225 for testing of chemicals in spiked

sediment relies on use of *Chironomus* species in testing. The larvae of *Chironomus sp.* live at the sediment water-interface and exposure is to the top layer of sediment only. The oligochaete *Lumbriculus variegatus* will contact sediment and pore water at greater depths and theoretically provides a complimentary assessment of sediment toxicity to *Chironomus sp.* testing.

2.2.3 Synchronization of Oligochaetes

Approximately two weeks prior to test initiation, the worms will be artificially fragmented and the posterior ends will be left to regenerate new heads. Worms are artificially fragmented using a scalpel to remove the anterior portion of each worm. The worms will then be held under test conditions (i.e., water and test sediment) for a period of 10 to 14 days to regenerate new heads. This provides organisms that are "synchronized" worms and are equally exposed to the treated sediment.

2.2.4 Origin

Lumbriculus variegatus cultures will be maintained at Springborn Smithers Laboratories. The oligochaetes will be cultured in aquaria under flow-through conditions. The water used in the main culture is unadulterated well water from a 100-meter bedrock well supplemented on demand with untreated Town of Wareham well water, and will be characterized as soft water with typical ranges as total hardness of 30 to 60 mg/L as $CaCO_3$, alkalinity of 25 - 45 mg/L as $CaCO_3$, pH of 6.9 to 7.7, and a specific conductance of 140 to 200 microohms/cm. Culture water will be maintained at 23 ± 1 °C. An appropriate number of oligochaetes from the main culture population will be artificially fragmented and placed in a small aquarium (e.g., 10 liter aquarium) with test sediment and unadulterated well water under flow-through conditions. The synchronized population will be acclimated to the test conditions by renewing the overlying water in the small aquarium with test dilution water at the rate of approximately one volume replacement per day. The test dilution water will consist of hard fortified well water with a total hardness of 160-180 mg/L as $CaCO_3$, alkalinity of 110-130 mg/L as $CaCO_3$, pH of 7.9-8.3 and a specific conductance of 400-600 micromhos/cm.

The origin of the culture and the background of the test culture will be described in the final report. The oligochaetes will be held in test sediment for a period of at least 10 to 14 days (synchronization period) to acclimate the organisms to the test sediment.

2.2.5 Feeding

The main culture of oligochaetes will be fed 15 mL of a flaked fish food suspension (100 mg/mL), weekly. The synchronized population will be fed a flaked fish food suspension after seven days of being acclimated to test conditions. The feeding rate of the synchronized oligochaetes will be modified based on the size of this population. Oligochaetes will be fed once during the study (see section 2.3.4).

2.2.6 Handling

Fine mesh nets and wide-bore pipettes will be used to remove and transfer the oligochaetes from the cultures to the test system, taking care to minimize possible stress due to handling. Oligochaetes that are damaged or dropped during transfer will not be used.

2.2.7 Loading

Prior to test initiation, a subsample of oligochaetes will be taken to determine dry weight of the organisms. The ratio of total organic carbon (TOC) of the sediment to organisms dry weight in each vessel will be calculated.

2.3 Physical System

2.3.1 Test Containers

The test chambers to be used in the 28-day static toxicity test will be 600-mL glass beakers. The test chambers will be chemically clean before the test is started. The test chambers will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with water. Each test chamber contains 1.5 cm (75 mL) of sediment and 6 cm (300 mL) of overlaying water. The total medium volume (sediment/water) is maintained at 375 mL and the ratio of sediment to water is 1:4. Each test chamber will be covered with a plexiglass plate to minimize evaporation. The test chambers will be labeled to identify the treatment/control and the replicate designation.

2.3.2 Replication and Control of Bias

Four replicates (A through D) will be included with each test concentration. The control and solvent control will have six replicates (A through F). Each replicate vessel will contain ten individuals, a total of either 40 or 60 oligochaetes per concentration or control. Oligochaetes will be added impartially to an intermediate beaker by adding no more than two oligochaetes to each vessel until all beakers contain two oligochaetes. This procedure will be repeated until each intermediate beaker contains ten oligochaetes. The test will be initiated when each intermediate beaker of 10 oligochaetes is added to each respective test vessel. Four additional replicate test vessels (E through H for each test concentration and G through J for the controls) will be established for analytical measurements during the test. These vessels will be maintained under the same conditions as the exposure vessels and will contain test organisms. These additional replicates will not be included in the biological observations for the study.

2.3.3 Overlying Water

Dilution water will consist of hard fortified well water with a total hardness of 160 to 180 mg/L as $CaCO_3$. The well water (total hardness about 30 mg/L as $CaCO_3$) will be fortified according to the formulation for hard water presented in "*Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians*" (U.S. EPA, 1975). Dilution water will be filtered through an amberlite XAD-7 resin column prior to delivery to the diluter. The column is 30 cm long and 1.6 cm wide. This filtration will effectively remove any potential organic contaminants from the water. Total hardness, total alkalinity, pH and specific conductance of the diluent water will be monitored on each batch prior to use to assure that these parameters are within the normal acceptable ranges. Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1995). Ranges for these parameters will generally be: total hardness, 160 to 180 mg/L as $CaCO_3$; alkalinity, 110 to 130 mg/L as $CaCO_3$; specific conductance, 400 to 600 µmhos/cm; and pH, 7.9 to 8.3.

New batches of diluent water will be prepared when either the previous batch is exhausted, when a water quality parameter (total hardness, alkalinity, etc.) differs from the normal ranges, or after two weeks of holding. The diluent water will be aerated with an air pump and air stones to bring the pH and dissolved gases into equilibrium with the atmosphere. Fiberglass containers will be used to hold the diluent water, and water will be pumped from this holding tank to the diluter. Periodic analysis of representative samples of the dilution water source will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP 7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to oligochaetes.

2.3.4 Sediment

The test will be conducted using natural freshwater sediment of medium organic carbon content (e.g., 2 – 5% OC). The freshwater sediment used in this study will be collected from a site known to be free from contamination. The natural sediment will be wet pressed through a 2 mm sieve to remove large particles and will be characterized for total organic carbon content, pH, percent sand, silt, clay (particle size distribution) and percent water holding capacity by Agvise Laboratories, Northwood, North Dakota. The ammonia concentration in the sediment pore water will be analyzed prior to the use of the sediment. Periodic analyses of representative samples of the sediment will be conducted using U.S. EPA standard methods by GeoLabs, Inc., Braintree, Massachusetts, in accordance with Springborn Smithers' SOP 7.92, to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations that may be harmful to oligochaetes.

In addition, *Urtica* sp. powder will be added to the sediment at a rate of 0.25% of the sediment dry weight as a food source for the oligochaetes during the test, along with alpha-cellulose at 0.25% of the sediment dry weight.

2.4 Test Conditions

2.4.1 Temperature

Water temperature of the test solutions will be maintained at 20 ± 2 °C by conducting the test in a water bath maintained at the appropriate test temperature. The temperature between replicates vessels at any one measurement interval should be ± 1 °C.

2.4.2 Lighting

The test will be conducted in a light controlled laboratory. The test will be illuminated to a light intensity of 100 to 500 lux using fluorescent bulbs. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer. Light intensity will be measured once during the study.

2.4.3 Dissolved Oxygen

Total dissolved oxygen will not be allowed to drop below 30% of the air saturation value (ASV). Aeration (with oil free air) will be initiated prior to test initiation to maintain the dissolved oxygen concentration at or above 30% of the ASV.

2.4.4 Preparation of Test Vessels

The sediment and overlying water will be added to the test vessels two days prior to the addition of the test organisms. During this period the test vessels will be held under test conditions. The amount of sediment is added to each test vessel to a layer of 1.5 cm deep (75 mL). To avoid separation of the sediment ingredients, a turbulence reducer is used to add the 6 cm of overlying water (300 mL of water). The turbulence reducer consists of a plastic disk that floats as the water is poured in the test vessel, taking care not to disturb the sediment. The initial water level in each test vessel will be marked so as to evaluate evaporation.

Each test vessel is covered with a plastic plate and gentle aeration is provided through a glass pipette fixed approximately 2 to 3 cm above the sediment surface. Aeration is delivered at a rate of 1 to 3 bubbles per second. If necessary, the water level in each test vessel will be topped off with deionized water (i.e., twice weekly) to prevent a change of more than 10% (i.e., 30 mL) of the total overlying water volume.

2.4.5 Initiation of Test Organisms

Two days before exposure initiation (day -2) the treated and control sediments will be added to the replicate test vessels and the overlying water will be added (section 2.4.4). This allows the sediment and water to equilibrate prior to addition of the test organisms.

The exposure will be initiated (Day 0) when all vessels contain ten synchronized oligochaetes and the exposure will terminate 28 days thereafter.

2.5 Sampling and Observations

2.5.1 Sampling

Prior to test initiation and before the sediment is allocated to the replicate test vessels, a sample of sediment from each treatment level and controls will be removed and analyzed. In addition, a sample of the stock solution used to apply the test substance will be sampled and analyzed.

During the in-life phase, samples of the sediment from each treatment level and controls will be removed on test day 0, test day 7 and at test termination (test day 28) for determination of test substance concentration. The sample volume of sediment will be dependent upon the analytical methodology employed for determination of test substance concentrations. Three quality control (QC) samples will be prepared at each sampling interval and stored and analyzed with the set of study samples. The QC samples will be prepared in sediment at test substance concentrations similar to the treatment level range. Results of these analyses indicate the accuracy of the analytical method for measuring test substance concentration at each sampling period. The analytical method used to measure test substance concentrations in the exposure solutions will be validated by Springborn Smithers Laboratories at the expected nominal concentration range prior to test initiation.

2.5.2 Sampling Devices

The entire volume of overlying water will be removed from each test vessel carefully by decanting or pipetting. After removal of the overlying water the sediment will be mixed thoroughly. An appropriate size sample of the mixed bulk sediment will then be removed with a stainless steel spatula for determination of sediment test substance concentrations.

2.5.3 Water Quality Measurements

On the day of addition of the test organisms (day 0) and test termination (day 28), temperature, dissolved oxygen (DO) concentration and pH will be measured and recorded in each test vessel established for biological observations (replicates A through D for treatment levels and A through F for controls). In addition, temperature, dissolved oxygen (DO) concentration and pH will be measured and recorded daily in a replicate test vessel established for biological observations of each concentration and control(s). Daily measurements will be alternated among replicate vessels. Total hardness, alkalinity, and specific conductance will be determined on day 0 and at test termination in a sample from the highest concentration tested and the solvent control. Total ammonia will be measured in the highest concentration and the solvent control at test initiation and three times weekly throughout the duration of the test. Overlying water removed for water quality determination on day 0 will be taken from additional replicates set-up for chemical analysis. Temperature will be monitored continuously in an auxiliary vessel, maintained in the water bath with all test vessels, by using a minimum-maximum thermometer. Readings of temperature extremes will be recorded daily.

2.5.4 Biological Observations

Following addition of the oligochaetes and daily thereafter, until termination, observations of oligochaete mortality and abnormal behavior (e.g., leaving sediment) will be made and recorded.

2.5.5 Test Termination

On test day 28 of the exposure, the replicate vessels from each exposure level (four replicates) and controls (six replicates) will be terminated to determine number of surviving oligochaetes and biomass of surviving oligochaetes.

Surviving worms will be assigned to one of three groups:

a) large complete worms (adults) without regenerated body regions

b) complete worms with regenerated, lighter colored body regions

c) incomplete worms (i.e., recently fragmented worms with non-regenerated body regions)

The biomass is determined by placing the entire number of surviving oligochaetes in each replicate vessel in a tared weigh tin and then drying in an oven at 100 ± 5°C overnight (18 to 24 hours). Following drying, the tins are cooled to room temperature in desiccators and then weighed to the nearest 0.01 mg on a calibrated analytical balance.

2.5.6 Acceptability Criteria

For the test, the following criteria have to occur in the control or the test will be considered unacceptable:

- Based on the results of a ring-test, the average number of living worms per replicate vessel in the control should have increased by a factor of 1.8 at the end of the exposure compared to the number of worms per replicate at the start of the exposure.
- The dissolved oxygen concentration should not be < 30% of the air saturation value (ASV) at the end of the test.
- The pH range in overlying water at the beginning and end of the test should be between 6 and 9.

3.0 STATISTICAL ANALYSES

3.1 Endpoints

The endpoints used for determination of significant effects by statistical evaluation include number of surviving oligochaetes per replicate vessel and biomass of oligochaetes per replicate vessel. All concentration-effect relationships are based on the nominal concentrations of test substance in the sediment.

3.2 Statistical Methods (Determination of LOEC/NOEC)

If a solvent is used as a carrier for the test substance, a t-Test ($p \leq 0.05$) will be used to compare the results (survival) of the solvent control to the control data. If the data are similar, they will be pooled for further analyses. If the data are not similar, the remaining statistical analyses will be performed comparing the treatment data to the solvent control data.

The data will be tested for normality and homogeneity of variance using Shapiro-Wilks' Test or Chi-Square Test (depending on the number of replicates in the analysis) and Bartlett's Test or Cochran's Test. If the data passes these two tests, then a parametric method will be used to evaluate the results of test, e.g., William's Test or Dunnett's Test. If the data fails the test for normality and homogeneity of variance, then a non-parametric method will be used to evaluate the results of the test, e.g., Dunn's (Sokal and Rohlf, 1981) or Steel's One-Many Rank Test (Weber et al, 1989). If necessary, mean values will be transformed using square root, arcsine square root, or log conversion procedures. The Lowest-Observed-Effect-Concentration (LOEC) is defined as the lowest test concentration that shows a statistically significant effect and the No-Observed-Effect-Concentration (NOEC) is the highest test concentration that shows no statistically significant difference from the control.

3.3 Transformations

Transformation of data is performed with data representing endpoint estimates obtained as a proportion (e.g., survival). Prior to analyzing data of this type, the observed proportion in each vessel is transformed by using the arcsine square-root transformation.

4.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

5.0 REPORTING

The raw data and final draft of the report will be reviewed by the Quality Assurance Unit and the Study Director. Chemical and water quality measurements will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The draft report will be initially submitted to the Study Sponsor for review. Upon acceptance by the Sponsor, the final report will be submitted. The final report will meet the formatting requirements of EPA's PR Notice 86-5. All reports will include, but will not be limited to, the following information:

- The report and project numbers from Springborn Smithers Laboratories and Sponsor study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Study Monitor, Study Director and Principal Investigator.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).
- Characterization and origin of the dilution water.
- Characterization and origin of the sediment.
- Scientific name of the test organisms, source and culturing information.
- Test container volume, sediment and water volume, number of replicates used per concentration, and mass of oligochaetes used per treatment/control.
- Description of exposure system and application of test substance to sediment.
- Test temperatures, dissolved oxygen concentration, and pH; and photoperiod and light intensity used, as well as specific conductance, total ammonia, total alkalinity and total hardness measured.
- Description or reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- The nominal test substance concentration in sediment.

- Number of surviving worms and biomass per replicate vessels for each treatment level and control.
- Data evaluation and conclusions.
- Deviations from the protocol not addressed in protocol amendments will be listed, together with a discussion of the impact on the study.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of raw data and final report.

6.0 PROTOCOL CHANGES

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any. If necessary, amendments other than the one providing the information required by page one of this protocol, may initially be verbally authorized, followed by Springborn Smithers' written documentation. In such cases, the effective date of the amendment will be the date of verbal authorization.

7.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records, and reports will comply with the Organization for Economic Co-operation and Development (OECD) Good Laboratory Practices.

8.0 REFERENCES

APHA, AWWA, WPCF. 1995. Standard Methods for the Examination of Water and Wastewater. 19th Edition, Washington, DC.

OECD, 1997. Good Laboratory Practice in the Testing of Chemicals. Paris, France.

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Sokal, R.R. and F.J. Rohlf. 1981. Biometry. 2nd Edition. W.H. Freeman and Company, New York. 859 pp.

U.S. EPA. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Ecological Research Series (EPA-660/3-75-009). 61 pp.

U.S. EPA. 40 CFR, Part 160. Federal Insecticide, Fungicide, and Rodenticide Act. Good Laboratory Practices Standards; Final Rule. Office of the Federal Register, National Archives and Records Administration. U.S. Government Printing Office, Washington, D.C.

Weber, C.I. et al. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH.

Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics, 27: 103-117.

Williams, D.A. 1972. A comparison of several dose levels with a zero dose control. Biometrics, 28: 519-531.

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PROTOCOL AMENDMENT

Amendment No.: 1

Effective Date: 17 December 2008

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937.6103

Amendment:

1. Protocol Cover Page, Study Specific Information
The following information is being provided as requested on the protocol cover page:

Internal Standard Tetrakis (Trimethylsiloxy) Silane Lot Number: 8D-12648 Purity· 99.8%

Test Concentrations: 100, 50, 25, 13 and 6.3 mg/kg plus controls

Proposed Experimental Dates (Start) January 2009 (Termination) February 2009

2. Page 2, Section 2.1.3 Solvent Control
The protocol states that an organic solvent (DMF) will be used as a carrier to solubilize the test substance. In this study, acetone will be the organic solvent used to solubilize the test substance

3. Page 3, Section 2.1.4 Application of Test Substance to the Sediment
The protocol states that the sediment will be dosed with a solution containing D4 dissolved in DMF. In this study, the sediment will be dosed with a solution containing D4 dissolved in acetone.

4. Page 9, Section 3.1 Endpoints
The protocol states that all concentration-effect relationships will be based on nominal concentrations of the test substance in the sediment. In this study, all concentration-effect relationships will be based on measured concentrations of the test substance in the sediment

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None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R. Picard
Springborn Smithers Study Director

12/22/08
Date

Study Sponsor Representative

12/22/08
Date

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PROTOCOL AMENDMENT

Amendment No.: 2

Effective Date: 13 February 2009

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937 6103

Amendment:

1. Page 3, Section 2.1.4 – Application of the Test Substance

The protocol states that the dosed sediment/water mixture will be allowed to settle for four hours and the water will then be decanted from the sediment. In this study, the dosed sediment/water mixture will be allowed to settle overnight (approximately 16 hours) before decanting the water from the sediment.

2. Page 8, Section 2.5.2 – Sampling Devices

The protocol states that the sediment in the test vessel will be mixed thoroughly and an appropriate sized sample of mixed bulk sediment will be removed with a stainless steel spatula for analysis. In this study, an appropriate sized sediment sample will be removed from the test vessels on days 0, 7 and 28 and centrifuged at approximately 1200 *g* for 15 to 30 minutes. After centrifuging, the pore water is decanted or removed by pipette An appropriate size sample of sediment will then be removed from the centrifuge tube with a stainless steel spatula for determination of sediment concentrations.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R Picard — 2/19/09
Springborn Smithers Study Director — Date

Study Sponsor Representative — 2/19/09
Date

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PROTOCOL AMENDMENT

Amendment No.: 3

Effective Date: 8 May 2009

Protocol Title: Sediment-Water Lumbriculus Toxicity Test using Spiked Sediment, Following OECD Guideline 225

Protocol Number: 062608/OECD/Oligochate/Spiked Sediment

Species: *Lumbriculus variegatus*

Study Sponsor: Cefic

Test Substance: D4

Study No.: 13937.6103

Amendment:

1. Protocol Cover Page Information and Amendment 1

An additional test concentration of 3.1 mg/kg is being added to the range of concentrations listed in Amendment 1.

2. Amendment 2

Amendment 2 states that sediment samples will be centrifuged at approximately 1200 *g* for 15 to 30 minutes. In this study, sediment samples will be centrifuged at approximately 10,000 *g* for 15 to 30 minutes.

None of the above changes will have a negative impact on the study.

Approval Signatures:

Christian R. Picard
Springborn Smithers Study Director — Date: 5/11/09

Study Sponsor Representative — Date: 5/11/09

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APPENDIX 2 – ANALYTICAL METHODOLOGY (ARTIFICIAL SEDIMENT)

SUMMARY

Methodology was validated (25 March 2009) to quantify the amount of D4 present in artificial sediment. Recovery samples containing the test substance were extracted twice with acetonitrile and hexane. The hexane extracts were combined and diluted with hexane prior to analysis. An aliquot of the final extracts were analyzed by automated injection on a gas chromatographic system equipped with mass selective detection (GC/MSD).

This method was validated by fortification of artificial sediment with D4 at nominal concentrations of 0.600, 10.0 and 100 µg a.i./g. Recoveries of D4 averaged 82.1 ± 9.02%. Due to instrumental carryover, the LOQ is set to the lowest validated concentration, i.e., 0.600 µg/g. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

EXPERIMENTAL

Equipment

1. Instrument:	Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector series 5973, Hewlett Packard autosampler, Hewlett Packard Model 7683 injector and Hewlett Packard ChemStation Version C.00.000 for data acquisition.
2. Balances:	Sartorius BL210S, Orion 0960, Sartorius MA45C moisture balance
3. Shaker table:	Lab-Line orbit shaker
4. Centrifuge:	Beckman GS-6R
5. Laboratory equipment:	syringes, volumetric flasks, volumetric pipets, Pasteur pipets, autosampler vials, 45-mL glass vials with PTFE-lined caps, and amber glass bottles with Teflon®-lined caps

Reagents

1. Acetone:	Burdick & Jackson, reagent grade
2. Hexane:	Burdick & Jackson, reagent grade
3. Acetonitrile:	Burdick & Jackson, reagent grade
4. Nitric acid:	JT Baker, reagent grade
5. Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Synonym:	Trimethylsiloxysilicate (M_4Q)
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

PROCEDURES

Preparation of Stock Solutions

A 10.0 mg a.i./mL D4 stock solution was prepared by placing 0.2514 g of D4 (0.2508 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with acetone. The 100 mg a.i./L secondary stock solution was used to fortify the low-level recovery samples. The 1000 mg a.i./L secondary stock solution was used to fortify the mid- and high-level recovery samples.

A 1.00 mg a.i./mL D4 stock solution was prepared by placing 0.1005 g of the test substance (0.1003 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with hexane. Two secondary stock solutions (10.0 and 100 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 1.00 mg a.i./L primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with hexane. The 10.0 and 100 mg a.i./L secondary stock solutions were used to prepare calibration standards.

A 10.0 mg/mL tetrakis(trimethylsiloxy)-silane internal standard stock solution was prepared by placing 0.2504 g of the internal standard in a 25.0-mL volumetric flask and bringing it to volume with acetone. A 1000 mg/L secondary stock solution was prepared by placing 5.00 mL of the 10.0 mg/mL primary stock solution in a 50.0 mL volumetric flask and bringing it to volume with acetone. The 1000 mg/L secondary internal standard stock solution was added to the mid- and high-level recovery samples.

A second 100 mg/L secondary internal standard stock solution was prepared by placing 0.500 mL of the 10.0 mg/mL primary internal standard stock solution in a 50.0-mL volumetric flask and bringing it to volume with hexane. The 100 mg/L secondary internal standard stock solution was added to the low-level recovery samples, the control samples and the calibration standards.

All stock solutions were stored in a refrigerator in glass amber bottles fitted with Teflon®-lined caps.

Preparation of Calibration Standards

Calibration standards were prepared in hexane by fortifying with the 10.0 and 100 mg a.i./L secondary stock solutions to yield concentrations of 0.0200, 0.0500, 0.100, 0.200, 0.300 and 0.500 mg a.i./L. Each calibration standard was fortified with 150 µL of the 100 mg/L secondary stock solution using the scheme outlined in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Calibration Standard Concentration (mg a.i./L)
NA[a]	NA	100	0.150	50.0	0.00
10.0	0.100	100	0.150	50.0	0.0200
10.0	0.250	100	0.150	50.0	0.0500
10.0	0.500	100	0.150	50.0	0.100
100	0.100	100	0.150	50.0	0.200
100	0.150	100	0.150	50.0	0.300
100	0.250	100	0.150	50.0	0.500

[a] NA = Not Applicable.

Sample Fortification and Preparation

All glassware used for the preparation and extraction of recovery samples was subjected to rigorous cleaning before use. The glassware was soaked and rinsed well with 20% nitric acid in water. This was followed by a water rinse to remove the nitric acid, and then a rinse with acetone to remove residual organics. Glassware was rinsed with copious amounts of hexane prior to use.

The recovery samples were prepared by fortifying artificial sediment (5.00 g, dry weight) with D4 at concentrations of 0.600, 10.0 and 100 µg a.i./g. The low-level recovery samples were prepared by fortifying 5.00 g (dry weight) of artificial sediment using the 100 mg a.i./L secondary stock solution. The mid- and high-level recovery samples were prepared by fortifying 5.00 g (dry weight) of artificial sediment using the 1000 mg a.i./L primary stock solution. Each

concentration level was produced in triplicate. In addition, three recovery samples 5.00 g (dry weight) were left unfortified to serve as controls.

A 0.600-mL aliquot of the 1000 mg/L secondary internal standard stock solution was added to the high-level recovery samples. A 0.0600-mL aliquot of the 1000 mg/L secondary internal standard stock solution was added to the mid-level recovery samples. A 0.150-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low-level recovery samples.

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Sample Mass (g)	Sample Concentration (µg a.i./g)
NA	NA	100	0.150	5.00	0.00
100	0.0300	100	0.150	5.00	0.600
1000	0.0500	1000	0.0600	5.00	10.0
1000	0.500	1000	0.600	5.00	100

[a] NA = Not Applicable.

Extraction

All recovery samples (5.00 g, dry weight) were prepared in 45-mL glass vials with PTFE-lined caps. An aliquot (6.00-mL) of acetonitrile was added to each sample. An aliquot of hexane (12.0 mL) was added to the vials that contained both sediment and acetonitrile. The samples were placed on a shaker table at 150 rpm for 30 minutes and centrifuged at 1500 rpm for 15 minutes to separate the layers. At this point the samples contained three layers: the top layer was hexane, the middle was acetonitrile and water and the bottom was the sediment. The upper hexane layer was transferred to 50.0-mL volumetric flasks. An addition aliquot of hexane (12.0-mL) was added to the acetonitrile and sediment in the vials. The samples were placed on a shaker table at 150 rpm for an additional 30 minutes and centrifuged at 1500 rpm for 15 minutes. The extracts were combined and taken to volume with hexane. The mid- and high-level samples were further diluted into the calibration standard range with hexane. An aliquot from each recovery sample was collected for analysis by gas chromatographic analysis with mass selective detection and negative chemical ionization (GC-MSD/NCI).

ANALYSIS

GC Parameters:

Column: DB-624 30m x 0.32mm x 1.8 µm
Temperature: 40 °C (initial) and held for 2.00 minutes
Ramps:

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
10.0	250	0.00
30.0	280	2.33

Run Time: 26.33 min
Injection Volume: 2.0 µL
Gas Flows: Carrier gas: Helium, at a constant flow of 65.2 mL/min
Inlet Mode: Splitless
Purge Time: on at 1.00 min at 60 mL/min
Inlet Temperature: 260 °C
Retention Time: approximately 11.3 minutes

MSD parameters

Solvent delay: 8.0 minutes
Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
281	100	quantitation D4
369	100	quantitation internal standard

Temperatures: MSD Transfer Line - 280 °C
MS Quad – 150 °C
MS Source – 230 °C

Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the system was performed by programmed automated injection.

CALCULATIONS

A calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (mg a.i./L) in the calibration standards against the natural logarithm (ln) of the peak area of the calibration standards. The equation of the line (equation 1) was algebraically

manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the natural logarithm (ln) of the peak area of the recovery sample and the dilution factor. Equations 2, 3 and 4 were then used to calculate measured concentrations and analytical results.

(1) $$\ln y = a(\ln x)^2 + b(\ln x) + c$$

(2) $$\ln x = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

(3) $$DC\,(x) = \text{inverse}(\ln x)$$

(4) $$A = DC \text{ x } DF$$

where:

y	=	detector response (peak area) for analyte
a, b, c	=	regression constants
x	=	detected concentration (µg a.i./L) in the sample
C	=	constant c minus the natural log of the peak area; C = (c - lny)
DC (x)	=	detected concentration in the sample
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

(5) $\ln(Area_{MIN}) = \ln(0.5 \times A_{LS})$

(6) $\ln(LOQ_{INST}) = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(7) $LOQ_{INST} = \text{inverse}(\ln LOQ_{INST})$

(8) $LOQ = LOQ_{INST} \times DF_{CTRL}$

where:

$Area_{MIN}$	=	one half of the mean detector response (peak area) of the low concentration calibration standard (two injections)
A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
a, b, c	=	regression constants
C	=	regression constant c – $Area_{MIN}$; $C = (c - \ln(Area_{MIN}))$
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

RESULTS AND DISCUSSION

The mean recovery was 82.1% ± 9.02%. Due to instrumental carryover the LOQ was set at 0.600 µg a.i./g, the lowest validated concentration. Measured test sample concentrations < 0.600 µg/g are reported as < 0.600 µg/g. The low concentration recoveries are reported even if they fall below 0.600 µg/g, because that is their nominal concentration. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70.0 to 120%.

Analytical results for the recovery of D4 from artificial sediment are presented in Table 2A. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample are presented in Figure 2A, Figure 2B and Figure 2C, respectively. A typical regression analysis for D4 is presented in Figure 2D.

Table 2A. Analytical results for the recovery of D4 from artificial sediment during the method validation study.

Sample ID	Fortified Concentration (µg a.i./g)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
Ctrl A	0.00	10.0	< 0.600	NA
Ctrl B	0.00	10.0	< 0.600	NA
Ctrl C	0.00	10.0	< 0.600	NA
Low A	0.600	10.0	0.512	85.4
Low B	0.600	10.0	0.452	75.4
Low C	0.600	10.0	0.366	61.0
Mid A	10.0	40.0	8.07	80.7
Mid B	10.0	40.0	8.92	89.2
Mid C	10.0	40.0	8.35	83.5
High A	100	400	88.1	88.1
High B	100	400	87.8	87.8
High C	100	400	87.5	87.5
MEAN				**82.1**
STD DEV				**9.02**
% CV				**11.0**

[a] Concentrations expressed as less than values were below the limit of quantitation (LOQ). Due to instrumental carryover the LOQ for this analysis is set at 0.600 µg/g, the lowest validated concentration. Measured test samples concentrations <0.600 µg/g are reported as < 0.600 µg/g.

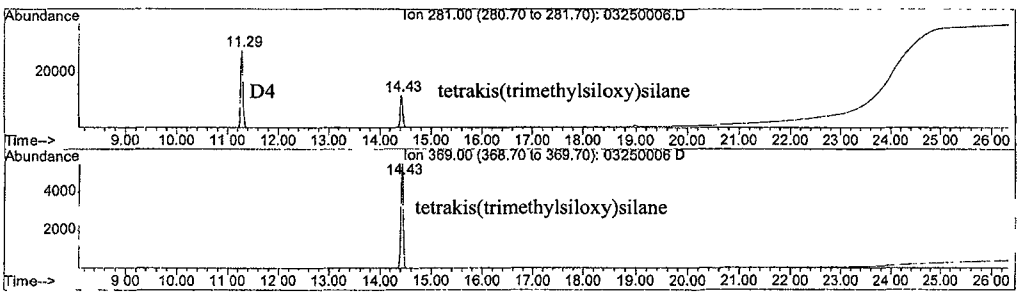
[b] NA = Not Applicable.

Note: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Figure 2A. Representative chromatogram of a 0.2 mg a.i./L calibration standard used for quantification during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250006.D
Operator    : MED
Acquired    : 25 Mar 2009  19:17     using AcqMethod D4SIM
Sample Name: std 0.2mg/l
Misc Info   :
Vial Number: 6
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```

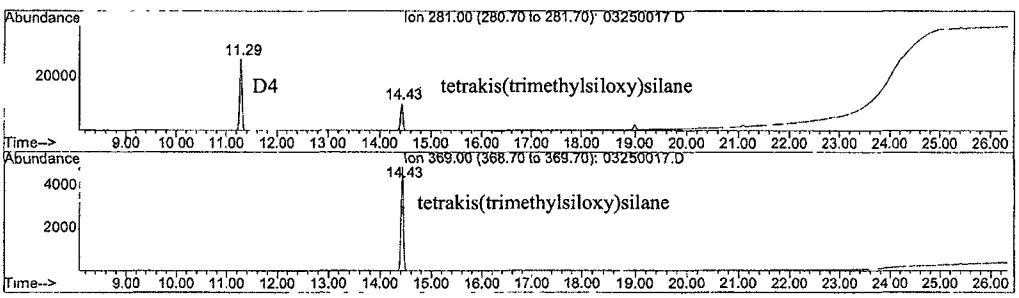


Retention Time	Area	Area %	Ratio %
281.00 amu			
11.290	905398	70.974	100.000
14.428	370270	29.026	40.896
369.00 amu			
14.429	173970	100.000	100.000

Figure 2B. Representative chromatogram of a 10.0 µg a.i./g recovery sample used during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250017.D
Operator    : MED
Acquired    : 26 Mar 2009   1:55     using AcqMethod D4SIM
Sample Name: MID B
Misc Info   :
Vial Number: 17
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```

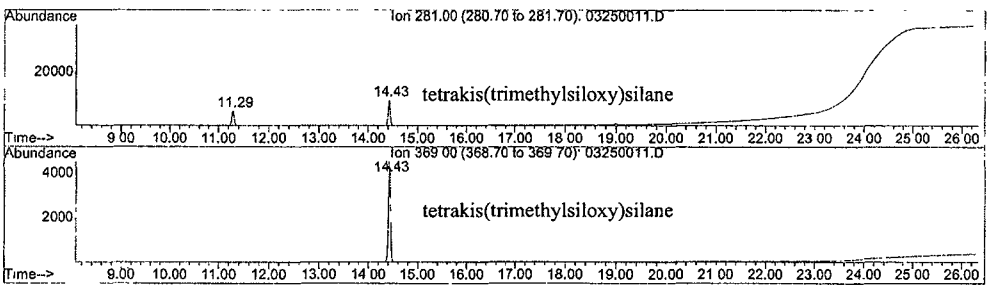


Retention Time	Area	Area %	Ratio %
281.00 amu			
11.289	866427	72.749	100.000
14.429	324553	27.251	37.459
369.00 amu			
14.432	153533	100.000	100.000

Figure 2C. Representative chromatogram of a control sample during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\MSDCHEM\1\DATA\D40325\03250011.D
Operator    : MED
Acquired    : 25 Mar 2009  22:22     using AcqMethod D4SIM
Sample Name: CTRL B
Misc Info   :
Vial Number: 11
CurrentMeth: C:\MSDCHEM\1\METHODS\D4SIM.M
```



Retention Time	Area	Area %	Ratio %
281.00 amu			
11.293	183597	37.211	59.262
14.429	309804	62.789	100.000
369.00 amu			
14.430	145441	100.000	100.000

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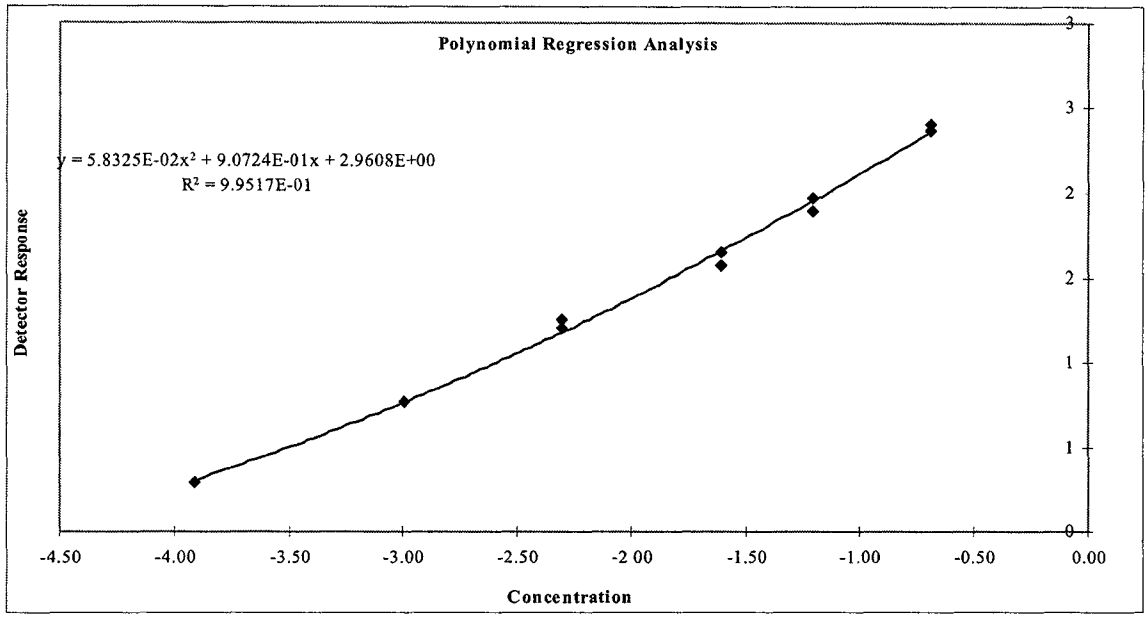
Note: The small peak observed at 11.3 minutes has an area that is below that of the lowest standard and is below the LOQ for this analysis. It is likely due to instrumental carryover from residual silicones in the system.

Figure 2D. A typical regression analysis for the calibration standards used to quantitate D4 during the method validation study.



Regression Analysis

$r^2 = 0.99517$
$y = 0.058325x^2 + 0.90724 + 2.9608$

ln Standard Concentration mg a.i./L	Standard Concentration mg a.i./L	ln Response Area	Standard Response Area	Internal Standard Response Area
-3.912	0.0200	--[a]	368592	167565
-2.996	0.0500	--[a]	444997	166994
-2.303	0.100	1.2528	599001	171133
-1.609	0.200	1.6495	905398	173970
-1.204	0.300	1.9713	1242092	172997
-0.693	0.500	2.4071	1942742	174992
-3.912	0.0200	0.2891	253442	189805
-2.996	0.0500	0.7647	412187	191858
-2.303	0.100	1.2058	601713	180176
-1.609	0.200	1.5649	963981	201573
-1.204	0.300	1.8918	1334634	201269
-0.693	0.500	2.3646	2063702	193960

[a] Standard not used due to extreme variance.

APPENDIX 3 – METHOD VALIDATION (NATURAL SEDIMENT)

SUMMARY

Methodology was validated (11 December 2008) to quantify the amount of D4 present in freshwater sediment. Recovery samples containing the test substance were extracted twice with acetonitrile and hexane. The hexane extracts were combined and diluted with hexane prior to analysis. An aliquot of the final extracts were analyzed by gas chromatography with mass selective detection and negative chemical ionization (GC-MSD/NCI).

This method was validated by fortification of freshwater sediment with D4 at nominal concentrations of 0.600, 10.0 and 100 µg a.i./g. Recoveries of D4 averaged 101 ± 18.1%. Due to instrumental carryover, the limit of quantitation (LOQ) was set at 0.600 µg a.i./g, lowest fortified sample concentration. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

EXPERIMENTAL

Equipment

1. Instrument:	Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector series 5973, Hewlett Packard autosampler, Hewlett Packard Model 7683 injector and Hewlett Packard ChemStation Version A.01.00 for data acquisition.
2. Balances:	Orion 0960, American Scientific Products SP182, Mettler PJ3000, Sartorius MA45C moisture balance
3. Shaker table:	Lab-Line orbit shaker
4. Centrifuge:	Beckman GPR
5. Laboratory equipment:	syringes, volumetric flasks, volumetric pipets, Pasteur pipets, autosampler vials, 45-mL glass vials with PTFE-lined caps, and amber glass bottles with Teflon®-lined caps

Reagents

1. Acetone:	Burdick & Jackson, reagent grade
2. Hexane:	Burdick & Jackson, reagent grade
3. Acetonitrile:	Burdick & Jackson, reagent grade
4. Nitric acid:	JT Baker, reagent grade
5. Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

Test Substance

The test substance, D4, was received on 8 July 2008 from Dow Corning Corporation, Midland, Michigan. The following information was provided:

Name:	D4
Synonyms:	octamethylcyclotetrasiloxane (Supplied as Dow Corning® 244 Fluid)
Batch No.:	LL084732
CAS No.:	556-67-2
Purity:	99.77%
Recertification Date:	17 June 2011

Upon receipt at Springborn Smithers, the test substance (SSL No. 130-24) was stored at room temperature in the original container in a dark ventilated cabinet. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

Internal Standard

The internal standard, tetrakis(trimethylsiloxy)silane, was received on 10 October 2008 from Gelest, Inc., Morrisville, Pennsylvania. The following information was provided:

Name:	tetrakis(trimethylsiloxy)silane
Synonym:	Trimethylsiloxysilicate (M_4Q)
Lot No.:	8D-12648
CAS No.:	3555-47-3
Purity:	99.8%
Recertification Date:	Not listed

Upon receipt at Springborn Smithers, the internal standard (SSL No. 132-03) was stored at room temperature in the original container in a dark ventilated cabinet. This sample was used in the quantitation of D4 in the exposure solutions and quality control samples during testing.

PROCEDURES

Preparation of Stock Solutions

A 10.0 mg a.i./mL primary stock solution was prepared by placing 0.2516 g of D4 (0.2510 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg a.i./L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with acetone. The 100 mg a.i./L secondary stock solution was used to fortify the low-level recovery samples. The 1000 mg a.i./L secondary stock solution was used to fortify the mid- and high-level recovery samples.

A 1.00 mg a.i./mL primary stock solution was prepared by placing 0.1007 g of D4 (0.1004 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with hexane. Two secondary stock solutions (10.0 and 100 mg a.i./mL) were prepared by placing 0.500 and 5.00 mL, respectively, of the 1.00 mg a.i./mL primary stock solution in separate 50.0-mL volumetric flasks and bringing each to volume with hexane. The 10.0 and 100 mg a.i./L secondary stock solutions were used to prepare calibration standards.

A 10.0 mg/mL tetrakis(trimethylsiloxy) silane internal standard stock solution was prepared by placing 0.2506 g of the internal standard in a 25.0-mL volumetric flask and bringing it to volume with acetone. Two secondary stock solutions (100 and 1000 mg/L) were prepared by placing 0.500 and 5.00 mL, respectively, of the 10.0 mg/mL primary internal standard stock solution in separate 50.0-mL volumetric flasks and bringing each volume with hexane and acetone, respectively. A 0.0500-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low recovery samples prior to analysis. Aliquots of 0.0200 and 0.200 mL of the 1000 mg/L secondary internal standard stock solution were added to the mid- and high-level recovery samples, respectively, prior to analysis.

All stock solutions were stored in a refrigerator in glass amber bottles fitted with Teflon®-lined caps.

Preparation of Calibration Standards

Calibration standards were prepared in hexane by fortifying with the 10.0 and 100 mg a.i./L secondary stock solutions to yield concentrations of 0.0200, 0.0500, 0.100, 0.200, 0.300 and 0.500 mg a.i./L. A 50-µL aliquot of the 100 mg/L secondary internal standard stock solution was added to the calibration standards prior to analysis. The calibration standard fortification procedure is presented in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Calibration Standard Concentration (mg a.i./L)
NA[a]	NA	100	0.0500	50.0	0.00
10.0	0.100	100	0.0500	50.0	0.0200
10.0	0.250	100	0.0500	50.0	0.0500
10.0	0.500	100	0.0500	50.0	0.100
100	0.100	100	0.0500	50.0	0.200
100	0.150	100	0.0500	50.0	0.300
100	0.250	100	0.0500	50.0	0.500

[a] NA = Not Applicable.

Sample Fortification and Preparation

All glassware used for the preparation and extraction of recovery samples was subjected to rigorous cleaning before use. The glassware was soaked and rinsed well with 20% nitric acid in water. This was followed by a water rinse to remove the nitric acid, and then a rinse with acetone to remove residual organics.

The recovery samples were prepared by fortifying freshwater sediment (5.00 g, dry weight) with D4 at concentrations of 0.600, 10.0 and 100 µg a.i./g. The low-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 100 mg a.i./L secondary stock solution. The mid- and high-level recovery samples were prepared by fortifying 5.00 g (dry weight) of freshwater sediment using the 1000 mg a.i./L secondary stock solution. Each concentration level was produced in triplicate. In addition, three recovery samples 5.00 g (dry weight) were left unfortified to serve as controls.

A 0.0500-mL aliquot of the 100 mg/L secondary internal standard stock solution was added to the control and low recovery samples prior to analysis. Aliquots of 0.0200 and 0.200 mL of the 1000 mg/L secondary internal standard stock solution were added to the mid- and high-level recovery samples, respectively, prior to analysis. The sample fortification procedure is presented in the following table:

D4 Stock Concentration (mg a.i./L)	Fortification Volume (mL)	Internal Standard Stock Concentration (mg/L)	Fortification Volume (mL)	Sample Mass (g)	Sample Concentration (µg a.i./g)
NA	NA	100	0.0500	5.00	0.00
100	0.0300	100	0.0500	5.00	0.600
1000	0.0500	1000	0.0200	5.00	10.0
1000	0.500	1000	0.200	5.00	100

[a] NA = Not Applicable.

Extraction

All recovery samples were prepared in 45-mL glass vials with PTFE-lined caps. An aliquot (6.00-mL) of acetonitrile was added to each sample. An aliquot of hexane (12.0 mL) was added to the vials that contained both sediment and acetonitrile. The samples were placed on a shaker table at 150 rpm for 30 minutes and centrifuged at 1500 rpm for 15 minutes to separate the layers. At this point, the samples contained three layers: the top layer was hexane, the middle was acetonitrile and water and the bottom was the sediment. The upper hexane layer was transferred to 50.0-mL volumetric flasks. An additional aliquot of hexane (12.0-mL) was added to the acetonitrile and sediment in the vials. The samples were placed on a shaker table at 150 rpm for an additional 30 minutes and centrifuged at 1500 rpm for 15 minutes. The hexane extracts were combined, taken to volume with hexane and mixed well. The mid- and high-level samples were further diluted into the calibration standard range with hexane. An aliquot from each recovery sample was collected for analysis by gas chromatographic analysis with mass selective detection (GC/MSD).

ANALYSIS

GC Parameters:

Column: Varian WCOT Fused Silica, CP-SIL 8 CB, 30m x 0.25 mm x 0.25 µm

Temperature: 40 °C (initial) and held for 2.00 minutes

Ramps:

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
10.0	250	0.00
30.0	300	2.33

Run Time: 27.0 min
Injection Volume: 2.0 µL
Gas Flows: Carrier gas: Helium, at a constant flow of 1.0 mL/min
Inlet Mode: Splitless
Purge Time: on at 1.00 min at 60 mL/min
Inlet Temperature: 260 °C
Retention Time: approximately 8.0 minutes

MSD parameters

Solvent delay: 6.0 minutes

Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
297	100	quantitation D4
311	100	quantitation internal standard

Temperatures: MSD Transfer Line - 280 °C
MS Quad – 106 °C
MS Source – 150 °C

Chemical Ionization gas: Methane

Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the system was performed by programmed automated injection.

CALCULATIONS

A calibration curve was constructed by plotting the natural logarithm (ln) of the analyte concentration (mg a.i./L) in the calibration standards against the natural logarithm (ln) of the peak area of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the natural logarithm (ln) of the peak area of the recovery sample and the dilution factor. Equations 2, 3 and 4 were then used to calculate measured concentrations and analytical results.

(1) $\ln y = a(\ln x)^2 + b(\ln x) + c$

(2) $\ln x = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(3) $DC(x) = \text{inverse}(\ln x)$

(4) $A = DC \times DF$

where:

y	=	detector response (peak area) for analyte
a, b, c	=	regression constants
x	=	detected concentration (µg a.i./L) in the sample
C	=	constant c minus the natural log of the peak area; C = (c - lny)
DC (x)	=	detected concentration in the sample
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

(5) $\ln(Area_{MIN}) = \ln(0.5 \times A_{LS})$

(6) $\ln(LOQ_{INST}) = \dfrac{-b + \sqrt{b^2 - 4aC}}{2a}$

(7) $LOQ_{INST} = \text{inverse}(\ln LOQ_{INST})$

(8) $LOQ = LOQ_{INST} \times DF_{CTRL}$

where:

$Area_{MIN}$	=	one half of the mean detector response (peak area) of the low concentration calibration standard (two injections)
A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
a, b, c	=	regression constants
C	=	regression constant c – $Area_{MIN}$; $C = (c - \ln(Area_{MIN}))$
LOQ_{INST}	=	limit of quantitation on the instrument
DF_{CTRL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

RESULTS AND DISCUSSION

The mean recovery was 101% ± 18.1%, with a limit of quantitation of 0.600 µg a.i./g. Due to instrumental carryover, the LOQ was set at lowest fortified sample concentration. These parameters, while relatively constant, do vary somewhat among runs and produce small variations in the LOQ. Defined limits for acceptance of quality control sample performance in subsequent studies were set at 70 to 120%.

Analytical results for the recovery of D4 from freshwater sediment are presented in Table 3A. Representative chromatograms from the analysis of a calibration standard, recovery sample and a control sample are presented in Figure 3A, Figure 3B and Figure 3C, respectively. A typical regression analysis for D4 is presented in Figure 3D.

Table 3A. Analytical results for the recovery of D4 from freshwater sediment during the method validation study.

Sample ID	Fortified Concentration (µg a.i./g)	Dilution Factor	Analytical Result (µg a.i./g)	Percent of Fortified
Ctrl A	0.00	10.0	< 0.600	NA
Ctrl B	0.00	10.0	< 0.600	NA
Ctrl C	0.00	10.0	< 0.600	NA
Low A	0.600	10.0	0.448	74.7
Low B	0.600	10.0	0.838	140
Low C	0.600	10.0	0.687	114
Mid A	10.0	40.0	10.5	105
Mid B	10.0	40.0	9.15	91.5
Mid C	10.0	40.0	9.21	92.1
High A	100	400	93.9	93.9
High B	100	400	97.9	97.9
High C	100	400	97.4	97.4
			MEAN	**101**
			STD DEV	**18.1**
			% CV	**18.0**

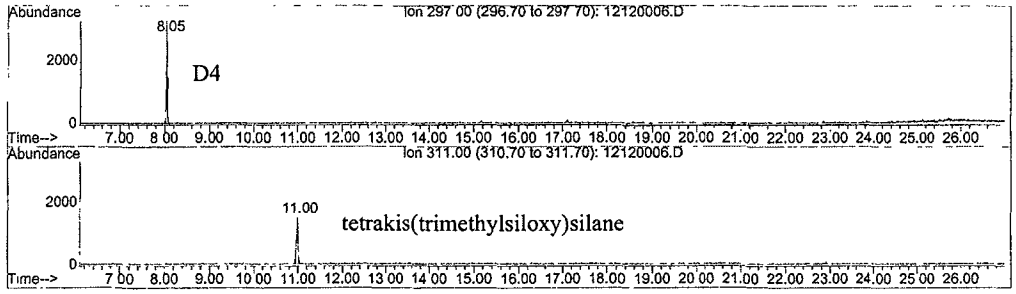
[a] Concentrations expressed as less than values were below the limit of quantitation (LOQ). The LOQ for the analysis was set at the lowest fortified sample concentration, 0.600 µg a.i./g, due to instrumental carryover.
[b] NA = Not Applicable.

Note: Results were calculated using the actual analytical (unrounded) results and not the rounded values presented in this table.

Figure 3A. Representative chromatogram of a 0.200 mg a.i./L calibration standard used for quantification during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120006.D
Operator    : MED
Acquired    : 12 Dec 2008  14:46     using AcqMethod D4CISIM
Sample Name: std 0.2mg/l
Misc Info   :
Vial Number: 6
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

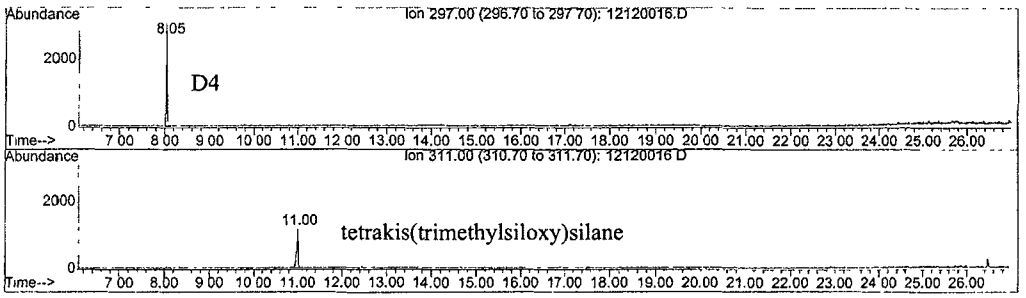


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.049	45339	100.000	100.000
311.00 amu			
10.999	37584	100.000	100.000

Figure 3B. Representative chromatogram of a 10 µg a.i./g recovery sample used during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120016.D
Operator    : MED
Acquired    : 12 Dec 2008  20:50     using AcqMethod D4CISIM
Sample Name: MID A
Misc Info   :
Vial Number: 16
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

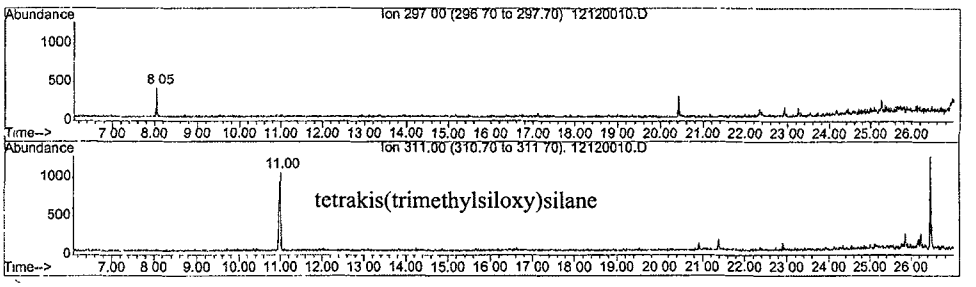


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.045	42937	100.000	100.000
311.00 amu			
11.000	28351	100.000	100.000

Figure 3C. Representative chromatogram of a control sample during the method validation study with D4.

Area Percent Report -- Sorted by Signal

```
Information from Data File:
File        : C:\HPCHEM\1\DATA\SSL1212\12120010.D
Operator    : MED
Acquired    : 12 Dec 2008  17:12     using AcqMethod D4CISIM
Sample Name: CTRL A
Misc Info   :
Vial Number: 10
CurrentMeth: C:\HPCHEM\1\METHODS\D4CISIM.M
```

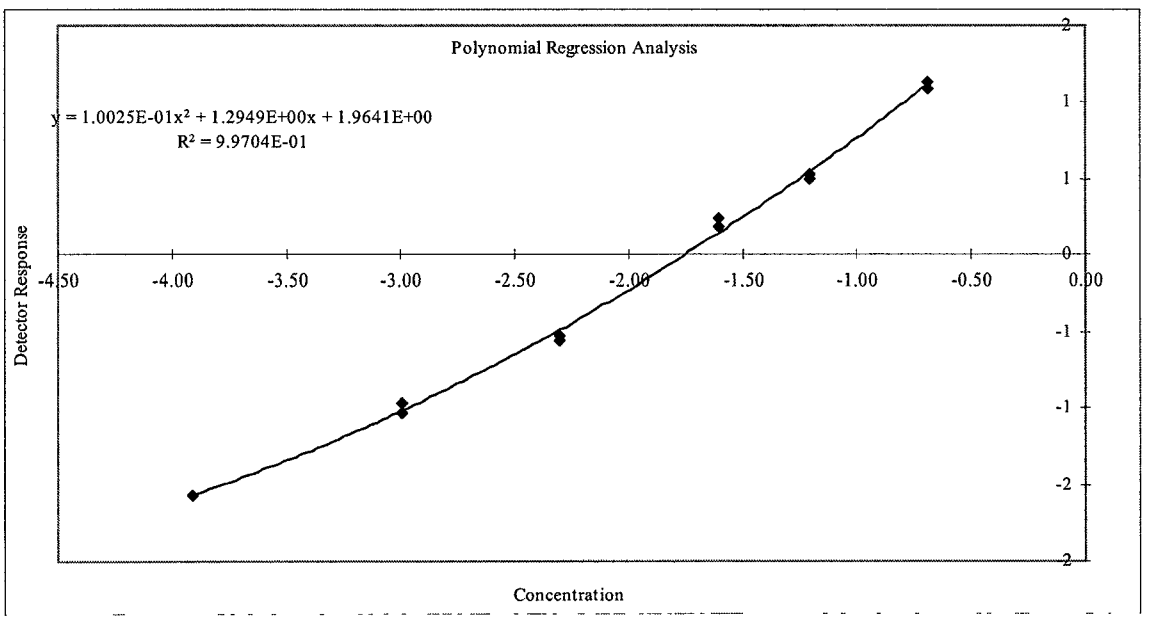


Retention Time	Area	Area %	Ratio %
297.00 amu			
8.047	6030	100.000	100.000
311.00 amu			
11.000	27235	100.000	100.000

12120010.D Tue Dec 16 15:10:33 2008 Page 1

Note: The peak at a retention time for D4, approximately 8.0 minutes, has an area below the lowest standard and is below the LOQ for the analysis. It is likely from instrumental carryover due to residual silicones in the system.

Figure 3D. A typical regression analysis for the calibration standards used to quantitate D4 during the method validation study.



Regression Analysis

$r^2 = 0.99704$

$y = 1.0025E\text{-}01x^2 + 1.2949E\text{+}00x + 1.9641E\text{+}00$

ln Standard Concentration mg a.i./L	Standard Concentration mg a.i./L	ln Response Area	Standard Response Area	IS Response Area
-3.912	0.0200		20941	44180
-2.996	0.0500	-0.9694	15814	41692
-2.303	0.100	-0.5545	23227	40441
-1.609	0.200	0.1876	45339	37584
-1.204	0.300	0.5018	63772	38610
-0.693	0.500	1.1380	96855	31037
-3.912	0.0200	-1.5684	6585	31600
-2.996	0.0500	-1.0258	11387	31762
-2.303	0.100	-0.5259	19046	32224
-1.609	0.200	0.2356	36943	29188
-1.204	0.300	0.5285	53895	31769
-0.693	0.500	1.0920	90338	30312

APPENDIX 4 – STUDY DATA

13937.6103 28 Day Oligochaete Results

Nominal Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes Recovered	Population Increase Factor	Tare Weight (g)	Tare Weight with Oligochaetes (g)	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
Control	A	17	12	1	30	3.0	1.23856	1.28094	42.38	30(2)	46(6)
	B	18	11	1	30	3.0	1.22723	1.27147	44.24		
	C	12	12	2	26	2.6	1.22901	1.26784	38.83		
	D	16	12	0	28	2.8	1.23434	1.28822	53.88		
	E	9	24	0	33	3.3	1.22692	1.27958	52.66		
	F	14	13	4	31	3.1	1.23580	1.28268	46.88		
Solvent Control	A	13	12	3	28	2.8	1.23938	1.28117	41.79	30(3)	51(9)
	B	16	10	0	26	2.6	1.22611	1.27226	46.15		
	C	23	6	2	31	3.1	1.24618	1.29514	48.96		
	D	21	10	2	33	3.3	1.23065	1.27887	48.22		
	E	20	11	1	32	3.2	1.22551	1.29258	67.07		
	F	16	9	3	28	2.8	1.23023	1.28523	55.00		
Pooled Control					356	3.0				30(2)	49(8)
3.1	A	19	12	0	31	3.1	1.22510	1.27475	49.65	31(1)	55(6)
	B	17	11	2	30	3.0	1.22090	1.28235	61.45		
	C	19	12	0	31	3.1	1.24622	1.30544	59.22		
	D	24	8	1	33	3.3	1.23232	1.28316	50.84		
6.3	A	10	14	6	30	3.0	1.21536	1.27088	55.52	31(3)	64(6)
	B	12	15	3	30	3.0	1.22850	1.29217	63.67		
	C	9	19	0	28	2.8	1.23838	1.30551	67.13		
	D	9	22	3	34	3.4	1.23233	1.30282	70.49		
13	A	16	10	2	28	2.8	1.23063	1.27541	44.78	28(1)	50(8)
	B	14	15	0	29	2.9	1.22938	1.2721	42.72		
	C	16	12	1	29	2.9	1.2229	1.27297	50.07		
	D	17	9	0	26	2.6	1.22521	1.28635	61.14		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation

13937.6103 28 Day Oligochaete Results (Continued)

Concentration (mg a.i./kg)	Replicate	LCW[a]	CW[a]	IW[a]	Total Number of Oligochaetes Recovered	Population Increase Factor	Tare Weight (g)	Tare Weight with Oligochaetes (g)	Biomass (mg)	Mean Number Of Oligochaetes per Replicate (SD[b])	Mean Biomass per Replicate per Concentration in Milligrams (SD)
25	A	10	16	0	26	2.6	1.23198	1.27633	44.35	29(3)	47(3)
	B	11	18	1	30	3.0	1.24069	1.29209	51.40		
	C	21	11	0	32	3.2	1.22556	1.27299	47.43		
	D	7	21	1	29	2.9	1.23199	1.27745	45.46		
50	A	11	10	4	25	2.5	1.22294	1.26058	37.64	26(3)	44(5)
	B	15	11	1	27	2.7	1.22783	1.27446	46.63		
	C	15	7	0	22	2.2	1.24010	1.28817	48.07		
	D	15	13	0	28	2.8	1.23125	1.27602	44.77		
100	A	11	11	0	22	2.2	1.22367	1.27355	49.88	22(1)	44(6)
	B	12	11	0	23	2.3	1.22541	1.26262	37.21		
	C	12	7	2	21	2.1	1.22857	1.27658	48.01		
	D	10	10	3	23	2.3	1.24487	1.28749	42.62		

[a] LCW = Large, complete worms; CW = Complete worms; IW = Incomplete worms
[b] SD = Standard Deviation

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OCTAMETHYLCYCLOTETRASILOXANE (D_4): A PROLONGED SEDIMENT TOXICITY TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED ARTIFICIAL SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-110B

ASTM Standard E 1706-00

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

ECB Technical Guidance Document on Risk Assessment

Draft OECD Guideline September 2006

AUTHORS:

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STUDY INITIATION DATE: July 13, 2007

STUDY COMPLETION DATE: June 25, 2009

SUBMITTED TO:

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D_4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-110B

STUDY COMPLETION: June 25, 2009

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exception:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods.

The characterization and the stability of the internal standard under conditions of storage at the test site were not determined in compliance with GLP standards.

STUDY DIRECTOR:

Henry O. Krueger, Ph.D
Director of Aquatic, Plant and Insect Toxicology

6/25/09
Date

SPONSOR APPROVAL:

Sponsor Representative

7/1/09
Date

Applicant/Submitter

Date

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM(98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: STUDY DIRECTOR	MANAGEMENT:
Initial Trial			
Test Substance Preparation	July 17, 2007	July 17, 2007	July 30, 2007
Protocol	July 19, 2007	July 19, 2007	October 9, 2007
Dissolved Oxygen Measurements	August 16, 2007	August 16, 2007	August 27, 2007
Calibration Standard Preparation	August 17, 2007	August 17, 2007	August 30, 2007
Second Trial			
Test Substanee Preparation	October 1, 2007	October 1, 2007	October 8, 2007
Observations	Oetober 31, 2007	October 31, 2007	November 12, 2007
Calibration Standard Preparation	November 1, 2007	November 1, 2007	February 25, 2008
Third Trial			
Dissolved Oxygen Measurements	December 27, 2007	Deeember 27, 2007	January 7, 2008
Analytical Data and Draft Report	January 29 – 30, 2009	February 3, 2009	February 16, 2009
Biological Data and Draft Report	January 30 – February 4, 2009	February 4, 2009	February 23, 2009
Final Report	June 25, 2009	June 25, 2009	June 25, 2009

All inspections were study-based unless otherwise noted.

Jeff H. Suzuki
Quality Assurance Representative

June 25, 2009
Date

REPORT APPROVAL

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D_4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-110B

STUDY DIRECTOR:

Henry O. Krueger, Ph. D.
Director of Aquatic, Plant and Insect Toxicology

Date 6/25/09

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Date 6/25/09

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

Willard B. Nixon, Ph. D.
Director of Chemistry

Date 6/25/09

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SUMMARY

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic)

TITLE: Octamethylcyclotetrasiloxane (D_4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-110B

TEST SUBSTANCE: Octamethylcyclotetrasiloxane (D_4); Dow Corning® 244 Fluid; Lot LL084732; CAS Number: 556-67-2; Purity: 99.75%; Expiration Date: 1/19/09

TEST DATES:

Study Initiation:	July 13, 2007
Experimental Start (OECD):	November 26, 2007
Experimental Start (EPA):	November 29, 2007
Biological Termination:	December 27, 2007
Experimental Termination:	January 2, 2008

LENGTH OF EXPOSURE: 28 Days

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

AGE OF TEST ORGANISMS: Adult

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
Superior, Wisconsin 54880
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
3.8 mg/Kg	0.73 mg/Kg
7.5 mg/Kg	1.5 mg/Kg
15 mg/Kg	3.1 mg/Kg
30 mg/Kg	5.8 mg/Kg
60 mg/Kg	11 mg/Kg
120 mg/Kg	38 mg/Kg

RESULTS: Based on mean measured test concentrations
28 Day EC50: 9.32 mg/Kg with 95% confidence limits of 4.38 and 25.4 mg/Kg
NOEC: <0.73 mg/Kg
LOEC: 0.73 mg/Kg

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Centre Europeen des Silicones, European Chemical Industry Council (Cefic) at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. An initial trial was conducted from July 20, 2007 to August 17, 2007, but was repeated at lower concentration in order to achieve a NOEC. A second trial was conducted from October 4, 2007 to November 1, 2007, but was repeated due to low reproduction. The in-life phase of the definitive test was conducted from November 29, 2007 to December 27, 2007 and dry-weight measurements were completed on January 2, 2008. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 570A-110B in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated D_4, on the oligochaete, *Lumbriculus variegatus* during a 28-day exposure period under flow-through test conditions. The measured endpoints of the test were survivorship (original organisms and/or offspring) and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of oligochaetes were exposed to a geometric series of six test concentrations and a negative control for 28 days under flow-through test conditions. Eight replicate test compartments were maintained in each treatment and control group, with 10 oligochaetes in each test compartment, for a total of 80 oligochaetes per test concentration. Each test compartment contained a quantity of sediment and overlying water. An additional five replicates were added in each treatment and control group for analytical sampling of sediment. Organisms were added at test initiation to one analytical replicate for the Day 7 and Day 28 analyses. The remaining three analytical replicates for Days 0, 7 and 28 were "abiotic" and did not contain organisms.

Nominal test concentrations selected in consultation with the sponsor were 3.8, 7.5, 15, 30, 60 and 120 mg/Kg of sediment based on the dry weight of the sediment. The results of the study are based on mean measured test concentrations. Due to the low solubility of D_4 in water (56.2 µg/L) only sediment samples were collected and analyzed from the "analytical replicates" of each treatment group and control (1). Samples were collected immediately after the addition of test organisms to the test

system on Day 0, on Day 7 and at the end of the test. Results of the analyses were used to verify the exposure over time.

Test compartments were indiscriminately positioned in a diluter unit according to treatment group approximately 48 hours prior to test initiation to condition the sediment. Oligochaetes were impartially assigned to exposure compartments at test initiation. Observations of mortality and abnormal behavior were made daily during the test. Survivorship/reproduction and growth (dry weights) were determined at the end of the 28-day test period. The percent reduction in the numbers of organisms present in the treatment groups at test termination in comparison to the control group was used to determine the 28-day EC50 value. The lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) were determined by the concentration-response pattern and statistical analysis of the survival/reproduction and dry weight data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Octamethylcyclotetrasiloxane (D_4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (2); the ASTM E 1706-00 Guideline: *Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (5), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (6). This study also generally meets OECD Guideline 225, Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment (7); although the guideline was not adopted when the protocol was signed (8).

Test Substance

The test substance was received from Dow Corning on June 22, 2007 and assigned Wildlife International identification number 8160. The test substance, a liquid, was identified as: Dow Corning® 244 Fluid; CAS Number 556-67-2; Batch No. LL084732. The reported purity was 99.75%, with an expiration date of January 19, 2009 (Appendix 2). The test substance was stored under ambient conditions.

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and was assigned Wildlife International, Ltd. identification number 7566 upon receipt. The internal standard, described as a liquid, was identified on the label as: Tetrakis (trimethylsilyloxy) silane; 97%; Lot #: 13222MB; CAS #: 3555-47-3. The standard had a reported purity of 97%. The standard was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (9) was used as the test sediment. The sediment was composed of approximately 10% peat, 20% kaolin clay, and 70% industrial quartz sand (Appendix 3). The dry constituents of the sediment were mixed in a mixer for approximately 20 minutes without the peat. The peat was mixed with the test material and added to the rest of the ingredients on a later date. The pH of the final mixture of sediment was found to be 7.3. The percent organic carbon of the sediment was 2.4. The sediment was stored under ambient conditions until used.

A 28-day ration of food (200 mg salmon starter) was dry mixed into the test compartments after the sediment was added, but immediately before adding the water to the test compartments, and 48 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. Just as in the OECD 218 Guideline, the food added to the test system during the test was not included in the measurement of organic carbon.

Preparation of Test Concentrations

Nominal test concentrations (3.8, 7.5, 15, 30, 60 and 120 mg/Kg) were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer. Second, a predetermined amount of sand and clay were added to the peat and test material in the Nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 30 minutes).

After mixing, approximately 100 mL of dosed formulated sediment was transferred to 13 replicate test beakers (300 mL glass beakers with stainless steel mesh covered holes on opposite

sides). The transfer time was kept to a minimum (approximately 10-20 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 13 beakers had approximately 100 mL of sediment added, a 28-day ration of food (approximately 200 mg of Salmon Starter) was added and gently stirred into the dry sediment. Finally, approximately 150 mL of well water was added to the beakers. After the water was added to the beakers, the beakers were placed in the water bath that was used for the study. The overlying water in the test beakers was exchanged two times daily.

Of the thirteen replicate test compartments prepared for each treatment and control group, eight test compartments were used for the evaluation of survival and growth and five compartments for analytical purposes. The sediment/water mixtures were allowed to acclimate for approximately 48 hours prior to the introduction of the test organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 14-day holding period immediately preceding the test, water temperature ranged from 22.1 to 24.7°C, while the pH ranged from 8.2 to 8.8 and dissolved oxygen ranged from 7.1 to 8.4 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, adult oligochaetes were collected from the culture and impartially added one and two at a time into transfer chambers until each chamber contained 10 organisms. Each group of ten organisms was impartially assigned and transferred to a test compartment. All transfers were made below the water surface using wide-bore pipettes. Ten replicate test compartments in each treatment and control group contained organisms (eight test compartments for the evaluation of effects plus two compartments for analytical purposes), while three test compartment per group were maintained without organisms for analytical purposes.

Oligochaetes were fed salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the holding period and during the test. A 200 mg aliquot of food (enough food for the entire study) was weighed for each test compartment that contained organisms during the test and was added to the test compartments after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. No additional food supplementation was made during the study.

Dilution Water

The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep loeated on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water. The specific conductance, hardness, alkalinity and pH measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The test apparatus consisted of test compartments placed in stainless steel diluter tanks. The tanks were placed in a temperature-controlled water bath in a Wildlife International, Ltd. diluter unit. One tank was assigned to each treatment or control group. Standpipes were placed in each tank to maintain the water height. Each diluter tank had above it a splitting chamber. Dilution water was delivered on a timer to the splitting chamber two times per day. Each splitting chamber contained 14 syringes. Each syringe delivered dilution water to individual test compartments which were indiscriminately and carefully positioned in diluter tanks according to treatment group.

Test compartments were 300-mL glass beakers with two stainless steel mesh-covered holes on opposite sides to allow for the flow of water through the test compartment. Each beaker contained approximately 100 mL of sediment (2.7 cm in a representative test chamber) and approximately 150 mL of overlying water. The depth of overlying water in each test compartment was maintained by the water levels in the diluter tanks (e.g. 7.0 cm in a representative test compartment). The diluter was adjusted so

that approximately 784 ml of water was delivered per minute for four minutes to each splitting chamber two times per day resulting in approximately two volume additions in each test compartment per day. Test compartments were labeled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 316 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test compartment at the beginning and end of the test, and daily during the test, using a liquid-in-glass thermometer. Temperature also was measured continuously in a beaker beside the test chambers in the negative control diluter tank using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one replicate (alternating) test compartment of each treatment and control group at the beginning and end of the test and daily during the test. Measurements of pH were made on samples of overlying water collected from one replicate (alternating) test compartment of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a composite of the overlying water from the control group replicates and the highest concentration treatment group (120 mg/Kg) replicates at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (10). Specific conductance was measured using a

Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test compartments at the beginning of the test, an additional 20 organisms were impartially selected at the beginning of the test and measured for dry weight. The test compartments were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). On Day 28 of the test, oligochaetes were removed from the sediment, and the numbers of live or dead worms were enumerated. Since *Lumbriculus variegatus* generally reproduces over a 28-day period, an increase in the number or size of worms in each test compartment is not unexpected. However, it is not possible to differentiate between adults and young. Therefore, survival and reproduction were considered one endpoint, i.e. the total number of organisms present at test termination.

Dry Weight Measurements

At test termination, each surviving worm was rinsed of excess sediment, placed in a pre-weighed labeled crucible and dried for approximately six days at approximately 60°C. The worms were weighed after allowing the crucibles to cool at room temperature for approximately one hour.

Statistical Analyses

The results of the test were based on the mean measured sediment concentrations (calculated using the measured concentrations in the biotic replicates only). The 28-day EC50 value, based on survival and reproduction was determined using the statistical program TOXSTAT version 3.5 (11).

The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival/reproduction and dry weight data. Using the statistical program TOXSTAT Version 3.5 the survival/reproduction and dry weight (growth) data were evaluated for normality (Chi-Square Test) and homogeneity of variances (Levene's Test or Bartlett's Test). After the data was deemed normal with homogeneous variance, the survival/reproduction and dry weight data were analyzed using Dunnett's t-test to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group (11,12).

Analytical Sampling

Analyses of sediment were conducted using separate test compartments collected from the control group and from each concentration treatment groups just after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28. The replicate used for sample collection on Day 0 did not contain oligochaetes. The Day 7 and Day 28 analytical replicates each had a biotic and an abiotic replicate. The sediment and overlying water were mixed to create a slurry and the slurry was used for analysis. The samples were analyzed on the day of collection.

Analytical Method

The analytical method used for the analysis of D_4 was developed by Wildlife International, Ltd. Sediment was sampled from the test beakers and approximately 5 mL of sediment was added to a centrifuge tube. To each sample, 10.0 mL of HPLC grade bottled water and 250 µL of internal standard was added (225 µL was added to the high level matrix fortification). Samples were vortexed and shaken after the addition of approximately 20 mL of ethyl acetate and the samples were centrifuged for approximately five minutes at 60% speed. The top layer was transferred into a 50 mL volumetric flask containing 2.5 g of sodium sulfate. Samples were extracted with an additional 20 mL of ethyl acetate. The extracts (1.00 mL) were transferred into autosampler vials and 50.0 µL N-methyl, N-trimethylsilyl trifluoroacetamide was added to each vial. Samples were then submitted for analysis.

Concentrations of D_4 in the samples were determined using an Agilent Model 6890 Gas Chromatograph (GC) with an Agilent Model 5975 Mass Selective Detector operated in SIM mode. Chromatographic separations were achieved using a Phenomen ZB-5 column (30 m x 0.25 mm, 0.25 µm film thickness). A method flowchart for the analysis of D_4 is presented in Appendix 6.1 and typical instrumental parameters are summarized in Appendix 6.2.

Calibration standards of D_4 analytical standard, ranging in concentration from 0.0500 to 6.00 mg/L were prepared using stock solutions of D_4 analytical standard in 80% dimethylformamide (DMF): 20% toluene. Each standard was fortified with 50.0 or 45.0 µL of the internal standard prior to final volume (Appendix 6.3). Calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the ratio of the peak area responses versus the respective concentrations of the calibration standards. The concentration of D_4 in the samples was determined by substituting the ratio 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 6.4.

The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (0.0500 mg a.i./L) and the dilution factor of the negative control samples. Each sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample.

Sediment samples were fortified at 3.80, 15.0 and 120 mg/Kg using stock solutions of D_4 in 80% DMF : 20% toluene (Appendix 6.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 55.4 to 110% of nominal concentrations (Appendix 6.5).

A representative calibration curve is presented in Appendix 6.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 6.7 and 6.8, respectively. A representative chromatogram of a matrix fortification sample is presented in Appendix 6.9. A representative chromatogram of a test sample is presented in Appendix 6.10.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of D_4 in samples collected during the definitive test are presented in Table 1 and Appendix 6. Nominal concentrations seleeted for use in this definitive test were 3.8, 7.5, 15, 30, 60 and 120 mg/Kg.

Samples of sediment were collected from the negative eontrol and each of the test concentrations on Day 0, Day 7 and Day 28. The purpose of these samples was to verify the concentrations in sediment at test initiation and to monitor the test substance over the course of the study. Results are based on nominal eoneentrations. Negative control samples on Days 0, 7, and 28 were all below the limit of quantitation (LOQ).

Measured concentrations of D_4 in sediment are presented in Table 1. On Day 0, there was 1.00, 2.66, 3.02, 6.99, 11.9 and 45.1 mg/Kg measured in the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg treatment groups, respeetively. Measured concentrations of D_4 in sediment from the "biotic" replicates from the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg nominal test concentrations on Day 7 were 0.390, 0.620, 3.95, 5.27, 9.62 and 35.7 mg/Kg, respectively. Measured concentrations of D_4 in sediment from the "abiotic" replicates from the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg nominal test concentrations on Day 7 were 0.148, 0.492, 1.76, 5.36, 8.07 and 27.6 mg/Kg, respectively. Measured concentrations of D_4 in sediment from the "biotic" replicates from the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg nominal test concentrations on Day 28 were 0.791, 1.24, 2.24, 5.24, 11.6 and 34.4 mg/Kg, respectively. Measured concentrations of D_4 in sediment from the "abiotic" replicates from the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg nominal test concentrations on Day 28 were 0.795, 1.83, 2.56, 4.41, 7.86 and 40.8 mg/Kg, respectively. All concentrations showed a stable trend in the sediment throughout the study. The mean measured test concentrations (using the results of the biotic replicates only) for the 3.8, 7.5, 15, 30, 60 and 120 mg/Kg treatment groups were 0.73, 1.5, 3.1, 5.8, 11 and 38 mg/Kg respectively, representing 19.1, 20.1, 20.5, 19.4, 18.4 and 32.0 percent of nominal, respectively.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4 respectively. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 78% (6.6 mg/L) of saturation throughout the test. Measurements of pH ranged from 8.4 to 8.6 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 5 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatment and control groups. At test termination, there was an increase in the numbers of oligochaetes present in some test compartments from the 10 worms originally placed in each replicate, indicating that reproduction had occurred. The mean number of worms in the negative control group at test termination was 24.1 worms (Table 6). The mean number of worms in the 0.73, 1.5, 3.1, 5.8, 11 and 38 mg/Kg treatment groups was 16.6, 18.1, 15.1, 13.5, 11.4 and 9.5, respectively, at test

termination. There were statistically significant differences ($p < 0.05$) in all treatment groups when compared to the negative control group, therefore the no observed effect concentration (NOEC) was estimated to be <0.73 mg/Kg, the lowest concentration tested and the lowest observed test concentration (LOEC) was determined to be 0.73 mg/Kg.

At the beginning of the test, a group of 20 organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the average individual dry weight of those twenty organisms was 0.58 mg (Appendix 8). The average individual dry weights for the surviving worms in each replicate at test termination are presented in Appendix 8. The average dry weight per worm in the negative control group was 0.70 mg (Table 6). The average dry weight per worm in the 0.73, 1.5, 3.1, 5.8, 11 and 38 mg/Kg treatment groups was 0.62, 0.67, 0.62, 0.72, 0.64 and 0.52 mg, respectively. The dry weights of the treatment groups were not significantly different ($p > 0.05$) from the negative control weights, therefore the NOEC was determined to be 38 mg/Kg and the LOEC was determined to be >38 mg/Kg.

CONCLUSIONS

The 28-day EC50 value for oligochaetes (*Lumbriculus variegatus*) exposed to D_4 in sediment was 9.32 mg/Kg, with a 95% confidence interval of 4.38 and 25.4 mg/Kg. Determination of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) was based on an evaluation of the survival/reproduction and dry weight data. Based on the survival/reproduction results of this study, the LOEC was 0.73 mg/Kg dry weight of sediment and the NOEC was <0.73 mg/Kg dry weight of sediment.

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Table 1

Measured Concentrations of D_4 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-110B-)	Sampling Time (Days)	Measured Concentration (mg/Kg)	Percent of Nominal [1]	Mean Measured Concentration (Percent of Nominal)
Negative Control	1 (abiotic)	0	<0.387	--	--
	8 (biotic)	7	<0.380	--	
	9 (abiotic)	7	<0.409	--	
	22 (biotic)	28	<0.510	--	
	23 (abiotic)	28	<0.487	--	
3.8	2 (abiotic)	0	1.00	26.4	0.73 (19.1)
	10 (biotic)	7	0.390	10.3	
	11 (abiotic)	7	0.148	3.90	
	24 (biotic)	28	0.791	20.8	
	25 (abiotic)	28	0.795	20.9	
7.5	3 (abiotic)	0	2.66	35.4	1.5 (20.1)
	12 (biotic)	7	0.620	8.27	
	13 (abiotic)	7	0.492	6.56	
	26 (biotic)	28	1.24	16.5	
	27 (abiotic)	28	1.83	24.3	
15	4 (abiotic)	0	3.02	20.1	3.1 (20.5)
	14 (biotic)	7	3.95	26.3	
	15 (abiotic)	7	1.76	11.7	
	28 (biotic)	28	2.24	15.0	
	29 (abiotic)	28	2.56	17.1	
30	5 (abiotic)	0	6.99	23.3	5.8 (19.4)
	16 (biotic)	7	5.27	17.6	
	17 (abiotic)	7	5.36	17.9	
	30 (biotic)	28	5.24	17.5	
	31 (abiotic)	28	4.41	14.7	

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[2] Analytical "biotic" replicates contained organisms, while "abiotic" replicates did not. Mean measured concentrations were calculated using the Day 0 analytical results and the "biotic" replicate results from Days 7 and 28, only.

Table 1 (Continued)

Measured Concentrations of D_4 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-110B-)	Sampling Time (Days)	Measured Concentration (mg/Kg)	Percent of Nominal [1]	Mean Measured Concentration (Percent of Nominal)
60	6 (abiotic)	0	11.9	19.9	11 (18.4)
	18 (biotic)	7	9.62	16.0	
	19 (abiotic)	7	8.07	13.4	
	32 (biotic)	28	11.6	19.4	
	33 (abiotic)	28	7.86	13.1	
120	7* (abiotic)	0	45.1	37.6	38 (32.0)
	20* (biotic)	7	35.7	29.7	
	21* (abiotic)	7	27.6	23.0	
	34* (biotic)	28	34.4	28.6	
	35* (abiotic)	28	40.8	34.0	

[1] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[2] Analytical "biotic" replicates contained organisms, while "abiotic" replicates did not. Mean measured concentrations were calculated using the Day 0 analytical results and the "biotic" replicate results from Days 7 and 28, only.
* Sample reanalyzed.

Table 2

Temperature of Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Day	Temperature (°C)													
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13
	Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control		23.1	22.9	22.9	22.9	23.0	22.8	23.0	22.9	22.8	23.1	23.1	22.8	23.2	23.3
0.73		23.1	22.8	23.0	23.0	22.9	22.9	23.1	23.1	22.9	23.1	23.2	22.8	23.2	23.3
1.5		23.1	22.8	22.8	23.0	22.9	22.8	23.0	23.0	22.8	23.0	23.1	22.8	23.1	23.3
3.1		23.0	22.9	22.9	23.0	22.9	23.0	22.9	23.0	22.9	23.1	23.1	22.8	23.0	23.3
5.8		23.1	22.8	22.8	23.0	22.9	23.0	22.9	23.0	22.8	22.9	23.0	23.0	23.1	23.3
11		23.1	22.9	22.9	23.1	22.9	23.0	23.1	23.1	22.9	23.1	23.0	22.8	23.0	23.3
38		23.0	22.8	22.9	23.0	22.9	22.8	22.8	22.9	23.0	23.0	22.9	22.8	23.0	23.3

[1] Temperature measured continuously in the negative control diluter tank ranged from approximately 23.0 to 24.0°C during the test.

Table 2 (Continued)

Temperature of Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Day	Temperature (°C)														
		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E
Negative Control		23.0	23.0	22.7	23.1	22.9	23.0	23.0	22.9	23.0	23.0	23.2	23.1	23.0	22.8	23.1
0.73		23.1	22.9	22.9	23.0	22.8	23.0	23.1	23.1	23.1	23.1	23.2	23.1	23.1	22.9	23.0
1.5		22.9	22.9	22.8	23.2	22.8	22.9	23.0	22.7	22.9	22.8	23.2	23.1	23.0	22.8	22.8
3.1		23.0	23.0	22.9	23.1	22.9	23.0	23.1	22.9	22.9	23.1	23.2	23.1	23.0	23.1	22.9
5.8		22.8	23.0	23.0	23.1	22.8	22.8	22.9	22.8	23.0	22.9	23.3	23.2	23.1	22.8	22.8
11		23.1	23.1	22.9	23.2	22.9	23.0	23.2	23.1	23.1	23.2	23.3	23.2	23.0	22.9	23.0
38		22.9	23.0	22.9	23.1	22.8	22.9	23.0	22.7	22.9	23.0	23.3	23.2	23.0	22.8	22.8

Table 3

Dissolved Oxygen in Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Day	Dissolved Oxygen (mg/L)													
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13
	Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control		8.1	7.8	7.8	8.0	7.7	7.8	7.8	7.5	8.1	8.0	8.1	7.8	7.9	7.3
0.73		8.1	8.0	7.9	7.9	7.8	7.7	7.8	7.3	8.5	8.0	8.0	7.6	7.8	7.2
1.5		8.1	7.9	7.9	7.9	7.5	7.6	7.7	7.5	8.3	7.8	8.0	7.6	7.6	7.1
3.1		8.1	8.1	7.9	7.9	7.7	7.7	7.7	7.4	8.3	8.0	8.0	7.8	7.8	7.2
5.8		8.1	8.0	7.9	7.9	7.5	7.6	7.8	7.3	8.2	7.9	7.9	7.5	7.7	7.1
11		8.0	8.0	7.9	7.9	7.8	7.5	7.8	7.5	8.4	7.8	8.0	7.7	7.9	7.1
38		8.1	7.9	7.9	8.1	7.7	7.7	7.8	7.5	8.2	8.0	8.0	7.9	7.8	7.1

[1] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

Table 3 (Continued)

Dissolved Oxygen in Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Day	Dissolved Oxygen (mg/L)														
		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E
Negative Control		7.8	7.9	7.3	6.6	8.2	7.9	8.0	8.1	8.5	8.2	8.0	7.4	8.1	6.8	7.9
0.73		7.7	7.9	7.2	6.7	8.1	7.6	8.0	8.0	8.4	8.0	8.1	7.5	8.2	6.9	7.8
1.5		7.7	7.9	7.3	6.7	8.0	8.0	7.8	7.9	8.3	8.0	8.1	7.6	7.1	7.4	7.8
3.1		7.8	8.1	7.2	6.8	8.3	8.4	8.1	8.0	8.3	8.1	8.2	7.7	8.0	7.6	7.8
5.8		7.7	7.8	7.2	7.1	8.0	7.9	8.1	8.0	8.3	8.1	8.1	7.7	8.0	7.4	7.9
11		7.8	7.9	7.3	7.1	8.1	8.2	8.3	7.9	8.3	8.1	8.1	7.7	6.7	7.6	7.7
38		7.9	8.1	7.3	7.0	8.2	8.2	8.1	8.1	8.4	8.2	8.0	7.7	8.0	7.7	7.8

[1] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

Table 4

pH of Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Replicate:	pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
		A	H	G	F	E
Negative Control		8.5	8.5	8.5	8.4	8.4
0.73		8.5	8.5	8.5	8.5	8.4
1.5		8.5	8.5	8.6	8.4	8.5
3.1		8.5	8.5	8.6	8.5	8.5
5.8		8.5	8.5	8.5	8.5	8.5
11		8.5	8.5	8.6	8.5	8.5
38		8.5	8.5	8.6	8.5	8.5

Table 5

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/cm)	Day 0 Ammonia (mg/L)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)	Day 28 Ammonia (mg/L)
Negative Control	150	189	335	<0.17[1]	128	166	305	<0.17[1]
38	146	187	345	<0.17[1]	124	168	315	<0.17[1]

Measurements were taken in a composite made from each of the replicates of the respective treatment groups.
[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Table 6

Mean Survival/Reproduction and Growth of Oligochaetes
(*Lumbriculus variegatus*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Mean Number of Surviving Worms (± Stdev)[1,3]	Percent Reduction[2] (%)	Mean Individual Dry Weight (mg)	Percent Reduction[2,4] (%)
Negative Control	24.1 (± 5.4)	-	0.70	--
0.73	16.6 (± 3.8)	31[3]	0.62	11
1.5	18.1 (± 3.5)	25[3]	0.67	4.3
3.1	15.1 (± 2.4)	37[3]	0.62	11
5.8	13.5 (± 4.6)	44[3]	0.72	-2.9
11	11.4 (± 2.6)	53[3]	0.64	8.6
38	9.5 (± 0.76)	61[3]	0.52	26

[1] Each replicate contained 10 oligochaetes at test initiation. The mean number of surviving worms is based on the total number of living worms at test termination since offspring cannot be differentiated from adults.
[2] Percent reduction was calculated in relation to the mean of the negative control.
[3] There were statistically significant differences ($p<0.05$) in the mean number of surviving worms in comparison to the negative control group using Dunnett's test.
[4] There were no statistically significant differences ($p>0.05$) in the mean individual dry weight of surviving worms in comparison to the negative control group using Dunnett's test.

Appendix 1

Study Protocol, Amendments and Deviation

PROTOCOL

OCTAMETHYLCYCLOPENTASILOXANE (D_4): A PROLONGED SEDIMENT TOXICITY TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

Guidelines:

Draft OECD Guideline
September 2006

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
570A-110

SPONSOR

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

PERFORMING LABORATORY

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601 USA
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July 5, 2007

Wildlife International, Ltd.

OCTAMETHYLCYCLOPENTASILOXANE (D_5): A PROLONGED SEDIMENT TOXICITY TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

SPONSOR: Silicones Environmental, Health and Safety Council
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TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
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STUDY DIRECTOR: Henry O. Krueger Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants and Insects
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Willard B. Nixon, Ph. D.
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 7/13/07

LABORATORY MANAGEMENT — DATE 7/10/07

SPONSOR'S REPRESENTATIVE — DATE 7/6/07

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570

Wildlife International, Ltd.

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 20 July 07 Experimental Termination Date: 17 Aug 07

Project No.: 570A-110

Test Concentrations: 31, 63, 125, 250, 500, 1000 mg/kg

Test Substance No.: 8160 Reference Substance No. (if applicable): ---

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1); the ASTM Standard E 1706-00: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (2); the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (3), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (4), and the Federal Environment Agency: Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (5). Raw data for all work performed at Wildlife International, Ltd. and 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. A copy of the final report will be archived at Dow Corning.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions. The sediment used in the test will have an organic content of approximately 5%. A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after the sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding organisms. The measured endpoints of the test are survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of oligochaetes will be exposed to a geometric series of at least five test concentrations and a negative (untreated sediment) control for approximately 28 days. Eight replicate test compartments will be maintained in each treatment and control group, with 10 oligochaetes in each compartment for a total of 80 individuals per test concentration. Each test compartment will contain a quantity of sediment and overlying water. Additional replicate test compartments will be included for analytical sampling of sediment. No oligochaetes will be placed in "analytical"

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replicate(s) sampled at the beginning of the test. However, the "analytical" replicate(s) sampled after the beginning of the test (e.g., on Day 7 and at the end of the test) will contain oligochaetes. An additional two " abiotic replicates" (analytical replicates without oligochaetes) will be added to each treatment group for analysis on Day 7 and Day 28 of the test.

Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate.

The water solubility of D4 is 56.2 µg/L (6). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of D4 in water. Therefore, only sediment samples from the analytical sampling test compartments will be collected from each control and treatment group at specified intervals for analysis of the test substance. Results of the analyses will be used to verify the exposure over time. The results of the study will be based on the nominal test concentrations in sediment, when appropriate.

To control bias, oligochaetes will be impartially assigned to exposure compartments at test initiation. No other potential sources of bias are expected to affect the results of the study. Survivorship and growth (dry weight) will be determined at the end of the test period (maximum of 28 days). Mortality in the treatment groups will be used to calculate, when possible, the EC50 value. The dose-response pattern and appropriate statistical analyses of survivorship and growth will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC).

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MATERIALS AND METHODS

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	Octamethylcyclotetrasiloxane (D4), (Supplied as Dow Corning® 244 Fluid)
Lot No.:	LL084732
Expiration Date:	January 19, 2009
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	556-67-2
Physical Description:	Colorless liquid
Chemical Stability:	Stable
Purity:	99.75 ± 0.003 area percent purity
Solubility:	Soluble in common organic solvents such as acetone
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1.5 kg batch of sediment to 5%. The peat and test substance will be mixed overnight (approximately 12 hours) on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately

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30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data. Approximately 100 mL of the dry mix will then be transferred to 13 replicate test beakers. The test replicates will be 300-mL glass beakers with stainless steel mesh-covered holes on opposite sides of the beaker. The transfer time will be kept to a minimum (approximately 10-20 minutes) and one batch of sediment will be completely processes before the other to prevent the potential for volatilization of the test substance.

After all 13 beakers have been filled with sediment, a 28 day ration of food (approximately 200mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 150mL of well water will be added to the beakers. After the beakers have been filled with water, they will be transferred to a water bath that will be used for the test. In the water bath the overlying water will be exchanged twice per day by adding one volume addition every 12 hours to each beaker and allowing water to passively flow out the holes in the sides of the beakers. After approximately 48 hours of acclimation and settling, organisms will be added to the beakers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study. Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd. Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test. Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (7) will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20%silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon

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content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon.

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report.

Test Apparatus

The test apparatus will consist of a Wildlife International, Ltd. diluter unit that is designed to hold up to 14 tanks in a temperature controlled water bath. Each tank can receive renewal rates that can range from static to greater than 10 tank volume additions per day.

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Test compartments will consist of 300 mL glass beakers with stainless-steel mesh-covered holes on opposite sides of the beaker. The compartments will be labeled with the project number, test concentration and replicate. All replicate beakers in each treatment group will be indiscriminately positioned in one or more diluter tanks. Each beaker will contain approximately 100 ml of sediment and approximately 150 ml of overlying water. Dilution water will be delivered directly into each beaker, passively forcing water out through the holes in the sides of the beakers and exchanging the water overlying the sediment. Each replicate beaker will receive approximately two-volume additions of water per day. Dilution water delivery will be verified by calibration prior to initiation of the test. Proper system operation will be verified at least one time daily during the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test compartment of each experimental group at the beginning and end of the test and at least three times per week during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test compartments or in the negative control diluter tank. Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least three times per week during the test. Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

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Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week. Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a composite sample of overlying water from the control group replicates and the highest concentration treatment group replicates at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (8). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two adult oligochaetes will be impartially distributed to transfer containers (e.g., glass beakers) until each container holds its complement of 10 individuals. The organisms in each transfer container then will be transferred below the air/water interface to the test compartments. An additional batch of 20 individuals will be impartially selected at the beginning of the test and will be measured for dry weight. After completing the transfer of organisms, the test compartments will be maintained in temperature-controlled diluter tanks. Test compartments will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming).

Observations will be made at test termination to determine the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior. On Day 28, oligochaetes will be segregated from the sediment and live and dead organisms will be enumerated. When the total numbers of individuals found in each replicate at test termination are fewer than the number initially placed in each replicate, then those missing will be considered dead. Dry weights of surviving oligochaetes in each replicate will be measured at the end of the test to evaluate effects on growth.

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Sampling for Analytical Measurements

Sediment samples from the analytical sampling test compartments will be collected from each concentration at the beginning of the test (no more than one hour after completing the addition of organisms to the test system), on Day 7, and at the end of the test. There will be two additional abiotic replicates in each test group so that additional samples can be collected on Day 7 and Day 28. The overlying water will be removed from the test compartment. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

	Day 0	Day 7		Termination (28)	
Experimental Group	Sed	Sed	Abiotic	Sed	Abiotic
Control	1	1	1	1	1
Solvent Control (if needed)	1	1	1	1	1
Level 1-Low Concentration	1	1	1	1	1
Level 2	1	1	1	1	1
Level 3	1	1	1	1	1
Level 4	1	1	1	1	1
Level 5-High Concentration	1	1	1	1	1
	7	7	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test compartments and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor

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and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the mortality data will be analyzed using the computer software of C.E. Stephan (9). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (10, 11, 12). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test.

Growth (dry weight) data will be evaluated for normality and homogeneity of variances using TOXSTAT Version 3.5 (13). If the data are deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe) will be used (12,13). If the data fail the tests for normality or homogeneity, then data transformations will be tried in an attempt to correct the condition. When the data transformations fail to correct for non-normality or heterogeneity of variances, nonparametric procedures will be used to identify statistically significant differences among the experimental groups. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Lumbriculus variegatus* culture records.
5. Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations.
6. Biological observations.
7. Test conditions (light intensity, photoperiod, etc.).

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8. Water chemistry results (e.g., hardness and alkalinity).
9. Statistical calculations, if applicable.
10. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and compartments per chamber per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.

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14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

1. Phipps, G.L., G.T. Ankley, D.A. Benoit, and V.R. Mattson. 1993. Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants. Environ. Toxicol. Chem. 12:269-279.

2. ASTM Standard E 1706-00. 2000. *Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates.* American Society for Testing and Materials.

3. U.S. Environmental Protection Agency. 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater.*

4. European Chemicals Bureau. 2003. *Technical Guidance Document on Risk Assessment, Part II.* Institute for Health and Consumer Protection.

5. Federal Environment Agency. 2001. *Validation of the Preliminary EU- Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances.* Institute of Hydrobiology Dresden University of Technology.

6. Varaprath, S., C.L. Frye and J. Hamelink. 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263 – 1265.

7. OECD Guideline 218. 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Organization for Economic Cooperation and Development. Adopted 13 April 2004.

8. 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.

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11. 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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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclopentasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegates* Using Spiked Sediment

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570 **AMENDMENT NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-110

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Study Title, Pages 1 and 2:

CHANGE: Octamethylcyclopentasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegates* Using Spiked Sediment

TO: Octamethylcyclotetrasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegates* Using Spiked Sediment

REASON:

The test material was incorrectly identified in the protocol title. The correct identification of the test material being used in the study was given on page 6 of the protocol and was verified with the documentation that accompanied the test material in shipment.

STUDY DIRECTOR — DATE 9/21/07

LABORATORY MANAGEMENT — DATE 9/24/07

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegates* Using Spiked Sediment

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570 **AMENDMENT NO.:** 2

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-110

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Page 3:

CHANGE:
Experimental Start Date: July 20, 2007
Experimental Termination Date: August 17, 2007
Test Concentrations: 31, 63, 125, 250, 500, 1000 mg/Kg

TO:
Experimental Start Date: October 4, 2007
Experimental Termination Date: November 1, 2007
Test Concentrations: 3.8, 7.5, 15, 30 and 60 mg/Kg

REASON:

The study will be repeated using lower concentrations in order to achieve a NOEC.

STUDY DIRECTOR DATE 10/1/07

LABORATORY MANAGEMENT DATE 10/3/07

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegates* Using Spiked Sediment

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570 **AMENDMENT NO.:** 3

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-110

EFFECTIVE DATE: October 12, 2007

AMENDMENT: Page 1 and 2:

CHANGE: **SPONSOR:** Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

TO: **SPONSOR:** Centre Européen des Silicones
European Chemical Industry Council (Cefic)
Avenue E. van Nieuwenhuyse 4
B-1160 Brussels, Belgium

REASON: Request of Sponsor.

STUDY DIRECTOR DATE 10/16/07

LABORATORY MANAGEMENT DATE 10/16/07

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclotetrasiloxane (D4): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570 **AMENDMENT NO.:** 4

SPONSOR: Centre Européen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 570A-110

EFFECTIVE DATE: November 26, 2007

AMENDMENT: Page 3:

CHANGE:
Experimental Start Date: October 4, 2007
Experimental Termination Date: November 1, 2007
Test Concentrations: 3.8, 7.5, 15, 30 and 60 mg/Kg

TO:
Experimental Start Date: November 29, 2007
Experimental Termination Date: December 27, 2007
Test Concentrations: 3.8, 7.5, 15, 30, 60 and 120 mg/Kg

REASON:
The study will be repeated due to low reproduction in the second trial. An additional test concentration will be added at the sponsor's request.

STUDY DIRECTOR — DATE 11/26/07

LABORATORY MANAGEMENT — DATE 11/26/07

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DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Octamethylcyclopentasiloxane (D4). A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070507/LUM-SED 28a/SUB570 **DEVIATION NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-110

DATE OF DEVIATION: November 26, 2007

DEVIATION: The percent organic carbon content of the formulated sediment was 2.4 instead of 5.0 ± 1.0 as stated in the protocol.

REASON: Due to a calculation error for the moisture content of peat in the preparation of the batch sediment, the incorrect amount of peat was added to the mixture resulting in a lower percent carbon than desired. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

STUDY DIRECTOR — DATE 2-18-09

LABORATORY MANAGEMENT — DATE 2/18/09

Appendix 2

Certificate of Analysis

Date of Analysis:	August 3, 2006
Identification:	Octamethylcyclotetrasiloxane (D4)
Lot Number:	LL084732
CAS Number:	556-67-2
Expiration Date:	January 19, 2009
Physical Description:	Colorless liquid
Storage Conditions:	Room Temperature
Purity:	99.75 ± 0.003 area % purity by GC/FID (n=3)
Composition:	The GC-MS data supports the identification of the material as octamethylcyclotetrasiloxane (D4)

Paul S. Larson — August 15, 2007

Paul S. Larson
Associate Analytical Specialist

Date

Appendix 3

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used. The peat was mixed with neat test material and added at a later date. The pH of the final sediment mixture was 7.3.

Appendix 4

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

	Mean	Range
Specific Conductance (µmhos/cm)	305 (N = 4)	295 – 310
Hardness (mg/L as $CaCO_3$)	136 (N = 4)	132 – 140
Alkalinity (mg/L as $CaCO_3$)	181 (N = 4)	178 – 184
pH	8.2 (N = 4)	8.2 – 8.2

Appendix 5

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.020	Heptachlor	< 0.010
Alpha BHC	< 0.010	Heptachlor Epoxide	< 0.024
Alpha Chlordane	< 0.010	Kepone	< 0.20
Beta BHC	< 0.024	Malathion	< 3.0
Bolstar	< 2.0	Merphos	< 6.0
Chlordane	< 0.50	Methoxychlor	< 0.10
Coumaphos	< 3.0	Methyl Parathion	< 2.0
Delta BHC	< 0.024	Mevinphos	< 4.0
Demeton-O	< 2.0	Mirex	< 0.11
Demeton-S	< 3.0	Naled	< 3.0
Diazinon	< 4.0	o,p-DDD	< 0.020
Dichlorvos	< 3.0	o,p-DDE	< 0.020
Dieldrin	< 0.020	o,p-DDT	< 0.020
Disulfoton	< 2.0	p,p-DDD	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDE	< 0.020
Endosulfan I	< 0.010	p,p-DDT	< 0.020
Endosulfan II	< 0.020	PCB-1016	< 0.50
Endosulfan Sulfate	< 0.040	PCB-1221	< 0.50
Endrin	< 0.020	PCB-1232	< 0.50
Endrin Aldehyde	< 0.10	PCB-1242	< 0.50
Endrin Ketone	< 0.040	PCB-1248	< 0.50
EPN	< 4.0	PCB-1254	< 0.50
Ethion	< 2.0	PCB-1260	< 0.50
Ethoprop	< 3.0	Phorate	< 2.0
Ethyl Parathion	< 2.0	Ronnel	< 2.0
Famphur	< 3.0	Stirophos	< 2.0
Fensulfothion	< 15	Telodrin	< 0.010
Fenthion	< 2.0	Tokuthion	< 2.0
Gamma BHC – Lindane	< 0.010	Toxaphene	< 1.0
Gamma Chlordane	< 0.010	Trichloronate	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Trithion	< 2.0
HCB	< 0.10		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 5

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water[1]
Page 2

Metals

Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum	< 0.200	Magnesium	12.8
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	7.55
Calcium	34.9	Selenium	< 0.0200
Chloride	4.8	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.0
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	1.2	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 6

The Analysis of D_4 in Sediment

Appendix 6.1

Analytical Method Flowchart for the Analysis of D_4 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF D_4 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Decant all the water from beaker. Homogenize the sediment.

↓

Sample sediment from the test container. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.

↓

Fortify recovery samples with the appropriate stock solution.

↓

Add 10.0 mL HPLC-grade bottled water and 250 µL of internal standard to each sample and QC. (225 µL was added to the high level matrix fortification.)

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately five minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50 mL volumetric flask containing 2.5 g sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining the ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Transfer 1.00 mL of extracts into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit samples for analysis.

Appendix 6.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Agilent Model 5975 Mass Selective Detector operated in SIM mode
ANALYTICAL COLUMN:	Phenomen ZB-5 column (30 m x 0.25 mm, 0.25 µm)
INJECTOR TEMPERATURE:	360°C
RUN TIME:	15.25 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 8.00°C/minute Final temperature 1: 150°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 220°C Final hold time 2: 0.00 minute
DETECTOR TEMPERATURE:	310°C
INJECTION VOLUME:	1.00 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	Approximately 12 psi
APPROXIMATE D_4 RETENTION TIME:	6.6 minutes

Appendix 6.3

Analytical Stocks and Standards Preparation

A stock solution of D_4 was prepared by weighing 1.0002 g of the test substance (weight corrected for purity) on an analytical balance. The test substance was transferred to a 100-mL class A volumetric flask and brought to volume using 80% DMF : 20% toluene. This primary stock solution (10.0 mg/mL) was diluted in 80% DMF : 20% toluene to produce 1.00 and 0.100 mg/mL stock solutions. The 1.00 and 0.100 mg/mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in ethyl acetate using the 0.100 mg/mL stock solution. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg/mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.000500	1000	10.0	0.0500
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
0.100	250	10.0	2.50
0.100	400	10.0	4.00
0.100	600	10.0	6.00

A stock solution of Tetrakis (trimethylsilyloxy) silane (D_4 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% (DMF): 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% (DMF): 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to all samples and calibration standards (45 and 50 µL, respectively).

Appendix 6.4

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-110B-3, with a nominal concentration of 7.5 mg/Kg, were calculated using the following equations:

$$D_4 \text{ in sample (mg/Kg)} = \frac{\text{Sum of Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of D4 in sample (mg/Kg)}}{\text{nominal concentration of D4 in sample (mg/Kg)}} \text{ X } 100$$

Ratio of Peak Area = 0.90450
Y-intercept = -0.19
Slope = 3.95
Dilution factor =9.63

$$D_4 \text{ in sample (mg/Kg)} = \frac{0.90450 + 0.19}{3.95} \text{ X } 9.63$$

Concentration of D_4 in sample (mg/Kg) = 2.66 mg/Kg

$$\text{Percent of nominal concentration} = \frac{2.66 \text{ mg/Kg}}{7.5 \text{ mg/Kg}} \text{ X } 100$$

Percent of nominal concentration = 35.4%*

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

Appendix 6.5

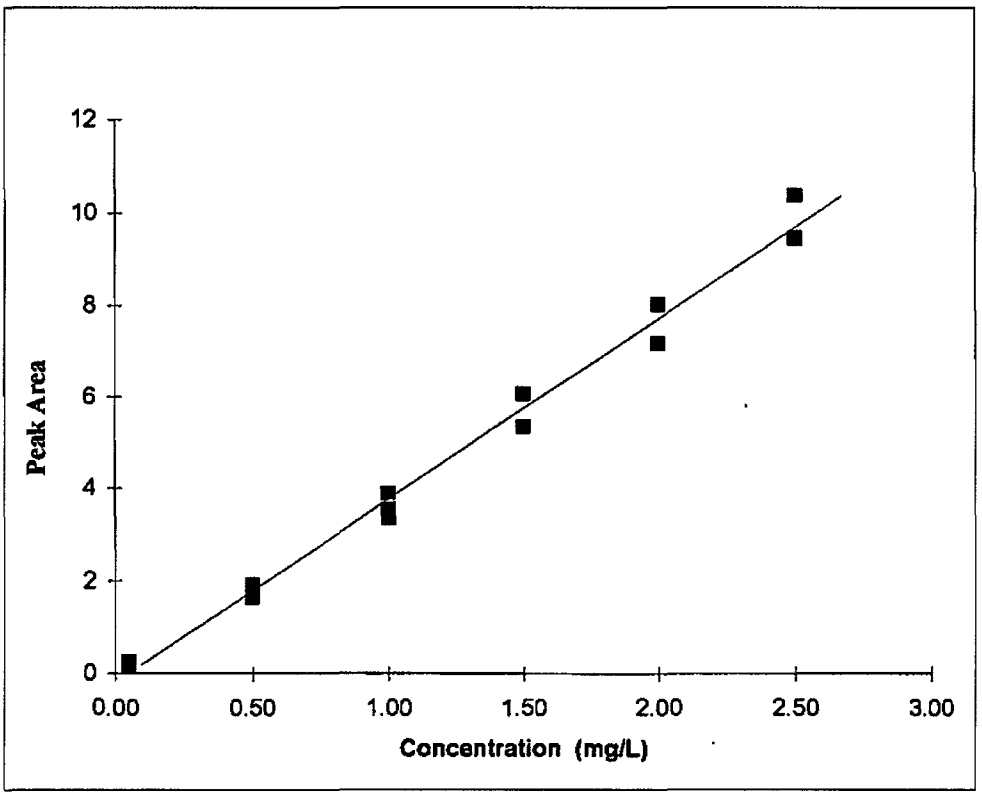
Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (570A-110B-)	Sampling Interval (Day)	Concentrations of D_4		Percent Recovery[1]
		Fortified (mg/Kg)	Measured (mg/Kg)	
MAS-1	0	3.80	3.98	105
MAS-2	0	15.0	14.2	94.7
MAS-3	0	120	119	99.4
MAS-4	7	3.80	2.10	55.4[2]
MAS-5	7	15.0	15.9	106
MAS-6	7	120	124	103
MAS-7	28	3.80	4.19	110
MAS-8	28	15.0	12.8	85.1
MAS-9	28	120	103	86.1
			Mean =	93.9
			Standard Deviation =	16.8
			CV =	17.9%

[1] Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.
[2] Result falls outside of normal range of 80 – 120%.

Appendix 6.6

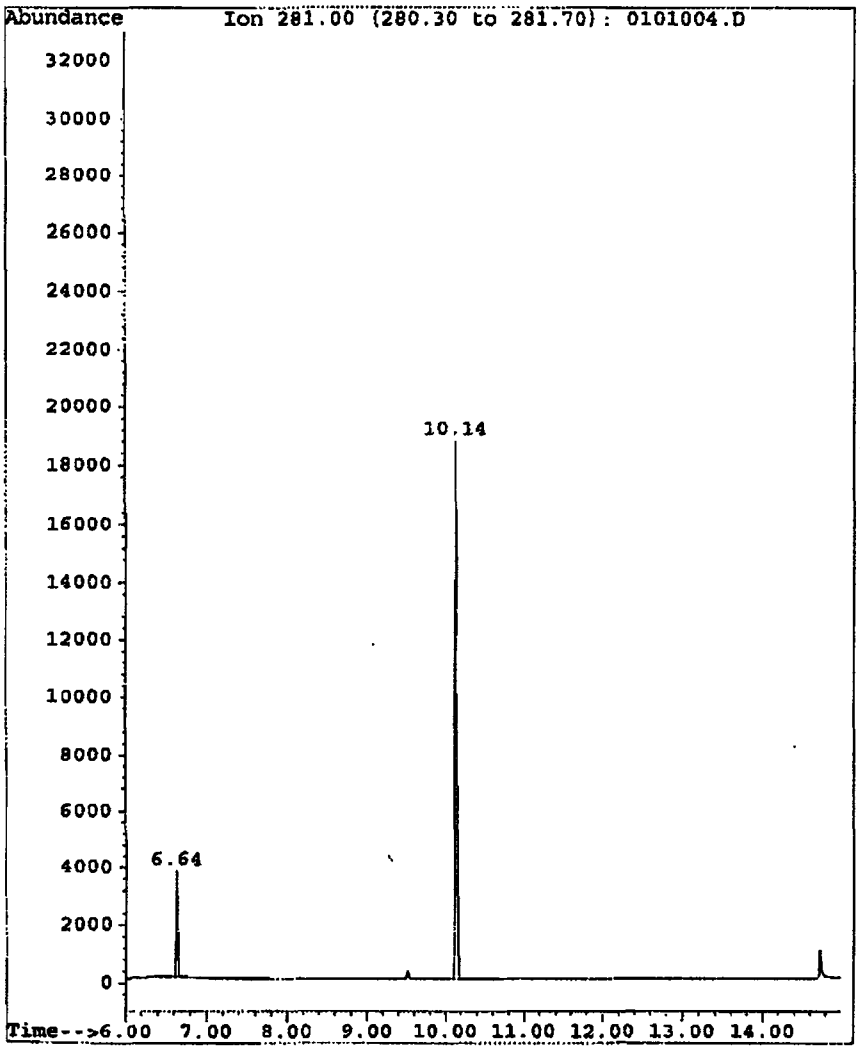
Representative Calibration Curve for D_4 in Sediment



Slope = 3.95; Y-Intercept = -0.19; $R^2 = 0.9888$

Appendix 6.7

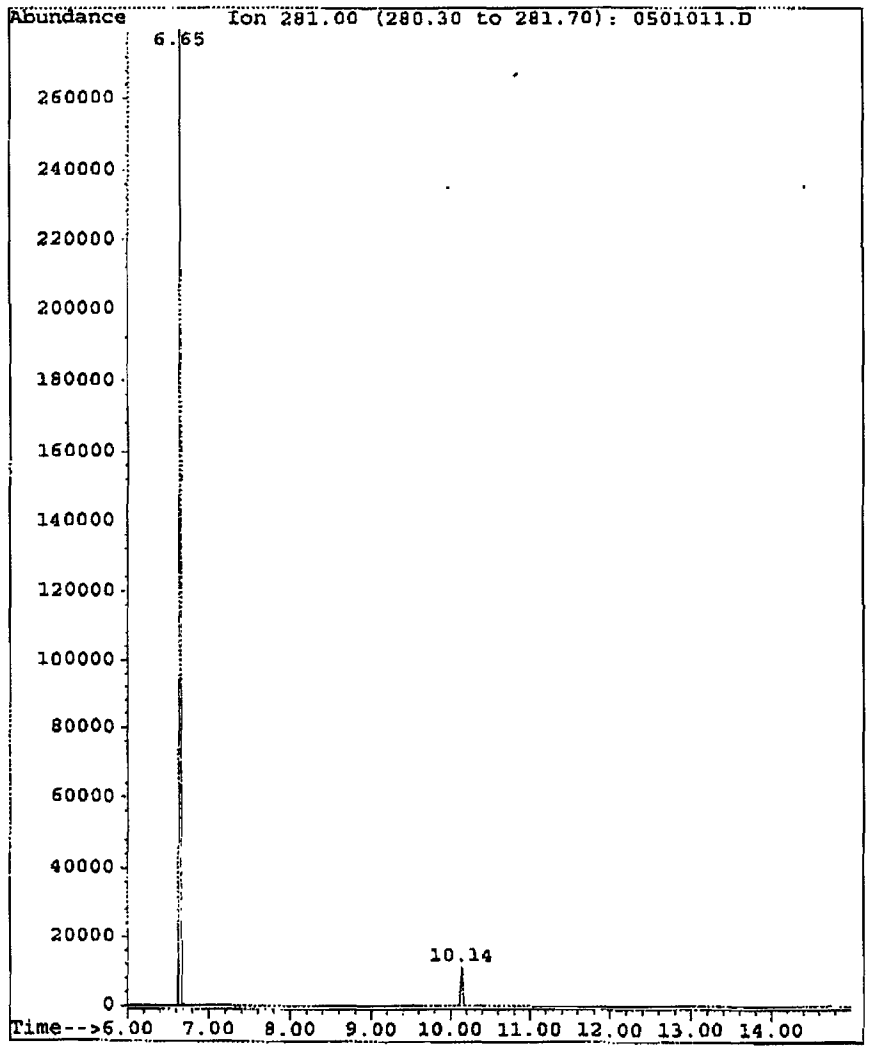
Representative Chromatogram of a Low-level D_4 Calibration Standard



Nominal concentration: 0.0500 mg/L

Appendix 6.8

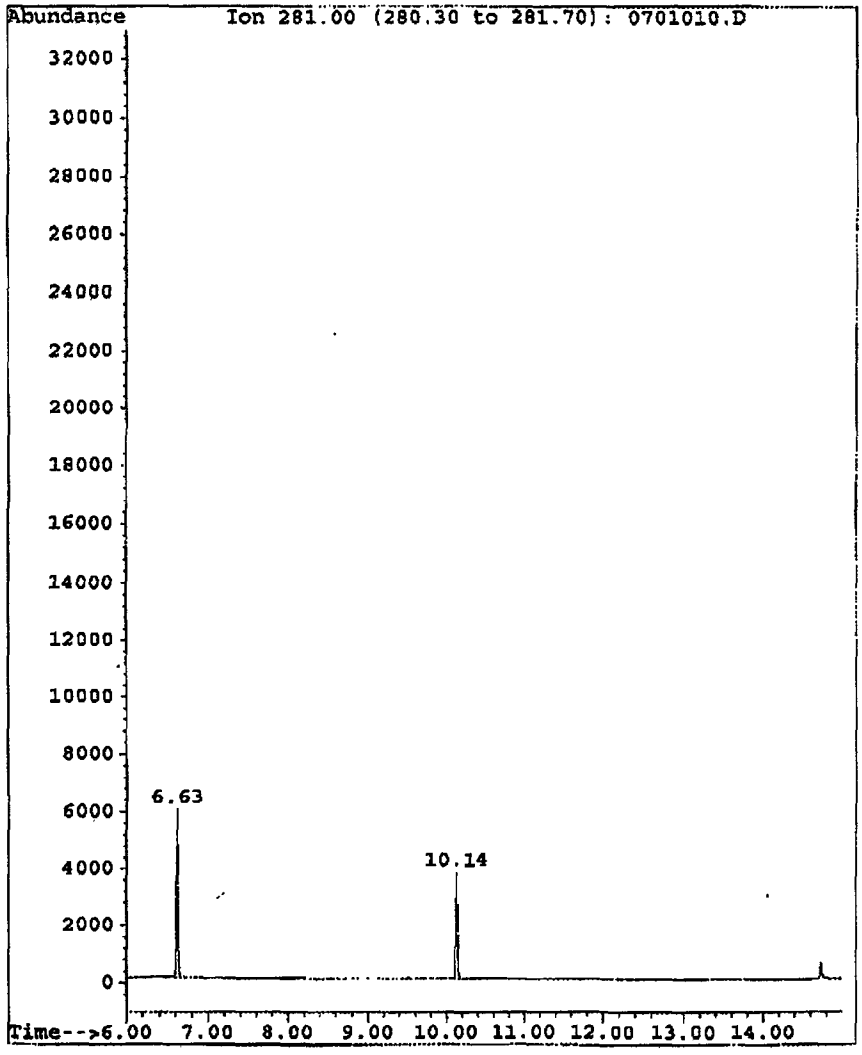
Representative Chromatogram of a High-level D_4 Calibration Standard for Sediment



Nominal concentration: 6.00 mg/L

Appendix 6.9

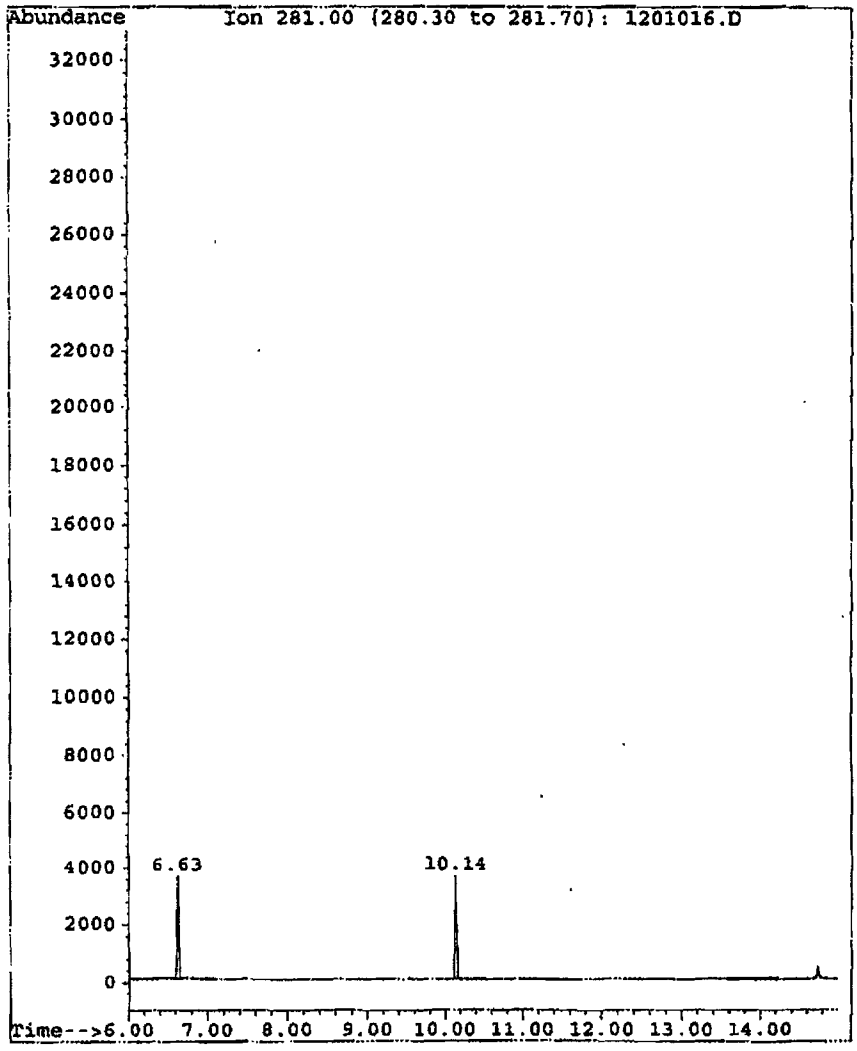
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-110B-MAS-1; 3.80 mg/Kg, nominal concentration.

Appendix 6.10

Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-110B-3; 7.5 mg/Kg nominal concentration

Appendix 7

Observations[1] of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
Negative Control	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1H; AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
Negative Control	A	10	AN	AN	1H; AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	1H; AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

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Page 2
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
0.73	A	10	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
0.73	A	10	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN
	E	10	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	3G; AN	1G; AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
1.5	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	1H; AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
1.5	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN
	F	10	AN	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

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Page 4
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
3.1	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
3.1	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Appendix 7
Page 5
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
5.8	A	10	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
5.8	A	10	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	2G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

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Page 6
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	1G; AN	AN	AN	AN	2G; AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
11	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Appendix 7
Page 7
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Rep.	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
38	A	10	AN	AN	1G;AN	AN	AN	AN	AN	2G;AN	AN	AN	2H; AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	1G;AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	3G;AN	1G;AN	AN	2G;AN	AN	AN	AN
	F	10	AN	1G;AN	AN	2G;AN	AN	1G;AN	AN	AN	AN	AN	AN	AN	1G;AN	AN
	G	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

Mean Measured Concentration (mg/Kg)	Rep.	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
38	A	10	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	1G; AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
Negative Control	A	0	AN	33
	B	0	AN	23
	C	0	AN	26
	D	0	AN	27
	E	0	AN	27
	F	0	AN	21
	G	0	AN	15
	H	0	AN	21
			Mean (± Stdev)	24.1 (± 5.4)
0.73	A	0	AN	17
	B	0	AN	17
	C	0	AN	18
	D	0	AN	14
	E	0	AN	13
	F	0	AN	18
	G	0	AN	12
	H	0	AN	24
			Mean (± Stdev)	16.6 (± 3.8)

[1]Observations: AN = appear normal.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
1.5	A	0	AN	18
	B	0	AN	18
	C	0	AN	16
	D	0	AN	20
	E	0	AN	21
	F	0	AN	24
	G	0	AN	14
	H	0	AN	14
			Mean (± Stdev)	18.1 (± 3.5)
3.1	A	0	AN	15
	B	0	AN	15
	C	0	AN	19
	D	0	AN	12
	E	0	AN	13
	F	0	AN	15
	G	0	3J; AN	18
	H	2	2J; AN	14
			Mean (± Stdev)	15.1 (± 2.4)

[1]Observations: AN = appear normal; J = observed outside the test chamber and placed back in.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
5.8	A	0	AN	13
	B	0	AN	11
	C	0	AN	11
	D	0	AN	24
	E	0	AN	10
	F	0	AN	15
	G	0	AN	14
	H	0	AN	10
			Mean (± Stdev)	13.5 (± 4.6)
11	A	0	AN	10
	B	0	AN	10
	C	0	AN	14
	D	0	AN	14
	E	0	AN	10
	F	3 MAD	AN	7
	G	0	AN	12
	H	0	AN	14
			Mean (± Stdev)	11.4 (± 2.6)

[1]Observations: AN = appear normal; MAD = missing and assumed dead.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
38	A	0	AN	10
	B	2 MAD	AN	8
	C	0	AN	10
	D	1 MAD	AN	9
	E	0	AN	10
	F	0	AN	10
	G	1 MAD	AN	9
	H	0	AN	10
			Mean (± Stdev)	9.5 (± 0.76)

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; MAD = missing and assumed dead.

Appendix 8

Worm Dry Weights by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

	Average Individual Dry Weight (mg) by Mean Measured Concentration						
Replicate	Negative Control	0.73 mg/Kg	1.5 mg/Kg	3.1 mg/Kg	5.8 mg/Kg	11 mg/Kg	38 mg/Kg
A	0.75	0.48	0.75	0.83	0.68	0.63	0.67
B	0.86	0.44	0.77	0.66	0.60	0.63	0.45
C	0.71	0.63	0.52	0.53	1.01	1.21	0.44
D	0.84	0.69	0.65	0.43	0.74	0.61	0.40
E	0.77	0.51	0.88	0.60	0.81	0.54	0.99
F	0.59	0.55	0.83	0.39	0.77	0.44	0.55
G	0.53	0.50	0.51	0.91	0.56	0.57	0.34
H	0.58	1.18	0.46	0.61	0.62	0.46	0.30
Mean ± Std. Dev.	0.70 ± 0.12	0.62 ± 0.24	0.67 ± 0.16	0.62 ± 0.18	0.72 ± 0.14	0.64 ± 0.24	0.52 ± 0.22

On Day 0, a subset of 20 organisms were impartially removed from the culture and used to determine dry weight. The average individual dry weight was determined to be 0.58 mg.

Appendix 9

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, Plant and Insect Toxicology
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

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D4: A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-107

OECD Guideline 218

AUTHORS:

Henry O. Krueger, Ph.D.
Susan T. Thomas, B.S.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: July 13, 2007

STUDY COMPLETION DATE: March 24, 2008

SUBMITTED TO:

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-107

STUDY COMPLETION: March 24, 2008

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999), with the following exceptions:

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

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

STUDY DIRECTOR:

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

3/24/08
Date

SPONSOR APPROVAL:

Sponsor Representative

3/27/08
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 Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. 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	July 30, 2007	July 30, 2007	August 9, 2007
Test Substance Preparation	August 6, 2007	August 7, 2007	August 21, 2007
Test Substance Preparation	September 17, 2007	September 17, 2007	October 4, 2007
Observations	October 16, 2007	October 16, 2007	October 22, 2007
Calibration Standard Preparation	October 18, 2007	October 18, 2007	November 8, 2007
Analytical Data and Draft Report	January 15 – 16, 2008	January 16, 2008	January 31, 2008
Biological Data, Draft Report & Protocol	January 14 – 15, 2008	January 15, 2008	February 4, 2008
Final Report	March 24, 2008	March 24, 2008	March 24, 2008

Linda R. Mitchell

Linda R. Mitchell
Director of Regulatory and Ecotox Operations

24 March 2008
Date

REPORT APPROVAL

SPONSOR. Silicones Environmental. Health and Safety Council

TITLE: D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD PROJECT NUMBER: 570A-107

STUDY DIRECTOR

Henry O. Krueger. Ph.D.
Director of Aquatic Toxicology. Non-Target Plants
And Invertebrates

Date 3/24/08

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Date 3/24/08

Timothy Z. Kendall, M.S.
Supervisor

Date 3-24-08

WILDLIFE INTERNATIONAL, LTD MANAGEMENT

For William N. Nixon

Willard B. Nixon, Ph.D
Director of Chemistry

Date 24 MAR 2008

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SUMMARY

SPONSOR Silicones Environmental, Health and Safety Council

TITLE. D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD PROJECT NUMBER: 570A-107

TEST SUBSTANCE Octamethylcyclotetrasiloxane (D4), supplied as Dow Corning® 244 Fluid (Batch No. LL084732; 99.75%)

TEST DATES:

Experimental Start (OECD):	September 6, 2007
Experimental Start (EPA)	September 20, 2007
Biological Termination:	October 18, 2007
Experimental Termination:	October 19, 2007

LENGTH OF EXPOSURE. 28 Days

TEST ORGANISM: Midge (*Chironomus riparius*)

AGE OF TEST ORGANISMS 1 - 3 Days

SOURCE OF TEST ORGANISMS. Environmental Consulting and Testing
1423 N. 8th St. Suite 118
Superior, WI 54880

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
31 mg/Kg	6.5 mg/Kg
63 mg/Kg	7.9 mg/Kg
125 mg/Kg	19 mg/Kg
250 mg/Kg	44 mg/Kg
500 mg/Kg	131 mg/Kg
1000 mg/Kg	355 mg/Kg

ENVIRONMENTAL CONDITIONS:

Water Temperature:	19.7 – 20.5°C
Dissolved Oxygen:	≥7.2 mg/L (80% of saturation)
Water pH:	8.2 – 8.6

RESULTS: Based on mean measured test concentrations

28-Day LC50:	114 mg/Kg with 95% confidence limits of 96 and 136 mg/Kg
NOEC:	44 mg/Kg
LOEC:	131 mg/Kg, based on survival and emergence ratio

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Silicones Environmental, Health and Safety Council at the Wildlife International. Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from September 20, 2007 to October 18, 2007. Raw data generated by Wildlife International. Ltd.. and a copy of the final report are filed under Project Number 570A-107 in archives located on the Wildlife International, Ltd. site. The original final report is archived at Silicones Environmental. Health and Safety Council.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated test substance on the midge. *Chironomus riparius*. during a 28-day exposure period under semi-static test conditions. The measured endpoints of the test were total number of adults emerged and the development time.

EXPERIMENTAL DESIGN

Groups of midges were exposed to a series of six nominal test concentrations in sediment (31, 63, 125. 250, 500 and 1000 mg/Kg, dry weight) and a negative control for 28 days under semi-static test conditions. Four replicate test chambers were maintained in each treatment and control group, with 20 midges in each test chamber, for a total of 80 midges per test concentration. Each test chamber contained a quantity of sediment and overlying water. Three additional replicates were added in each treatment and control group for analytical sampling of sediment and one additional replicate was added to each treatment and control group for Day 0 water quality measurements. The "analytical" and "water quality" replicates sampled on Day 0 contained no midges, while midges were added at test initiation to the "analytical" replicates sampled on Days 7 and 28. Biological observations were not made for Day 7 and 28 "analytical" replicates.

The results of the study are based on mean measured test concentrations in sediment. Sediment samples were collected and analyzed from the "analytical replicates" of each concentration. Sediment samples were collected on Day 0, Day 7 and at the end of the test.

Test chambers were arranged in a randomized pattern in a temperature controlled environmental chamber approximately 48 hours prior to test initiation to condition the sediment prior to introduction of organisms. Midges were impartially assigned to exposure chambers at test initiation.

Observations of mortality and abnormal behavior were made daily during the test. The total number of adults emerged at the end of the test period was recorded.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment" (Appendix 1). The protocol was based on the OECD Guideline 218: *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment* (1)

Test Substance

The test substance used to prepare the dosed sediment, matrix fortifications and analytical calibration standards was received from Dow Corning on June 22, 2007. It was assigned Wildlife International, Ltd. identification number 8160 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as Dow Corning 244 Fluid; Lot number LL084732; Chemical Abstract (CAS) number 556-67-2. The test substance contained 99.75% active ingredient by analysis and had an expiration date of January 19, 2009

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and was assigned Wildlife International, Ltd. identification number 7566 upon receipt. The standard, described as a liquid, was identified on the label as: Tetrakis (trimethylsilyloxy) silane; 97%; Lot #: 13222MB; CAS #: 3555-47-3 The standard had a reported purity of 97%. The standard was stored under ambient conditions.

Test Sediment

The sediment used in the study was formulated sediment based on the recommendations of OECD Guideline 218 (1). The sediment was composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand (Appendix 3). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the peat, since the peat was added later. The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry soil was stored under ambient conditions until used The final pH of the sediment was 6.9 The percent organic carbon of the sediment was found to be 4.1 as determined by Agvise Laboratories (Northwood, ND) (Appendix 5). Analyses were performed to determine the concentrations of selected organic and inorganic

constituents in a representative sample of sediment similar to that which was used in the study and the results of the analyses are summarized in Appendix 6.

A 28-day ration of food (280 mg Tetramin Flake food) was dry mixed into the test chambers after the sediment was added. but immediately before adding the water to the test chambers. and 48 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. The food added to the test system during the test was not included in the measurement of organic carbon.

Preparation of Test Concentrations

The test substance was administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment dwelling organisms.

Nominal test concentrations (31. 63. 125. 250, 500 and 1000 mg/Kg) were prepared on a dry weight basis. (i.e . mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer Second, a predetermined amount of formulated sediment was added to the peat and test material in the Nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 20 minutes).

After mixing. dosed formulated sediment was transferred to eight replicate test beakers (2000 mL glass beakers) until reaching a depth of 2 cm. The transfer time was kept to a minimum (approximately 11 -13 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 8 beakers had approximately 2 cm of sediment added. a 28-day ration of food (approximately 280 mg of Tetramin flake food) was added to each beaker that was to contain organisms and gently stirred into the dry sediment. Finally, approximately 8 cm of well water was added to the beakers. After the water was added to the beakers. the beakers were placed in the environmental test chamber that was used for the study.

Of the eight replicate test chambers prepared for each treatment and control group. four test chambers were used for the evaluation of survival and emergence. three chambers were used for analytical confirmation and one test chamber was used for water quality measurements on Day 0. The sediment/water mixtures were allowed to acclimate for approximately 48 hours prior to the introduction

of the test organisms. At test initiation, the overlying water in all test chambers appeared slightly cloudy tan. At test termination the overlying water in the test chambers appeared clear and tan.

Test Organism

The midge, *Chironomus riparius*, was selected as the test species for this study. *Chironomus riparius* are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. Egg masses were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The supplier verified the identity of the species. The organisms were held for one day prior to the start of the test at approximately the same temperature used during the test and in water from the same source as the water used during the test.

At test initiation, the midges were collected from the culture and impartially added one and two at a time to test chambers. All transfers were made below the air/water interface using wide-bore pipettes. Six replicate test chambers in each treatment and control group contained organisms (four test chambers for the evaluation of effects plus two chambers for Day 7 and Day 28 analytical purposes), while two test chambers per group were prepared without organisms, one for Day 0 analytical purposes and one for Day 0 water quality measurements.

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 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 again was filtered (0.45 µm) and passed through an ultraviolet sterilizer to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 4. Hardness, alkalinity, pH and specific conductance were measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test are summarized in Appendix 2.

Test Apparatus

Test chambers consisted of 2000-mL glass beakers containing approximately 2 cm of sediment and 8 cm of overlying water. Loose plastic covers were placed over each test chamber during the test. Aeration was applied to each test chamber through a glass pipette that did not extend to a depth closer than 2 cm from the surface of the sediment. Air was bubbled into the test chamber at a rate greater than 1 bubble per second but not so great as to disturb the sediment. Test chambers were arranged in a randomized block order in an environmental chamber. Test chambers were labeled with project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50) 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 355 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 20 ± 2°C. Temperature was measured using a liquid-in-glass thermometer in the overlying water of alternating replicate test chambers of each treatment and control group daily during the study. Temperature also was measured continuously in a beaker of water adjacent to the test compartments using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH and ammonia were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, weekly during the test and at test termination. Hardness, alkalinity and specific conductance were measured in a sample of overlying water from the control group and the highest concentration treatment group (1000 mg/Kg) at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (2). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion Model 720Aplus pH/ISE meter.

Observations

The test chambers were observed daily during the test to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. During the period of expected emergence, the sex and number of fully emerged midges was recorded on a daily basis. After identification, the midges were removed from the test chambers. If the total number of emerged midges at the end of the test were fewer than what was initially placed in each replicate, then the number of midges not accounted for were considered dead. The numbers of emerged midges by day are found in Appendix 8

Statistical Analyses

The 28-Day LC50 was calculated using the computer software of C.E. Stephan (4). The program is designed to calculate the EC50 value and 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (5,6,7). The EC50 value was calculated using the mortality data collected at the end of the test. The no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC) were determined by statistical analyses of survival, mean development times, emergence ratios (rates) and development rates.

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, was calculated for each replicate of each treatment and control groups The emergence ratio and development rate were calculated for each replicate of each control and treatment group using the SAS System for Windows Version 8.2 (3). The emergence ratio, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_e}{n_a}$$

where: ER = emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The emergence ratio data were arcsine transformed using the following formula to normalize the data prior to statistical analyses:

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_e}{n_a}}\right)$$

where: ERarc = arcsin-transformed emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day. Development rate was calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where: i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment (= $\sum f_i$)
x_i = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

where: day_i = inspection day (days since application).

All statistical procedures were performed using SAS version 8.2 (3) and Stephan version 1.1 (4). The data were analyzed using Dunnett's test, as appropriate, to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group. An ANOVA procedure looking at the interaction between sexes was used to evaluate sensitivity between sexes for development rates and total number emerged. There were no statistically significant interactions found between sex and treatments

for development rate and therefore the data for each sex were pooled for this variable. There is no feasible way to determine the sex of one – four day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence ratio are not meaningful because calculations require knowing how many individuals of each sex are initially exposed. However, since there were no statistically significant interactions found between sex and treatment for the total number emerged, the emergence ratio data for both sexes were pooled for analysis.

Analytical Sampling

Sediment samples were collected from the analytical replicates from each test concentration and the control on Day 0, on Day 7 and at test termination on Day 28.

Analytical Method

The analytical method used for the analysis of D4 was developed by Wildlife International, Ltd. Sediment was sampled from the test beakers and approximately 5 mL of sediment was added to each centrifuge tube. To each sample, 10.0 mL of HPLC grade bottled water and 5 or 50 µL of internal standard was added. Samples were vortexed and shaken after the addition of approximately 20 mL of ethyl acetate and the samples were centrifuged for approximately ten minutes at 60% speed. The top layer was transferred into a 50 mL volumetric flask containing 2.5 g of sodium sulfate. Samples were extracted with an additional 20 mL of ethyl acetate. The extracts (1.00 mL) were transferred into autosampler vials and 50.0 µL N-methyl, N-trimethylsilyl trifluoroacetamide was added to each vial. Samples were then submitted for analysis.

Concentrations of D4 in the samples were determined using an Agilent Model 6890 Gas Chromatograph (GC) with a Flame Ionization Detector (FID). Chromatographic separations were achieved using a J&W DB-5MS column (30 m x 0.25 mm, 0.25 µm film thickness). A method flowchart for the analysis of D4 is presented in Appendix 7.1 and typical instrumental parameters are summarized in Appendix 7.2

Calibration standards of D4, ranging in concentration from 1.00 to 70.0 mg/L were prepared using stock solutions of D4 in 80% dimethylformamide (DMF)/ 20% toluene. Each standard was fortified with 0.750 mg a.i./L of the internal standard prior to final volume (Appendix 7.3). Fresh calibration standards were prepared and analyzed with each sample set. Linear regression equations

were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of D4 in the samples was determined by substituting 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 7.4.

The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg/L) and the dilution factor of the samples. Each sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample.

Sediment samples were fortified at 30.0, 100 and 1000 mg/Kg using stock solutions of D4 in 80% DMF : 20% toluene (Appendix 7.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 89.6 to 97.1% of nominal concentrations (Appendix 7.5)

A representative calibration curve is presented in Appendix 7.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 7.7 and 7.8, respectively. A representative chromatogram of a matrix fortification sample is presented in Appendix 7.9. A representative chromatogram of a test sample is presented in Appendix 7.10.

RESULTS AND DISCUSSION

Confirmation of Test Concentrations

Measured concentrations of D4 in the sediment samples collected during the definitive test are presented in Table 1. Measured concentrations of D4 in the sediment samples collected on Day 0 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were <LOQ, <LOQ, 21.2, 46.9, 120 and 450 mg/Kg respectively. Measured concentrations of D4 in the sediment samples collected on Day 7 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were <LOQ, <LOQ, 26.6, 50.3, 173 and 360 mg/Kg respectively. Measured concentrations of D4 in the sediment samples collected on Day 28 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were <LOQ, <LOQ, <LOQ, 36, 101 and 255 mg/Kg respectively. Measured concentrations of the test substance in the negative control sediment samples collected on Days 0, 7 and 28 were below the limit of quantification. Results of the study were based on mean measured test concentrations Samples that had measured concentrations less than the LOQ were assigned a value of one-half the LOQ for the determination of

mean measured concentrations. The mean measured concentrations for the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were 6.5, 7.9, 19, 44, 131 and 355 mg/Kg respectively.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4, respectively. Temperatures were within the 20 ± 2°C range established for the test. Dissolved oxygen concentrations remained ≥ 7.2 mg/L (80% of saturation) throughout the test, and measurements of pH ranged from 8.2 to 8.6. Measurements of ammonia in the overlying water are presented in Table 4. Since there was greater than 4.0 mg/L ammonia in the test chambers, the overlying water was partially renewed on Days 7, 14 and 21. Measurements of hardness, alkalinity and conductivity of the overlying water in the test chambers are presented in Table 5 and were consistent with typical water quality of Wildlife International, Ltd well water.

Observations of emergence in individual replicates at each observation interval are presented in Appendix 8. Adults that emerged appeared normal. There were a few observations of dead pupae or larvae, organisms on the surface of the sediment and partially emerged adults. These abnormal observations were few in number and were not considered to be treatment related. Emergence was first noted on Day 15 of the test. Percent mortality at test termination was 8.8, 13, 11, 2.5, 19, 55 and 88 in the negative control, 6.5, 7.9, 19, 44, 131 and 355 mg/Kg treatment groups, respectively (Table 6). The LC50 based on percent survival was 114 mg/Kg with 95% confidence limits of 96 and 136 mg/Kg. Dunnett's t-test showed that survival was significantly reduced in the 131 and 355 mg/Kg treatment groups in comparison to the negative control ($p < 0.05$). Therefore, the NOEC for survival was determined to be 44 mg/Kg and the LOEC for survival was determined to be 131 mg/Kg.

Mean development time in the negative control group was 18.4 days (Table 7). Mean development times in the 6.5, 7.9, 19, 44, 131 and 355 mg/Kg treatment groups were 17.5, 17.9, 17.9, 18.7, 18.0 and 21.1 days, respectively. There was a statistically significant difference ($p<0.05$) in development time between the negative control group and the 355 mg/Kg treatment group using a two-tailed Dunnett's t-test. Therefore, the NOEC for development time was determined to be 131 mg/Kg and the LOEC for development time was determined to be 355 mg/Kg.

The mean emergence ratios in the negative control, 6.5, 7.9, 19, 44, 131 and 355 mg/Kg treatment groups were 0.93, 0.88, 0.89, 0.98, 0.85, 0.48 and 0.13, respectively (Table 8). There were statistically significant reductions ($p<0.05$) in emergence ratios between the negative control and the 131 and 355 mg/Kg treatment groups using a Dunnett's t-test, that was considered to be treatment related. Therefore, the NOEC for emergence ratio was determined to be 44 mg/Kg and the LOEC for emergence ratio was determined to be 131 mg/Kg (Table 8).

Mean development rates in the negative control, 6.5, 7.9, 19, 44, 131 and 355 mg/Kg treatment groups were 0.0572, 0.0597, 0.0584, 0.0586, 0.0560, 0.0579 and 0.0496, respectively (Table 8). There was a statistically significant reduction ($p<0.05$) in development rate between the negative controls and the 355 mg/Kg treatment groups using a Dunnett's t-test, that was considered to be treatment related. Therefore, the NOEC for development rate was determined to be 131 mg/Kg and the LOEC for development rate was determined to be 355 mg/Kg (Table 8).

CONCLUSIONS

The 28-Day LC50 value based on mortality of midges (*Chironomus riparius*) exposed to sediment-incorporated D4 was 114 mg/Kg with 95% confidence limits of 96 and 136 mg/Kg. Midges exposed to 355 mg/Kg showed a statistically significant reduction in survival, development time, emergence ratio and development rate. In addition, midges exposed to 131 mg/Kg showed statistically significant reductions in survival and emergence ratio. Consequently, the LOEC, based on survival and emergence ratio, was 131 mg/Kg and the NOEC was 44 mg/Kg.

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Table 1

Measured Concentrations of D4 in Sediment Samples

Nominal Test Concentration (mg/Kg)	Sample Number (570A-107-)	Sampling Time (Day)	Measured Concentration (mg/Kg) [1]	Mean Measured Concentration (mg/Kg) [2]	Percent of Nominal [3]
Negative Control (0.0)	1	0	< 18.5	--	--
	8	7	< 12.0		
	15	28	< 12.9		
31	2	0	< 13.1	6.5	21
	9	7	< 11.5		
	16	28	< 14.1		
63	3	0	< 17.5	7.9	13
	10	7	< 15.0		
	17	28	< 14.6		
125	4	0	21.2	19	15
	11	7	26.6		
	18	28	< 15.9		
250	5	0	46.9	44	18
	12	7	50.3		
	19	28	36.0		
500	6	0	120	131	26
	13	7	173		
	20	28	101		
1000	7	0	450	355	36
	14	7	360		
	21	28	255		

[1] The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg/L) and the dilution factor of the samples. Each sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample.

[2] Samples that had measured concentrations less than the LOQ were assigned a value of one-half the LOQ for the determination of mean measured concentrations.

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

Table 2

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Temperature (°C) [1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep:	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Negative Control	20.4	20.3	20.4	20.4	20.2	20.3	20.3	20.5	20.4	20.3	20.0	20.4	20.3	20.4
6.5	20.2	20.0	20.2	20.2	20.1	20.0	20.2	20.3	20.3	19.9	19.8	20.3	20.1	20.2
7.9	20.3	20.2	20.3	20.3	20.3	20.3	20.1	20.3	20.4	20.3	19.9	20.2	20.3	20.4
19	20.3	20.2	20.2	20.2	20.2	20.3	20.4	20.3	20.4	20.3	19.7	20.2	20.2	20.4
44	20.2	20.2	20.3	20.4	20.0	20.3	20.3	20.3	20.3	20.3	19.9	20.1	20.0	20.3
131	20.3	20.2	20.3	20.3	20.2	20.2	20.3	20.3	20.4	20.2	19.9	20.3	20.5	20.3
355	20.2	20.2	20.2	20.2	20.1	20.3	20.3	20.4	20.3	20.3	19.9	20.2	20.2	20.4

[1] Temperature measured continuously during the test ranged from approximately 19.0 to 20.0°C.

Table 2(Continued)

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Rep.:	Temperature (°C)														
		Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
		C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Negative Control		20.4	20.5	20.3	20.3	20.4	20.5	20.3	20.3	20.4	20.5	20.3[1]	20.3	20.3	20.5	20.4
6.5		20.2	20.3	20.1	20.2	20.3	20.3	20.1	20.2	20.2	20.4	20.2	20.2	20.2	20.3	20.2
7.9		20.3	20.3	20.4	20.3	20.2	20.3	20.1	20.3	20.3	20.4	20.3	20.3	20.1	20.2	20.2
19		20.4	20.2	20.2	20.2	20.3	20.3	20.2	20.3	20.5	20.3	20.3	20.3	20.1	20.1	20.2
44		20.3	20.3	20.1	20.2	20.3	20.4	20.2	20.3	20.4	20.3	20.2	20.2	20.2	20.2	20.2
131		20.3	20.4	20.1	20.2	20.2	20.3	20.1	20.2	20.4	20.3	20.4	20.2	20.2	20.2	20.2
355		20.3	20.3	20.1	20.2	20.3	20.5	20.1	20.3	20.5	20.4	20.2	20.3	20.3	20.3	20.2

[1] Measurement taken in replicate B.

Table 3

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Dissolved Oxygen (mg/L)[1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep:	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Negative Control	8.5	8.1	8.4	8.5	8.7	8.3	8.2	8.2	8.4	8.1	8.6	8.4	8.4	8.2
6.5	8.4	8.4	8.4	8.6	8.3	7.8	8.2	8.2	8.1	7.7	8.2	8.2	8.2	7.9
7.9	8.6	8.3	8.4	8.7	8.6	8.1	8.2	8.2	8.3	7.7	8.3	8.2	8.3	7.9
19	8.4	8.4	7.7	8.2	8.2	7.9	7.5	7.7	8.0	7.5	8.3	7.8	8.2	7.8
44	8.5	8.1	8.0	8.1	8.2	8.1	7.8	7.6	7.9	7.2	8.1	7.7	8.1	7.9
131	8.4	8.2	8.1	8.2	7.8	7.6	8.0	7.6	7.8	7.7	8.2	7.7	7.9	7.9
355	8.2	8.0	7.8	8.2	8.1	8.0	7.8	7.7	7.8	7.9	8.3	8.0	8.0	7.8

[1] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20°C in freshwater.

Table 3 (Continued)

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Rep	Dissolved Oxygen (mg/L)														
		Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
		C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Negative Control		8.3	8.6	8.6	8.6	8.4	8.4	8.3	8.5	8.2	8.3	8.4[1]	8.4	8.4	8.5	8.8
6.5		8.3	8.5	8.5	8.5	8.4	8.3	8.3	8.4	8.1	8.4	8.5	8.5	8.1	8.4	8.7
7.9		8.4	8.6	8.1	8.4	8.3	8.3	8.0	8.3	8.2	8.3	8.0	8.4	8.2	8.1	8.2
19		7.9	8.2	8.3	8.3	8.2	8.0	8.1	8.3	8.0	8.2	8.2	8.4	8.0	8.0	8.4
44		8.2	8.3	8.3	8.3	8.0	8.0	8.1	8.2	8.1	8.0	8.5	8.3	8.1	8.1	8.5
131		8.1	8.1	8.2	8.4	8.1	8.0	8.1	8.2	8.1	8.1	8.3	8.4	8.1	8.1	8.5
355		7.8	8.2	8.2	8.3	8.0	8.0	8.1	8.0	7.8	8.0	8.3	8.2	7.6	8.2	8.2

[1] Measurement taken in replicate B.

Table 4

pH and Ammonia (mg/L) of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day									
	0		7		14		21		28	
	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia
Rep	A	H	D		C		B		A	
Negative Control	8.3	0.669	8.5	3.78	8.3	3.59	8.6	4.28	8.4	<0.17[1]
6.5	8.3	0.554	8.6	3.98	8.4	4.49	8.6	3.79	8.5	1.53
7.9	8.3	0.587	8.6	4.13	8.5	3.89	8.5	4.89	8.4	<0.17
19	8.3	0.781	8.5	4.31	8.2	3.92	8.5	6.50	8.5	<0.17
44	8.3	0.660	8.4	4.40	8.3	5.64	8.5	6.49	8.5	<0.17
131	8.3	0.628	8.4	4.38	8.4	3.92	8.6	5.79	8.5	<0.17
355	8.2	0.699	8.4	4.66	8.2	3.83	8.5	6.29	8.4	<0.17

[1] The lowest standard used for ammonia calibration was 0.17 mg/L

Table 5

Hardness. Alkalinity and Conductivity of the Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/cm)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)
Replicate:		H			A	
Negative Control	140	166	310	144	168	320
355	144	172	330	154	178	350

Table 6

Percent Emergence of Midges (*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Number of Emerged Midges - Day 28			Percent Emergence	Percent Mortality[1,2]
			Males	Females	Total		
Negative Control	A	20	9	11	20	100	0
	B	20	11	8	19	95	5
	C	20	6	11	17	85	20
	D	20	8	10	18	90	10
	Group	80	34	40	74	93	8.8
6.5	A	20	5	10	15	75	25
	B	20	8	12	20	100	0
	C	20	9	10	19	95	5
	D	20	9	7	16	80	20
	Group	80	31	39	70	88	13
7.9	A	20	6	11	17	85	15
	B	20	12	7	19	95	5
	C	20	9	10	19	95	5
	D	20	8	8	16	80	20
	Group	80	35	36	71	89	11
19	A	20	10	10	20	100	0
	B	20	10	10	20	100	0
	C	20	9	9	18	90	10
	D	20	9	11	20	100	0
	Group	80	38	40	78	98	2.5
44	A	20	8	11	19	95	5
	B	20	10	10	20	100	5
	C	20	7	8	15	75	30
	D	20	7	7	14	70	35
	Group	80	32	36	68	85	19
131	A	20	11	2	13	65	35
	B	20	9	4	13	65	45
	C	20	2	3	5	25	75
	D	20	4	3	7	35	65
	Group	80	26	12	38	48*	55*
355	A	20	1	0	1	5	95
	B	20	2	1	3	15	85
	C	20	1	2	3	15	85
	D	20	1	2	3	15	85
	Group	80	5	5	10	13*	88*

[1] Percent mortality includes organisms that died in the pupae or larval stages, organisms that emerged and died and organisms that did not emerge by the end of the study.

[2] The LC50 (calculated based on percent survival) was 114 mg/Kg with 95% confidence limits of 96 and 136 mg/Kg, using the probit method.

* There was a statistically significant difference ($p<0.05$) from the negative control using Dunnett's t-test.

Table 7

Mean Development Time of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Mean Development Time	
		Replicate Mean (days)	Group Mean[1,2] (days)
Negative Control	A	18.3	18.4
	B	17.6	
	C	19.9	
	D	17.7	
6.5	A	16.9	17.5
	B	17.4	
	C	18.9	
	D	16.8	
7.9	A	19.0	17.9
	B	17.2	
	C	17.8	
	D	17.5	
19	A	18.4	17.9
	B	17.5	
	C	16.3	
	D	19.4	
44	A	18.7	18.7
	B	18.8	
	C	18.9	
	D	18.3	
131	A	16.7	18.0
	B	18.6	
	C	18.4	
	D	18.1	
355	A	20.0	21.1*
	B	24.0	
	C	21.0	
	D	19.3	

[1] Calculated using Excel 2000. Manual calculations may differ slightly.
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
* There was a statistically significant difference from the negative control ($p < 0.05$), using a Dunnett's t-test.

Table 8

Emergence Ratio and Development Rate of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Emergence Ratio		Development Rate[1]	
		Replicate	Mean[2]	Replicate	Mean[2]
Negative Control	A	1.00	0.93	0.0573	0.0572
	B	0.95		0.0595	
	C	0.85		0.0531	
	D	0.90		0.0588	
6.5	A	0.75	0.88	0.0615	0.0597
	B	1.00		0.0603	
	C	0.95		0.0553	
	D	0.80		0.0619	
7.9	A	0.85	0.89	0.0546	0.0584
	B	0.95		0.0606	
	C	0.95		0.0588	
	D	0.80		0.0597	
19	A	1.00	0.98	0.0573	0.0586
	B	1.00		0.0593	
	C	0.90		0.0634	
	D	1.00		0.0543	
44	A	0.95	0.85	0.0556	0.0560
	B	1.00		0.0558	
	C	0.75		0.0553	
	D	0.70		0.0573	
131	A	0.65	0.48*	0.0622	0.0579
	B	0.65		0.0561	
	C	0.25		0.0563	
	D	0.35		0.0569	
355	A	0.05	0.13*	0.0513	0.0496*
	B	0.15		0.0442	
	C	0.15		0.0493	
	D	0.15		0.0536	

[1] The development rate represents that portion of larval development which takes place per day.
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
[3] There was a statistically significant reduction from the negative control ($p < 0.05$), using a Dunnett's t-test.

Appendix 1

Study Protocol and Amendments

PROTOCOL

D4: A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

Guidelines:

OECD Guideline 218

Submitted to

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2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.

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May 3, 2007

Wildlife International, Ltd.

D4. A PROLONGED SEDIMENT TOXICITY TEST
WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

SPONSOR: Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
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and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 7/13/07

LABORATORY MANAGEMENT — DATE 7/13/07

SPONSOR'S REPRESENTATIVE — DATE 6/4/07

PROTOCOL NO.. 570/050307/CHI-SEDa/SUB570

Wildlife International, Ltd.

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 30 July 07 Experimental Termination Date: 27 Aug 07

Project No.: 570A-107

Test Concentrations: 31, 63, 125, 250, 500 and 1000 mg/Kg

Test Substance No.: 8160 Reference Substance No. (if applicable): _____

PROTOCOL NO.: 570/050307/CIII-SEDa/SUB570

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INTRODUCTION

Wildlife International, Ltd will conduct a prolonged sediment toxicity test with the midge, *Chironomus riparius*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon the OECD Guideline 218: Sediment-Water Chironomid Toxicity Test Using Spiked Sediment (1). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the midge, *Chironomus riparius*, during a 20 to 28 day exposure period under static or semistatic test conditions. The measured endpoints of the test are emergence ratio (percent emergence), development rate, and the development time.

EXPERIMENTAL DESIGN

Groups of midges will be exposed to a geometric series of at least five test concentrations, a negative (untreated sediment) control, and if necessary, a solvent control for approximately 20 to 28 days. Four replicate test chambers will be maintained in each treatment and control group, with 20 midges in each chamber for a total of 80 individuals per test concentration. Each test chamber will contain a quantity of sediment and overlying water. Additional replicate test chambers may be included for analytical sampling of water and sediment and for water quality measurements. No midges will be placed in "analytical" or "water quality" replicate(s) sampled at the beginning of the test. However, replicate(s) sampled on Day 7 and at the end of the test will contain midges. Test concentrations in the sediment will be prepared on a mg/kg dry weight basis.

The formulated sediment will use peat as its source of organic matter in order to achieve an organic content of approximately 4 to 5%. Test substance will first be mixed with peat and then dry components of the formulated sediment (sand and clay). After mixing, dry formulated sediment will be transferred to each test chamber and a 28-day ration of food (tetramin) will be dry mixed into each test chamber Water will then be added to each chamber approximately 48 hours before adding organisms Partial renewals of overlying water will occur if weekly ammonia concentrations approach 4 mg/L.

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Nominal test concentrations will be selected in consultation with the Sponsor, and may be based upon the results of exploratory range-finding toxicity data. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate. Sediment samples from the analytical sampling test chambers will be collected from each of the test concentrations and controls at test initiation, on Day 7, and at the end of the test. Results of the analyses will be used to verify the exposure over time. Nominal or mean measured test concentrations will be used to calculate EC50 values, when appropriate.

To control bias, midges will be impartially assigned to exposure chambers at test initiation (e g 4 groups of 20 larvae per chamber) No other potential sources of bias are expected to affect the results of the study. The total number of adults emerged at the end of the test period (approximately 28 days) will be recorded. Percent emergence in the treatment groups will be used to calculate, when possible, the EC50. The dose-response pattern and appropriate statistical analyses will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC).

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to GLPs prior to its use in the study GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification.	Octamethylcyclotetrasiloxane (D4), (Supplied as Dow Corning® 244 Fluid)
Lot No :	LL084732
Expiration Date:	January 19, 2009
Source	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	556-67-2
Physical Description:	Colorless liquid

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Chemical Stability.	Stable
Purity:	99.75 ± 0.003 area percent purity
Solubility:	Soluble in common organic solvents such as acetone
Storage Conditions.	Room Temperature
Archive Requirements.	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a batch of sediment to 5% The peat and test substance will be mixed overnight on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 20 - 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data.

After mixing, sediment will be placed in the bottom of the test chambers to a depth of approximately 2 cm (approximately 150 ml). The weight of the sediment added to each chamber will be measured using a top-loading balance and recorded. A pre-weighed aliquot of Tetramin Flake Food (280 mg) will be added to the sediment prior to adding the overlying water and mixed into the sediment by hand using a glass stir rod. Approximately 8 cm of water will be slowly added into the test chamber in such a manner as to minimize the disturbance to the sediment. A 1:4 ratio of sediment to overlying water will be maintained throughout the study. A mark on each test chamber noting the level of the water will be made so that water lost due to evaporation can be replaced in the test chambers as needed with reverse osmosis water The transfer time will be kept to a minimum (approximately 10 – 20 minutes) and one batch of sediment will be completely processed before the

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other to prevent the potential for volatilization of the test substance. After the test chambers are prepared, they will be placed in a randomized block arrangement in an environmental chamber at the target temperature and the sediment-water mixtures will be allowed to settle for approximately 48 hours before adding the test organisms. A partial renewal of the overlying water will take place if ammonia concentrations exceed 4 mg/L.

Test Organism

The midge, *Chironomus riparius*, has been selected as the test species for this study. Midges represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Environmental Consulting and Testing (ECT), Superior, WI or some other source will supply identified egg masses. Midges to be used in the test will be first instar larvae (approximately 1-4 days after hatching) at test initiation. Larvae will be collected from at least three separate egg masses to start the test and will be batched in water from the same source and at approximately the same temperature as will be used during the test Midge larvae will be fed Tetramin Flake Food or equivalent incorporated into the sediment at the beginning of the test This ration of food is equivalent to feeding the organisms ten mg daily during the test. No other supplemental food will be added during the test.

Specifications for acceptable levels of contaminants in the food for midges have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

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A 28-day ration of food (tetramin fish food or equivalent) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. The food added to the test system during the test is not included in the measurement of organic carbon.

Dilution Water

Water used for the holding and testing of midges will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove microorganisms and fine particles and passed through an ultraviolet sterilizer. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the analyses will be summarized in the final report.

Test Apparatus

Test chambers will consist of 2-L glass beakers, or equivalent, containing approximately 2 cm of sediment and approximately 8 cm of overlying water. Loose plastic covers will be placed over each test chamber during the test. Aeration will be applied to each test chamber through a glass pipette that shall extend to a depth of not closer than 2 cm from the surface of the sediment. Air will be bubbled into the test chambers at a rate greater than 1 bubble/sec but not so great as to disturb the sediment. Test chambers will be arranged in a randomized block order in an environmental chamber.

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Test chambers will be labeled with the project number, test concentration and replicate. A check will be performed at least once daily to monitor the water levels in the test chambers, the temperature in the environmental chamber where the study is being conducted and the proper function of the aeration.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 20 ± 2°C. Temperature will be measured using a liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group daily during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in one alternating replicate of each experimental group daily during the test using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. Measurements of pH will be made at the beginning and end of the test and at least once per week on a sample of the overlying water from each experimental group. Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, then discontinued.

Ammonia measurements will be made in a sample of overlying water from one or more of the "water quality" replicates at the beginning of the test, and from one alternating biological replicate from each experimental group weekly during the test Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent. Hardness, alkalinity and specific conductance

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will be measured in a sample of the overlying water from one or more of the "water quality" replicates at the beginning of the test and from a biological replicate at the end of the test, from the controls and the highest treatment group Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (2). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two first instar larvae will be sequentially added to test chambers until each chamber contains its complement of 20 individuals. After adding the test organisms to the test chambers, the test chambers will be placed in a walk-in environmental chamber and organized in a randomized block arrangement.

Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming) During the period of expected emergence the sex and number of fully emerged midges will be recorded on a daily basis. After identification the midges will be removed from chambers. Egg masses deposited prior to the end of the test will be recorded and then removed to prevent re-introduction of larvae into the sediment. If the total number of individuals emerged in each replicate at the end of the test are fewer than what was initially placed in each replicate, then the number of individuals not accounted for will be considered dead. The test duration for *Chironomus riparius* is approximately 28 days. If midges emerge earlier, the study can terminate after a minimum of five days following emergence of the last adult in the control.

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from selected concentrations at test initiation, on Day 7, and at the end of the test. The overlying water will be removed from the test chamber. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen, refrigeration or ambient) until analyzed. The sampling scheme is summarized below:

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PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 7	Termination (28)
Control	1	1	1
Level 1-Low Concentration	1	1	1
Level 2	1	1	1
Level 3	1	1	1
Level 4	1	1	1
Level 5-High Concentration	1	1	1
	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

Analytical Chemistry

Chemical analysis of the samples will be perfomed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Statistical Analyses

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software 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 method, or binomial probability with nonlinear interpolation (4,5,6) The EC50 value will be calculated, when possible, using mortality data collected at the end of the test. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) will be determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence rates and development rates.

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, will be calculated for each replicate of each treatment and control group. The emergence rate and development rate will be calculated for each

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replicate of each control and treatment group using the SAS System for Windows Version 8.2 (7). The emergence rate, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_e}{n_a}$$

where: ER = emergence rate
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The emergence rate data were arcsine transformed using the following formula to normalize the data prior to statistical analyses.

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_e}{n_a}}\right)$$

where: ERarc = arcsin-transformed emergence rate
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day. Development rate will be calculated using the following formula.

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where: $\bar{x}$ = development rate
i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment (= Σf_i)
x_i = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

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where. day_i = inspection day (days since application).

The data will be analyzed using Student's t-test to determine any statistical differences between solvent and negative control groups. If there are no significant differences between the solvent and negative control, the controls will be pooled and all comparisons will be made between the pooled control and treatment groups. The data will be analyzed using Dunnett's test, as appropriate, to identify those treatment levels that are statistically different ($p<0.05$) from the pooled control group (5, 6). If data cannot be pooled, the most appropriate comparisons will be made to either the negative or solvent control, or both. If other statistical procedures are used to analyze the data, the procedures will be described in the raw data and final report.

An ANOVA procedure looking at the interaction between sexes will be used to evaluate sensitivity between sexes for development rates. The data will be analyzed for significant interactions between sex and treatments for development rate to determine if data can be pooled between sexes. There is no feasible way to determine the sex of three day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence rate are not possible because calculations require knowing how many individuals of each sex are initially exposed. If there are no significant interactions found between sex and treatments for the number of female and male midges that emerge, the emergence rate data for both sexes will also be pooled for analysis.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Chironimus riparius* culture records.
5. Stock solution calculation and preparation, if applicable.
6. Biological observations.
7. Water chemistry results (e.g., hardness and alkalinity).
8. Statistical calculations, if applicable
9. Test conditions (light intensity, photoperiod, etc.).

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10 Calculation and preparation of test concentrations.

11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4 Objectives and procedures, as stated in the approved protocol, including all changes to the protocol
5 The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6 Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions
10 The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11 A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13 A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses
14. Statistical methods used to evaluate the data.
15 The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.

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16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management
18. 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

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology) Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

1. **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Adopted 13 April 2004.

2. **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.

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

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

5. **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

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

7 **The SAS System for Windows.** 1999-2001. Release 8.2, (TS2M0). SAS Institute, Inc. Cary, North Carolina

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AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/050307/CHI-SEDa/SUB570 **AMENDMENT NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-107

EFFECTIVE DATE: September 13, 2007

AMENDMENT: Page 2:

CHANGE: Proposed Dates: Experimental Start Date: July 30, 2007
Experimental Termination Date: August 27, 2007

TO: Proposed Dates: Experimental Start Date: September 20, 2007
Experimental Termination Date: October 18, 2007

REASON: The study was rescheduled.

STUDY DIRECTOR DATE 9/20/07

LABORATORY MANAGEMENT DATE 9/20/07

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: D4: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/050307/CHI-SEDa/SUB570 **AMENDMENT NO.:** 2

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-107

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Page 3:

ADD: Reference Substance No.: 7566

REASON: The above information was not know at the time the study director signed the protocol.

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Final Report, Page 14:

ADD: 18. The amendment will clearly identify the part of the final report that is being amended and the reasons for the alteration.

REASON: To clarify the intent of the protocol regarding amendments to the final report.

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EFFECTIVE DATE: September 20, 2007

AMENDMENT: Sampling for Analytical Measurements, Page 10

CHANGE: The overlying water will be removed from the test chamber.

TO: A layer of overlying water will be maintained over the sediment during sampling to prevent loss of material due to volatility

REASON: The procedure will be changed to reduce the potential loss of material due to volatility, and will be based on the methodology developed by the sponsor

STUDY DIRECTOR — DATE 2/4/08

LABORATORY MANAGEMENT — DATE 2/4/08

Appendix 2

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

	Mean	Range
Specific Conductance (µmhos/cm)	315(N = 4)	310 - 320
Hardness (mg/L as $CaCO_3$)	132 (N = 4)	128 - 136
Alkalinity (mg/L as $CaCO_3$)	180 (N = 4)	180- 180
pH	8.2 (N = 4)	8.1 – 8.2

Appendix 3

Formulated Sediment Preparation [1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000

[1]The sand and clay were mixed in a PK Twinshell® mixer for 20 minutes and the dry sediment was stored under ambient conditions until used

[2] The peat moss was added separately to each individual batch (750 gram batch) of sediment prepared. The amount of peat moss added was adjusted for the percent moisture (75%).

The pH was measured in the negative control sediment once all ingredients were mixed together and found to be 6.9.

Appendix 4

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	< 0.020	Heptachlor	< 0.010
Alpha BHC	< 0.010	Heptachlor Epoxide	0.024
Alpha Chlordane	< 0.010	Kepone	0.20
Beta BHC	< 0.024	Malathion	3.0
Bolstar	< 2.0	Merphos	< 6.0
Chlordane	< 0.50	Methoxychlor	< 0.10
Coumaphos	3.0	Methyl Parathion	2.0
Delta BHC	< 0.024	Mevinphos	4.0
Demeton-O	< 2.0	Mirex	< 0.11
Demeton-S	3.0	Naled	3.0
Diazinon	< 4.0	o,p-DDD	0.020
Dichlorvos	< 3.0	o,p-DDE	0.020
Dieldrin	0.020	o,p-DDT	0.020
Disulfoton	< 2.0	p,p-DDD	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDE	0.020
Endosulfan I	< 0.010	p,p-DDT	0.020
Endosulfan II	0.020	PCB-1016	0.50
Endosulfan Sulfate	< 0.040	PCB-1221	< 0.50
Endrin	< 0.020	PCB-1232	0.50
Endrin Aldehyde	0.10	PCB-1242	< 0.50
Endrin Ketone	< 0.040	PCB-1248	< 0.50
EPN	4.0	PCB-1254	< 0.50
Ethion	2.0	PCB-1260	< 0.50
Ethoprop	3.0	Phorate	2.0
Ethyl Parathion	2.0	Ronnel	< 2.0
Famphur	< 3.0	Stirophos	< 2.0
Fensulfothion	< 15	Telodrin	< 0.010
Fenthion	< 2.0	Tokuthion	2.0
Gamma BHC – Lindane	< 0.010	Toxaphene	< 1.0
Gamma Chlordane	0.010	Trichloronate	2.0
Guthion (Azinphos-methyl)	< 4.0	Trithion	< 2.0
HCB	< 0.10		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 4 (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	12.8
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	7.55
Calcium	34.9	Selenium	0.0200
Chloride	4.8	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.0
Cobalt	0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	0.0200
Fluoride	1.2	Vanadium	< 0.0050
Iron	0.200	Zinc	< 0.0200
Lead	0.0150		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 5

Agvise Soil Characterization Report

AGVISE LABORATORIES

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P.O. Box 510
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AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INT.
Protocol or Study No = VARIOUS
Sample ID. = 570A-107
Trial ID. = NEGATIVE CONTRO
Date Received = 12-21-07
Date Reported = 01-02-2008

AGVISE Lab No	07-1147
Percent Sand	73
Percent Silt	10
Percent Clay	17
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	0.94
Cation Exchange Capacity (meq/100 g)	11.1
% Moisture at 1/3 Bar	25.9
% Organic Carbon --Walkley Black	4.1
% Organic Matter--Walkley Black	7.0
pH in 1:1 soil:water ratio	7.3

Base Saturation Data

Cation	Percent	ppm
Calcium	43.5	963
Magnesium	43.8	582
Sodium	0.9	24
Potassium	0.3	12
Hydrogen	11.6	13

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

1/2/08
Date

Appendix 6

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Sediment

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.85	Heptachlor	< 0.85
Alpha BHC	< 1.0	Heptachlor Epoxide	< 0.85
Alpha Chlordane	< 0.85	Kepone	< 7.1
Beta BHC	< 2.0	Malathion	< 68
Bolstar	< 68	Merphos	< 68
Chlordane	< 17	Methoxychlor	< 8.5
Coumaphos	< 68	Methyl Parathion	< 68
Delta BHC	< 0.85	Mevinphos	< 68
Demeton-O	< 68	Mirex	< 1.7
Demeton-S	< 68	Naled	68
Diazinon	< 68	o,p-DDD	< 1.7
Dichlorvos	68	o,p-DDE	< 1.7
Dieldrin	< 1.7	o,p-DDT	< 1.7
Disulfoton	< 77	p,p-DDD	1.7
Dursban (Chlorpyrifos)	< 68	p,p-DDE	< 1.7
Endosulfan I	< 0.85	p,p-DDT	< 1.7
Endosulfan II	< 1.7	PCB-1016	< 17
Endosulfan Sulfate	1.7	PCB-1221	43
Endrin	< 1.7	PCB-1232	17
Endrin Aldehyde	1.7	PCB-1242	23
Endrin Ketone	< 1.7	PCB-1248	17
EPN	< 68	PCB-1254	17
Ethion	< 68	PCB-1260	17
Ethoprop	< 68	Phorate	68
Ethyl Parathion	< 68	Ronnel	68
Famphur	< 68	Stirophos	68
Fensulfothion	68	Telodrin	< 1.2
Fenthion	< 68	Tokuthion	68
Gamma BHC - Lindane	< 0.85	Toxaphene	< 34
Gamma Chlordane	3.1	Trichloronate	68
Guthion (Azinphos-methyl)	68	Trithion	68
HCB	< 0.85		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 6 (Continued)

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

Metals			
Component	Measured Concentration (mg a.i./Kg)	Component	Measured Concentration (mg a.i./Kg)
Aluminum	9200	Magnesium	2710
Antimony	< 2.00	Manganese	4.05
Arsenic	< 2.00	Mercury	< 0.0968
Barium	61.0	Nickel	2.81
Beryllium	< 0.500	Nitrate by IC (solid)	< 1.5
Bromide by IC (solid)	5.1	Nitrite by IC (solid)	< 1.0
Cadmium	< 0.500	Potassium	89.7
Calcium	5000	Selenium	< 2.00
Chloride by IC (solid)	20.1	Silver	< 0.500
Chromium	8.25	Sodium	< 100
Cobalt	< 0.500	Sulfate by IC (solid)	91.9
Copper	1.33	Thallium	< 2.00
Fluoride by IC (solid)	4.3	Vanadium	4.97
Iron	422	Zinc	3.96
Lead	11.7		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 7

The Analysis of D4 in Sediment

Appendix 7.1

Analytical Method Flowchart for the Analysis of D4 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF D4 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample sediment from the test beakers. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.

↓

Fortify recovery samples with the appropriate stock solution.

↓

Add 10.0 mL HPLC-grade bottled water and 5 or 50 µL of internal standard to each sample and QC.

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately 10 minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50 mL volumetric flask containing 2.5 g sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining the ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Dilute samples further, as necessary, with ethyl acetate.

↓

Transfer 1.00 mL of extracts into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit samples for analysis.

Appendix 7.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Flame Ionization Detector (FID)
ANALYTICAL COLUMN:	J&W DB-5MS column (30 m x 0.25 mm, 0.25 µm film thickness)
INJECTOR TEMPERATURE:	280°C
RUN TIME:	15.25 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 8.00°C/minute Final temperature 1: 150°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 220°C Final hold time 2: 0.00 minute
DETECTOR TEMPERATURE:	310°C
TOTAL FLOW:	13.8 mL/min at split vent
HYDROGEN:	40 mL/min
AIR:	450 mL/min
INJECTION VOLUME:	1.0 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	12 psi
APPROXIMATE D4 RETENTION TIME:	7.2 minutes

Appendix 7.3

Analytical Stocks and Standards Preparation

A stock solution of D4 was prepared by weighing 1.0002 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 80% DMF : 20% toluene. This primary stock solution (10.0 mg/mL) was diluted in 80% DMF : 20% toluene to produce 1.00 and 0.100 mg/mL stock solutions. The 10.0 and 1.00 mg/mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in ethyl acetate using the 1.00 and 0.100 mg/mL stock solutions. 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	100	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D4 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% DMF : 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% DMF : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to all samples and calibration standards (5 or 50 µL).

Appendix 7.4

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-107-4, with a nominal concentration of 125 mg/Kg, were calculated using the following equations:

$$\text{D4 in sample (mg/Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of D4 in sample (mg/Kg)}}{\text{nominal concentration of D4 in sample (mg/Kg)}} \text{ X } 100$$

Peak Area = 1.70506
Y-intercept = 0.28392
Slope = 1.14
Dilution factor = 17.1

$$\text{D4 in sample (mg/Kg)} = \frac{1.70506 - 0.28392}{1.14} \text{ X } 17.1$$

Concentration of D4 in sample (mg/Kg) = 21.2 mg a.i./Kg*

$$\text{Percent of nominal concentration} = \frac{21.2 \text{ mg/Kg}}{125 \text{ mg/Kg}} \text{ X } 100$$

Percent of nominal concentration = 17.0%

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

Appendix 7.5

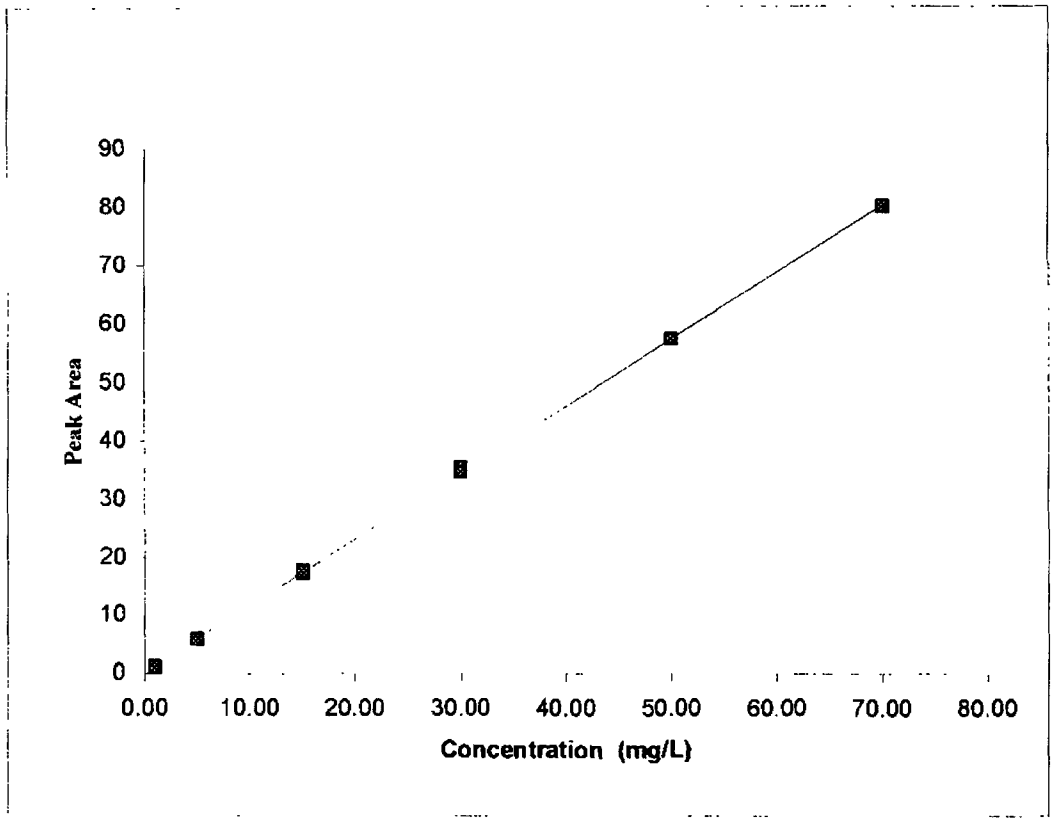
Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (570A-107-)	Sampling Interval (Day)	Concentrations of D4		Percent Recovery[1]
		Fortified (mg/Kg)	Measured (mg/Kg)	
MAS-1	0	30.0	29.1	97.1
MAS-2	0	100	92.3	92.3
MAS-3	0	1000	949	94.9
MAS-4	7	30.0	27.6	92.1
MAS-5	7	100	92.6	92.6
MAS-6	7	1000	910	91.0
MAS-7	28	30.0	26.9	89.7
MAS-8	28	100	89.6	89.6
MAS-9	28	1000	928	92.8
			Mean =	92.5
			Standard Deviation =	2.39
			CV =	2.58%

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

Appendix 7.6

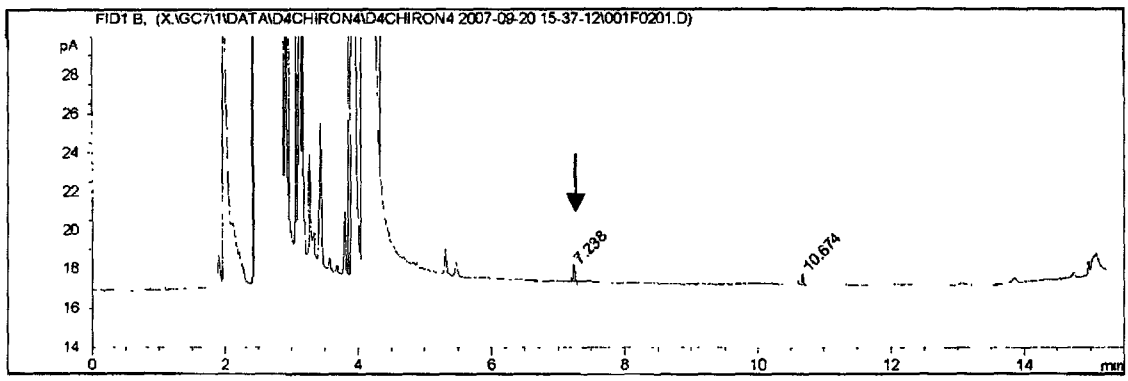
Representative Calibration Curve for D4 in Sediment



Slope = 1.14; Y-Intercept = 0.28392; R^2 = 0.9999

Appendix 7.7

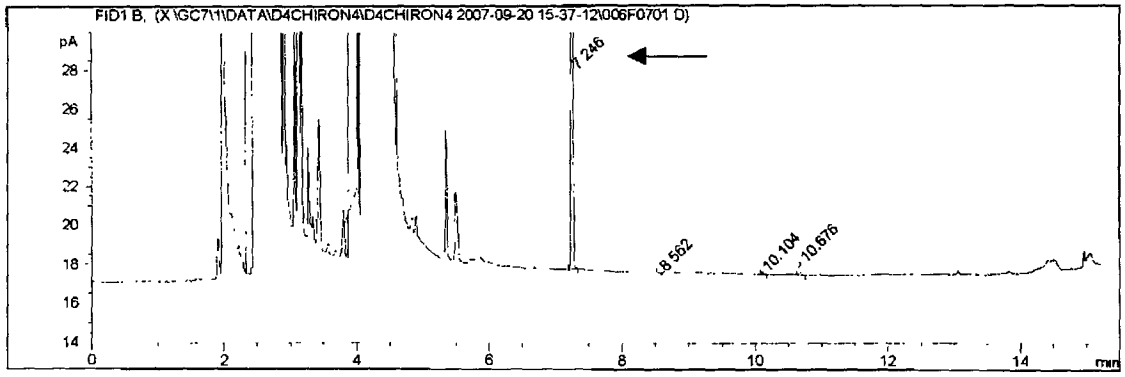
Representative Chromatogram of a Low-level D4 Calibration Standard



Nominal concentration: 1.00 mg/L. The arrow indicates the approximate retention time of D4.

Appendix 7.8

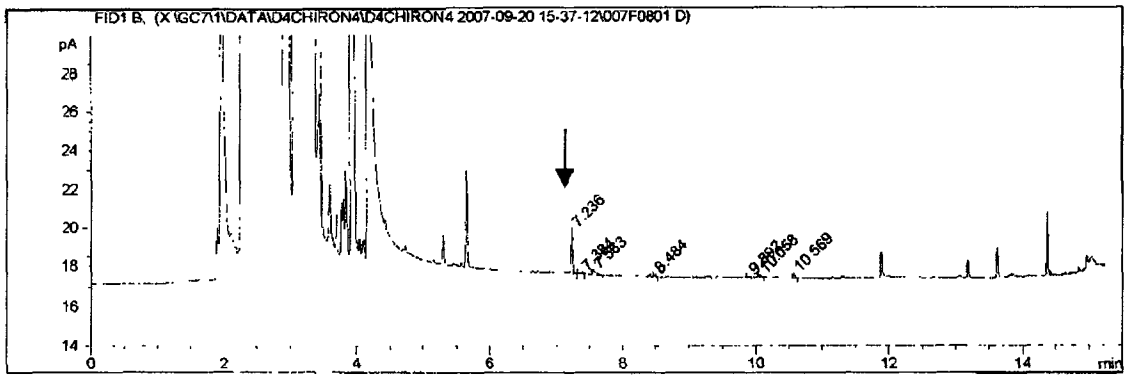
Representative Chromatogram of a High-level D4 Calibration Standard for Sediment



Nominal concentration: 70.0 mg/L. The arrow indicates the approximate retention time of D4.

Appendix 7.9

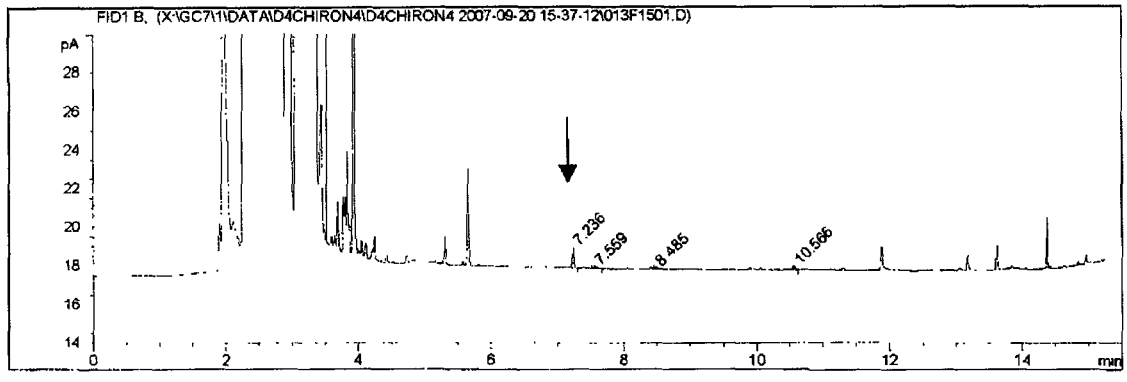
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-107-MAS-1; 30.0 mg/Kg, nominal concentration. The arrow indicates the approximate retention time of D4.

Appendix 7.10

Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-107-4; 125 mg/Kg nominal concentration. The arrow indicates the approximate retention time of D4.

Appendix 8

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measuredl Concentration (mg/Kg)		Numbers of Emerged Midges (Males and Females pooled) by Day of Test													
Replicate		15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	1	5	3	5	1	0	2	1	2	0	0	0	0	0
	B	2	5	4	5	1	0	1	0	0	0	0	1	0	0
	C	0	4	2	2	1	0	3	1	0	0	3	1	0	0
	D	0	5	5	5	1	0	0	2	0	0	0	0	0	0
6.5	A	1	7	4	1	0	2	0	0	0	0	0	0	0	0
	B	1	10	3	3	0	0	2	0	0	0	1	0	0	0
	C	1	2	3	5	2	0	3	1	1	0	0	1	0	0
	D	3	5	2	5	1	0	0	0	0	0	0	0	0	0
7.9	A	0	1	4	4	1	1	4	2	0	0	0	0	0	0
	B	3	6	4	1	3	0	1	1	0	0	0	0	0	0
	C	2	6	3	2	2	2	0	0	0	2	0	0	0	0
	D	3	3	4	1	3	0	1	0	1	0	0	0	0	0
19	A	2	2	5	6	2	0	0	0	0	2	0	1	0	0
	B	2	4	4	5	2	3	0	0	0	0	0	0	0	0
	C	4	7	4	3	0	0	0	0	0	0	0	0	0	0
	D	0	2	6	4	1	2	1	0	0	0	2	2	0	0
44	A	0	2	4	3	4	4	1	0	0	1	0	0	0	0
	B	1	2	5	4	3	1	1	0	0	2	0	1	0	0
	C	0	1	6	0	4	1	1	1	0	0	0	1	0	0
	D	0	4	3	2	2	1	1	0	0	0	0	1	0	0
131	A	1	7	3	1	0	0	1	0	0	0	0	0	0	0
	B	0	2	3	2	3	2	0	0	0	0	0	1	0	0
	C	0	1	0	2	1	0	1	0	0	0	0	0	0	0
	D	0	1	0	3	3	0	0	0	0	0	0	0	0	0
355	A	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	B	0	0	0	1	0	0	0	0	0	0	0	1	0	1
	C	0	0	0	0	1	1	0	0	0	1	0	0	0	0
	D	0	0	1	0	0	1	1	0	0	0	0	0	0	0

NC = negative control.

Appendix 8 (Continued)

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Males) by Day of Test													
Replicate		15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	1	4	0	4	0	0	0	0	0	0	--	--	--	--
	B	2	4	3	1	1	0	0	0	0	0	0	0	0	0
	C	0	3	2	1	0	0	0	0	0	0	0	0	0	0
	D	0	4	2	2	0	0	0	0	0	0	0	0	0	0
6.5	A	1	2	2	0	0	0	0	0	0	0	0	0	0	0
	B	1	5	1	1	0	0	0	0	0	0	0	--	--	--
	C	1	2	3	3	0	0	0	0	0	0	0	0	0	0
	D	3	4	1	1	0	0	0	0	0	0	0	0	0	0
7.9	A	0	0	4	1	0	0	1	0	0	0	0	0	0	0
	B	3	5	3	0	1	0	0	0	0	0	0	0	0	0
	C	2	4	2	0	0	1	0	0	0	0	0	0	0	0
	D	3	2	2	0	1	0	0	0	0	0	0	0	0	0
19	A	2	2	2	3	0	0	0	0	0	0	0	1	--	--
	B	2	3	4	1	0	0	--	--	--	--	--	--	--	--
	C	4	4	1	0	0	0	0	0	0	0	0	0	0	0
	D	0	1	5	2	0	1	0	0	0	0	0	0	--	--
44	A	0	2	3	2	1	0	0	0	0	0	0	0	0	0
	B	1	1	5	1	1	0	1X	0	0	0	0	0	--	--
	C	0	0	5	0	2	0	0	0	0	0	0	0	0	0
	D	0	3	3	1	0	0	0	0	0	0	0	0	0	0
131	A	1	6	3	0	0	0	1	0	0	0	0	0	0	0
	B	0	2	2	1	2X	1	0	0	0	0	0	1	0	0
	C	0	0	0	1	0	0	1	0	0	0	0	0	0	0
	D	0	0	0	3	1	0	0	0	0	0	0	0	0	0
355	A	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	B	0	0	0	1	0	0	0	0	0	0	0	0	0	1
	C	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	D	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Observations: X = emerged and died; -- = all emerged or dead.
NC = negative control.

Appendix 8 (Continued)

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Females) by Day of Test													
Replicate		15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	0	1	3	1	1	0	2	1	2	0	--	--	--	--
	B	0	1	1	4	0	0	1	0	0	0	0	1	0	0
	C	0	1	0	1	1	0	3	1	0	0	3	1X	0	0
	D	0	1	3	3	1	0	0	2	0	0	0	0	0	0
6.5	A	0	5	2	1	0	2	0	0	0	0	0	0	0	0
	B	0	5	2	2	0	0	2	0	0	0	1	--	--	--
	C	0	0	0	2	2	0	3	1	1	0	0	1	0	0
	D	0	1	1	4	1	0	0	0	0	0	0	0	0	0
7.9	A	0	1	0	3	1	1	3	2	0	0	0	0	0	0
	B	0	1	1	1	2	0	1	1	0	0	0	0	0	0
	C	0	2	1	2	2	1	0	0	0	2	0	0	0	0
	D	0	1	2	1	2	0	1	0	1	0	0	0	0	0
19	A	0	0	3	3	2	0	0	0	0	2	0	0	--	--
	B	0	1	0	4	2	3	--	--	--	--	--	--	--	--
	C	0	3	3	3	0	0	0	0	0	0	0	0	0	0
	D	0	1	1	2	1	1	1	0	0	0	2	2	--	--
44	A	0	0	1	1	3	4	1	0	0	1	0	0	0	0
	B	0	1	0	3	2	1	0	0	0	2	0	1	--	--
	C	0	1	1	0	2	1	1	1	0	0	0	1X	0	0
	D	0	1	0	1	2	1	1	0	0	0	0	1X	0	0
131	A	0	1	0	1	0	0	0	0	0	0	0	0	0	0
	B	0	0	1	1	1	1	0	0	0	0	0	0	0	0
	C	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	D	0	1	0	0	2	0	0	0	0	0	0	0	0	0
355	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	C	0	0	0	0	0	1	0	0	0	1	0	0	0	0
	D	0	0	0	0	0	1	1	0	0	0	0	0	0	0

Observations: X = emerged and died; -- all emerged or dead.
NC = negative control.

Appendix 9

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 & Insects
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

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D5: A BIOACCUMULATION TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-110

ASTM Standard E 1706-00

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

AUTHORS:

Henry O. Krueger, Ph.D.
Susan T. Thomas, B.S.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: April 12, 2007

STUDY COMPLETION DATE: August 15, 2008

AMENDED REPORT DATE: February 23, 2010

SUBMITTED TO:

Centre Europeen des Silicones
European Chemical Industry Council (Cefic)
Avenue E. van Nieuwenhuyse 4
B-1160 Brussels
Belgium

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

AMENDED

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Centre Europeen des Silicones European Chemical Industry Counell (Cefic)

TITLE: D5: A Bioaacumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-110

STUDY COMPLETION: August 15, 2008

AMENDED REPORT DATE: February 23, 2010

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exceptions:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods.

The stability of tissue samples during frozen storage was not determined.

Reference substance characterization and stability at the test site were not determined in compliance with Good Laboratory Practices.

STUDY DIRECTOR:

Henry O. Krueger, Ph.D — Date 2/23/10
Director of Aquatic Toxicology/Terrestrial Plants and Insects

SPONSOR APPROVAL:

Sponsor Representative — Date 3/2/10

Applicant/Submitter — Date

AMENDED

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM(98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR	MANAGEMENT:
Protocol	December 20, 2006	December 20, 2006	January 10, 2007
Test Substance Preparation	April 13, 2007	April 13, 2007	April 20, 2007
Temperature and Dissolved Oxygen Measurements	May 11, 2007	May 11, 2007	May 21, 2007
Observations	May 31, 2007	May 31, 2007	June 6, 2007
Biological Data and Draft Report	December 5 and 6, 2007	December 7, 2007	December 14, 2007
Analytical Data and Draft Report	December 3,4 and 5, 2007	December 5, 2007	December 6, 2007
Final Report	April 8, 2008	April 8, 2008	April 8, 2008
Amended Final Report	February 22, 2010	February 22, 2010	February 23, 2010

All inspections were study-based unless otherwise noted.

Marshall T. Hynson

Marshall T. Hynson, B.A.
Quality Assurance Program Supervisor

2/23/2010
Date

AMENDED

REPORT APPROVAL

SPONSOR: Centre Europeen des Siliconcs European Chemical Industry Council (Cefic)

TITLE: D5: A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-110

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AMENDED

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SUMMARY

SPONSOR: Centre Europeen des Silicones European Chemical Industry Council (Cefic)

TITLE: D5: A Bioaacumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-110

TEST DATES:		
	Study Initiation:	April 12, 2007
	Experimental Start (OECD):	April 13, 2007
	Experimental Start (EPA):	April 16, 2007
	Biological Termination:	June 5, 2007
	Experimental Termination:	June 5, 2007

LENGTH OF EXPOSURE: 50 Days (28 days Uptake; 22 days Depuration)

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

SOURCE OF TEST ORGANISMS: Aquatie Research Organisms
Hampton, NH 03842
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
100 mg a.i./Kg	20.8 mg/Kg
1000 mg a.i./Kg	336 mg/Kg

RESULTS:

	20.8 mg/Kg	336 mg/Kg
BAF[1]	4.27	0.46
k_1[2]	0.830	0.092
k_2[3]	0.193	0.201
BAFK[4]	4.29	0.46
$t_{1/2}$ (days)[5]	3.6	3.4
t_{90} (days)[6]	11.9	11.4

[1] BAF = Bioaccumulation Factor
[2] k_1 = Kinetic Uptake Rate
[3] k_2 = Depuration Rate
[4] BAFK based on Whole Tissue Concentrations (BAFK = Kinetic Bioaccumulation Factor)
[5] $t_{1/2}$ = Calculated time for 50% clearance of D5 in tissue
[6] t_{90} = Calculated time to reach 90% of steady-state in tissues.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Centre Europeen des Silicones European Chemical Industry Council (Cefic) at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from April 16, 2007 to June 5, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 583A-110 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the bioaccumulation potential of sediment-incorporated decamethylcyclopentasiloxane (D5) in the oligochaete, *Lumbriculus variegatus*. The formulated sediment used in the test had an organic content of approximately 3%. The sediment and a 28-day ration of food were dry mixed in the test chambers. Overlying water was added to the test chambers and the test chambers were allowed to settle for approximately 48 hours prior to the addition of the test organisms. The measured endpoints of the test were tissue concentrations after 14 and 28 days of uptake and during depuration.

EXPERIMENTAL DESIGN

The test consisted of a 28-day uptake phase followed by a 22-day depuration phase. During the uptake phase, oligochaetes were exposed to two sub-lethal test concentrations and a negative control. Eight replicate test chambers were maintained in each treatment and control group. Seven replicates were initiated with approximately one gram of tissue in each replicate. One replicate was used for analytical sampling of sediment on Day 0 and did not contain organisms. Each test chamber contained a quantity of sediment and overlying water.

Nominal test concentrations selected in consultation with the sponsor were 100 and 1000 mg a.i./Kg of sediment based on the dry weight of the sediment. The results of the study are based on the mean measured test concentrations. Sediment samples were collected and analyzed from one sacrificed replicate of each treatment group and control on days 0, 14 and 28 of the uptake phase. Results of the analyses were used to verify the exposure over time. Tissue samples were collected from the culture on Day 0 and from one sacrificed replicate from each treatment group and control on days 14 and 28 of the uptake phase and day 14 of the depuration phase. An additional replicate was sacrificed at the end of the depuration phase from each treatment group and control for the determination of lipid content in the

tissue. The tissue concentrations were used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioaccumulation factor (BAFK).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "D5: A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et. Al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (1); the ASTM E 1706-00 Guideline: *Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (2); the OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (3); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (4), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (5).

Test Substance

The test substance was received from Dow Corning on March 24, 2006 and assigned Wildlife International identification number 7556. The test substance, a liquid, was identified as: Dow Corning® 1693 Fluid; CAS Number 541-02-6; Batch No. 0000341832. The reported purity was 99.19%, with an expiration date of September 7, 2008. The test substance was stored under ambient conditions.

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and assigned Wildlife International identification number 7566. The internal standard, a liquid, was identified as: Tetrakis (trimethylsilyloxy) silane, 97%; Batch Number: 13222MB; CAS Number: 3555-47-3. The internal standard had a reported purity of 97% and was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guidelines 218 (6) was used. The sediment was composed of approximately 10% peat moss, 70% sand and 20% silt and clay (kaolin clay). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the

peat, since the peat was added later (Appendix 2). The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry constituents of the final mixture were stored under ambient conditions until used. The results of periodic analyses performed to measure the concentrations of selected contaminants in the formulated sediment used by Wildlife International, Ltd. are presented in Appendix 3.

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 measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The study was conducted in a temperature-controlled room. Each test chamber was a 9-L glass aquaria (approximately 15 x 30 x 20 cm high) holding 5 liters of water and 1 liter of sediment. Aquaria were arranged in groups of four according to treatment group.

A continuous-flow diluter was used to provide dilution water (well water) to each aquaria so that there were two volume additions per day. The flow of dilution water was controlled using rotameters. The rotameters were calibrated prior to the start of the test and approximately weekly during the test. The diluter system delivered dilution water to a mixing chamber from which one line was split into four separate supply lines that delivered water to each aquaria in the group. The proportion of water split to each replicate was checked prior to the test and approximately weekly during the test to ensure that flow rates varied by no more that 10% of the mean flow rate of the replicates. The general operation of the diluter was checked visually at least two times per day during the test and at least one time at the beginning and end of the test.

Preparation of Test Concentrations

Concentrations of the test substance were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment). Nominal test concentrations were 100 and 1000 mg a.i./Kg. An aliquot of 384.6 g of peat (74% moisture content) was weighed into 24 Nalgene bottles. Eight bottles were used to prepare the 100 mg a.i./Kg treatment level and eight were used to prepare the 1000 mg a.i./Kg treatment level. The remaining eight bottles were used to prepare the negative control. Neat test material was then added to the peat and mixed ovemight on a rotary mixer. After approximately 16 hours of mixing the treatment groups were completely processed one at a time by adding 900.0 g of the formulated sediment to each of the 24 Nalgene bottles (eight at a time) and mixed on the rotary mixer for approximately 40 minutes each.

Eight replicate test chambers were prepared for each treatment and control group for use in the uptake phase. For each replicate, 1000 mL of sediment was placed in a 9-L glass aquaria. Next, a pre-weighed amount of food (Salmon Starter), enough for 28 days (200 mg), was added to each chamber that would contain organisms during the test and was mixed into the dry sediment using a glass stir rod. Finally, approximately 5000 mL of overlying water (well water) was poured into the chamber over a baffle to minimize the disturbance to the sediment. Test chambers were indiscriminately positioned in an environmentally controlled room in groups of four according to treatment group approximately 48 hours prior to test initiation to condition the sediment prior to introduction of organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Three replicate test chambers were prepared for each treatment and control group for use in the depuration phase. For each replicate, 1000 mL of untreated sediment was placed in 9-L glass aquarium The untreated sediment used in the depuration phase of the study was prepared similarly to the treated sediment used in the uptake phase of the study, however 370.0 g of peat (with a 73% moisture content) were mixed with 900.0g of the sand/clay mixture. The reason the weight of peat added to the mixture changed between the sediment prepared for the uptake phase and the sediment prepared for the depuration phase is due to the fact that the moisture content of the peat was different. When corrected for the percent moisture, the same amount of peat was added to each mixture. The percent organic carbon for the sediment used in the uptake phase and in the depuration phase was 3.2 and 2.7% respectively, showing that the final mixtures were comparable

A pre-weighed aliquot of salmon starter was mixed into the dry sediment prior to adding the 5000 mL of overlying water. The chambers were allowed to settle in the environmental chamber approximately two days prior to transferring the test organisms from the uptake test chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Aquatic Research Organisms, Hampton, New Hampshire. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 14-day holding period immediately preceding the test, water temperature ranged from 20.0 to 24.4°C, while the pH ranged from 8.1 to 8.4 and dissolved oxygen ranged from 6.3 to 8.7 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, approximately one gram (wet weight) of oligochaetes was impartially distributed to transfer chambers. The transfer chambers were indiscriminately assigned to test chambers and the organisms were transferred to the test chambers below the air/water interface. Observations of mortality and abnormal behavior were made daily during the test. Seven replicate test chambers in each treatment and control group contained organisms, while one test chamber per group was maintained without organisms for day 0 analytical purposes.

Oligochaetes were fed a mixture of yeast, cereal grass and trout chow (YCT) during the holding period and salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the test. A 200 mg aliquot of food was weighed for each test chamber that contained organisms during the test and was added to the test chamber after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. An additional 200 mg aliquot of salmon starter was added to the test chambers prepared for the depuration phase of the test after adding the sediment, but prior to adding the overlying water and organisms.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 215 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test chamber daily during the test using a hand-held liquid-in-glass thermometer. Temperature also was measured continuously in a beaker of water adjacent to the test chambers using a Fulscope ER/C Recorder. The continuous recorder was verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a sample of overlying water collected from one alternating replicate test chamber from each treatment and control group at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (7). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test chambers at the beginning of the test, an additional 1 gram organisms were impartially selected at the beginning of the test and measured for dry weight.

The weight of the subset of organisms was used to determine the ratio of organic carbon in the sediment to the dry weight of organisms. The test chambers were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). The test was divided into two phases: uptake and depuration. The duration of the uptake phase was 28 days, and the depuration phase was 22 days. On Day 28 of the uptake phase, the organisms from three replicates of each treatment group were sieved from the sediment, enumerated and transferred to new test chambers containing untreated sediment and clean water for the duration of the test. At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group was measured.

Statistical Analyses

The equations used for BAF (bioaccumulation factor) and BAFK (kinetic bioaccumulation factor) calculations are described in draft OPPTS 850.1730 Guidance Document (8). The kinetic uptake rate (k_1) and depuration rate (k_2) were calculated for oligochaete tissue and then used to calculate a kinetic bioconcentration factor (BAFK = k_1/ k_2). These rate constants were also used to calculate both the half-life for clearance in tissue ($t_{½}$) and time to reach 90% of steady-state (t_{90}).

Analytical Sampling

Sediment samples were collected from one test chamber of each treatment group at the beginning of the test, on Day 14 and Day 28 of the uptake phase. The replicate used for sample collection on Day 0 did not contain oligochaetes, whereas at test initiation oligochaetes were placed in the replicates used for sample collection on Days 14 and 28. The samples were analyzed on the day of collection. Tissue samples were collected on Day 0 from the culture, on Days 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Tissue samples were also collected on Day 22 of the depuration phase from each treatment group for the determination of lipid content. The depuration phase was continued past Day 14 to Day 22 so that the analytical results of tissue concentrations could be evaluated. Tissue samples were collected by sieving the sediment to remove organisms. The organisms were rinsed free of as much sediment as possible and placed in a tared liquid scintillation vial. The water was removed from the vial (as much as possible) and a wet weight was obtained. Tissue samples were stored frozen for 0 to 57 days until analysis.

Analytical Methods

The methods used for the sediment and tissue analyses of the D5 samples were based on methodologies provided by the Sponsor and modified by Wildlife International, Ltd. For the sediment analysis, the analytical method consisted of extracting sediment with ethyl acetate (two times) and transferring to appropriate vessel containing sodium sulfate. The ethyl acetate extract (1 mL) was added to an autosampler vial containing 50.0 µL of MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to remove interferences. Samples were analyzed by gas chromatography equipped with flame ionization detection. For the tissue analysis, the method consisted of extracting the samples with tetrahydrofuran (THF). Samples were analyzed by gas chromatography equipped with mass selective detection or by gas chromatography equipped with flame ionization detection.

Concentrations of D5 in the sediment samples were determined using an Agilent 5890 Gas Chromatograph (GC) equipped with Flame Ionization Capture Detector (FID). Chromatographic separations were achieved using a J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness). Concentrations of D5 in the tissue samples were determined using an Agilent 5890 Gas Chromatograph (GC) equipped with an Agilent Model 5973 Mass Selective Detector operated in SIM mode. Chromatographic separations were achieved using a ZB-5 HT Column (0.25 mm x 30 m ID, 1.0 µm). Method flowcharts for the analyses of D5 in sediment and tissue are provided in Appendices 6.1 and 6.2, respectively. The instrumental parameters for the analyses are provided in Appendix 6.4 and 6.5, respectively.

For sediment samples, calibration standards of D5, ranging in concentration from 1.00 to 70.0 mg/L were prepared in ethyl acetate using stock solutions of D5 test substance in 80% dimethylformamide (DMF) : 20% toluene. For tissue samples, calibration standards of D5, ranging in concentration from 0.0500 to 0.500 mg/L were prepared in tetrahydrofuran (THF) using stock solutions of D5 in THF (Appendix 6.6). After the Day 14 tissue samples were analyzed, it was determined that the test samples should be analyzed GC/FID and the negative controls and QC's were analyzed by GC/MS to achieve the appropriate LOQ. All sample extracts from depuration were analyzed by GC/MS due to lower analyte concentrations. For tissue analyzed by GC/FID, the calibration standards ranged from 4.00 to 40.0 mg/L and were prepared in THF using stock solutions of D5 in THF (Appendix 6.6). Fresh standards were prepared and analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the

calibration standards. The concentration of D5 in the sediment samples was determined by substituting the peak area responses of the samples into the applicable linear regression equation. The concentration of D5 in the tissue samples was determined by substituting the ratio of the peak area of D5 to the peak area of the internal standards into the applicable linear regression equation. An example of the calculations for a representative sediment sample is included in Appendix 6.7.

For sediment, the method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg/L) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample. The method limit of quantitation (LOQ) for tissue was defined as 0.500 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.0500 mg/Kg) and the dilution factor of the matrix blank samples (10.0). No interferences were observed at or above the LOQ during the sample analyses (Appendices 6.8 and 6.9).

Sediment samples were fortified at 100 and 500 mg/Kg, using stock solutions of D5 in 80% (DMF) : 20% toluene (Appendix 6.6). The measured concentrations for the matrix fortification samples were 76.7 to 100% (Appendix 6.8).

Tissue samples were fortified at 1.00 and 10.0 mg/Kg, using stock solutions of D5 in THF (Appendix 6.6). The quality control samples were fortified with the internal standard at 250 µg a.i./L for the 1.00 mg/Kg concentration and 1000 µg a.i./L for the 10.0 mg/Kg concentration at the instrument. The measured concentrations for the matrix fortification samples were 97.4 to 133% (Appendix 6.9).

A representative calibration curve for sediment is presented in Appendix 6.10. Representative chromatograms of low and high-level sediment calibration standards are presented in Appendices 6.11 and 6.12, respectively. A representative chromatogram of a sediment matrix fortification sample is presented in Appendix 6.13. A representative chromatogram of a sediment test sample is presented in Appendix 6.14.

A representative calibration curve for tissue is presented in Appendix 6.15. Representative chromatograms of low and high-level tissue calibration standards are presented in Appendices 6.16 and

6.17, respectively. Representative chromatograms of a tissue matrix blank and a matrix fortification sample are presented in Appendices 6.18 and 6.19, respectively. A representative chromatogram of a tissue test sample is presented in Appendix 6.20.

Lipid Analyses

Vials containing tissue samples for lipid analysis were removed from the freezer and allowed to thaw. Tare weights of the vials had been pre-determined. HPLC-grade bottled water (10 mL) was added to each sample and samples were homogenized for approximately one minute using a hand-held tissue homogenizer. The homogenizer was rinsed with the appropriate solvent(s) in between samples. Each homogenate was transferred to a 250-mL separatory funnel containing 25 mL of chloroform and 50 mL of methanol. Each vial was rinsed with an additional 10 mL of bottled water and the rinse was poured into its respective separatory funnel. The funnels were then shaken with venting for approximately one minute, and 50 mL of chloroform followed by 50 mL of saturated sodium chloride was added to each separatory funnel. Funnels were swirled briefly with venting and the phases were allowed to separate. For each sample, the chloroform layer was drained through a powder funnel packed with glass wool and anhydrous sodium sulfate into a 250-mL roundbottom flask. An additional 50-mL aliquot of chloroform was added to each separatory funnel and the extraction and draining procedures were repeated. The extracts were rotary-evaporated to near dryness in a waterbath maintained at approximately 40°C. Each sample was then transferred to a pre-weighed, labeled scintillation vial. Each 250-mL roundbottom flask was rinsed with a small volume of chloroform and the rinse was transferred to its respective scintillation vial. The remaining solvent in each vial was evaporated under a gentle stream of nitrogen or clean dry air. Each vial was reweighed and the weight recorded. The lipid content was calculated from the difference in the vial tare weight and the vial weight containing the lipid. Percent lipids were calculated from the ratio of lipid content to total tissue sample weight. Lipid content determinations are presented in Table 4, and a method flowchart is presented in Appendix 6.3.

RESULTS AND DISCUSSION

Measurement of Test Concentrations in Sediment

Results of analyses to measure concentrations of D5 in samples collected during the definitive test are presented in Tables 1-4 and Appendix 6. Nominal concentrations selected for use in this definitive test were 100 and 1000 mg a.i./Kg.

Samples of sediment were collected from the negative control and each of the test concentrations on Day 0, Day 14 and Day 28. The purpose of these samples was to verify the concentrations in sediment at test initiation and to monitor the test substance in the sediment over the course of the study. Results are based on mean measured concentrations. Negative control samples on Days 0, 14, and 28 were all below the limit of quantitation (LOQ).

Mean measured concentrations of D5 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 0 were 14.2 and 376 mg/Kg, respectively (Table 1). Mean measured concentrations of D5 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 14 were 30.6 and 355 mg/Kg, respectively. Mean measured concentrations of D5 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 28 were 17.7 and 278 mg/Kg, respectively. All concentrations showed a low but constant trend in the sediment throughout the study. The overall mean measured concentrations for the 100 and 1000 mg a.i./Kg nominal treatment groups were 20.8 and 336 mg/Kg, or 21 and 34 % of nominal, respectively. The reason the nominal concentrations are presented as mg a.i./Kg and the measured concentrations are presented as mg/Kg is that the analytical standards were not corrected for the active ingredient of the test article (99.19%).

Measurement of D5 in Tissue

Tissue samples were collected from the negative control and each of the test concentrations on Day 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Negative control samples were below the limit of quantitation (LOQ) at each sampling interval.

The concentrations of D5 in tissues of oligochaetes exposed to 20.8 mg/Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 1. Mean measured concentrations of D5 in tissues of oligochaetes from the 20.8 mg/Kg nominal test concentration on Days 14 and 28 were 95 and 89 mg/Kg. The BAF and BAFK values for oligochaete tissue were 4.27 and 4.29 respectively (Table 5). The estimated time to reach 90% of steady state (t_{90}) in oligochaete tissue was 11.9 days (Table 5). The concentration of D5 depurated quickly in oligochaete tissue and the mean measured concentration of D5 in oligochaete tissue for the 20.8 mg/Kg treatment group by Day 14 of depuration was 5.93 mg/Kg, which is approximately 6.7% of

the mean tissue concentration at Day 28 of uptake (Figure 1). The estimated time to reach 50% clearance ($t_{1/2}$)for oligochaete tissue was 3.6 days (Table 5).

The concentrations of D5 in tissues of oligochaetes exposed to 336 mg/Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 2. Mean measured concentrations of D5 in tissues of oligochaetes from the 336 mg/Kg nominal test concentration on Days 14 and 28 were 171 and 155 mg/Kg. The BAF and BAFK values for oligochaete tissue were 0.46 and 0.46, respectively (Table 5). The calculation to estimate time to reach 90% of steady state in oligochaete tissue was 11.4 days (Table 5). As in the 20.8 mg/Kg treatment group, the concentration of D5 depurated quickly in oligochaete tissue and the mean measured concentration of D5 in oligochaete tissue by Day 14 of depuration was 9.21 mg/kg, which is approximately 5.9% of the mean tissue concentration at Day 28 of uptake (Figure 2). Estimate of time to reach 50% clearance for oligochaete tissue was 3.4 days (Table 5).

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 6 through 11. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 75% (6.4 mg/L) of saturation throughout the test. Measurements of pH ranged from 8.0 to 8.1 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 12 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatments and control group and no treatment-related signs of toxicity. At test termination, there was an increase in the numbers of oligochaetes present in some test chambers from the number of worms originally placed in each replicate, indicating that reproduction had occurred.

At the beginning of the test, a one-gram (wet weight) aliquot of organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the dry weight of those organisms was 0.1015 g. It was determined that the formulated sediment had an organic

content of 3%; therefore, the ratio of organic carbon to the dry weight of organisms was 30 g organic carbon : 1 g of dry tissue.

CONCLUSIONS

A kinetic BAF value for D5 in oligochaetes (*Lumbriculus variegatus*) was calculated for both the 20.8 and 336 mg/Kg treatment groups. The BAF and BAFK values for oligochaete tissue in the 20.8 mg/Kg treatment group were 4.27 and 4.29, respectively. The BAF and BAFK values for oligochaete tissue in the 336 mg/Kg treatment group were 0.46 and 0.46, respectively. During depuration, D5 was eliminated quickly and the Day 14 depuration tissue concentrations for the two treatment groups were 6 – 6.7% of the Day 28 uptake tissue concentrations. The estimated time to reach 50% clearance for oligochaete tissue concentrations was 3.6 days for the 20.8 mg/Kg treatment group, and 3.4 days for oligochaete tissue for the 336 mg/Kg treatment group.

REFERENCES

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Table 1

Measured Concentrations of D5 in Sediment Samples

Nominal Test Concentration (mg a.i./Kg)	Sample Number (583A-110-)	Sampling Time (Day)	Measured Concentration (mg/Kg)	Percent of Nominal[1]	Mean Measured	Mean % of Nominal
Negative Control (0.0)	S1	0	< 7.82	--	--	--
	S2	0	< 8.83	--		
	S9	14	< 11.3	--	--	--
	S10	14	< 8.80	--		
	S17	28	< 19.3	--	--	--
	S18	28	< 14.3	--		
100	S3	0	11.4	11.4	20.8	21
	S4	0	16.4	16.4		
	S5	0	14.8	14.8		
	S11	14	34.4	34.4		
	S12	14	29.9	29.9		
	S13	14	27.5	27.5		
	S19	28	17.4	17.4		
	S20	28	18.7	18.7		
	S21	28	17.1	17.1		
1000	S6	0	342	34.2	336	34
	S7	0	426	42.6		
	S8	0	361	36.1		
	S14	14	417	41.7		
	S15	14	275	27.5		
	S16	14	373	37.3		
	S22	28	266	26.6		
	S23	28	298	29.8		
	S24	28	271	27.1		

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

Table 2

Measured Concentrations of D5 in Tissue Samples

Mean Measured Test Concentration (mg/Kg)	Sample Number (583A-110-)	Sampling Time (Day)	Measured Concentration[1] (mg/Kg)	Mean Measured
Negative Control (0.0)	T1A	0	<LOQ	--
	T1B	0	<LOQ	
	T2A	14	<LOQ	
	T2B	14	<LOQ	
	T5A	28	<LOQ	
	T5B	28	<LOQ	
20.8	T3A	14	96.5	95
	T3B	14	102	
	T3C	14	86.1	
	T6A	28	75.4	89
	T6B	28	101	
	T6C	28	90.5	
336	T4A	14	156	171
	T4B	14	147	
	T4C	14	210	
	T7A	28	156	155
	T7B	28	173	
	T7C	28	135	

[1] The limit of quantitation (LOQ) was 40.0 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (4.00 mg/L) and the dilution factor of the matrix blank samples (10.0).

Table 3

Measured Concentrations of D5 in Tissue Samples Analyzed by GC/MS
During the Depuration Phase

Mean Measured Test Concentration (mg/Kg)	Sample Number (583A-110-)	Sampling Time (Day)	Measured Concentration[1] (mg/Kg)	Mean Measured
Negative Control	T8A	14	< LOQ	
(0.0)	T8B	14	< LOQ	
20.8	T9A	14	8.00*	5.93
	T9B	14	5.79*	
	T9C	14	4.00*	
336	T10A	14	12.0*	9.21
	T10B	14	6.43*	
	T10C	14	9.21*	

[1] The limit of quantitation (LOQ) was 0.500 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.0500 mg/Kg) and the dilution factor of the matrix blank samples (1.00).

* Extrapolated values, the sample slightly exceeded the highest calibration standard.

Table 4

Lipid Determination in Oligochaete Tissue

Sample Number (583A-110-)	Tissue Weight (grams)	Lipid Weight (grams)	Percent Lipids[1] (%)
L-1	0.9391	0.0190	2.02
L-2	1.1225	0.0200	1.78
L-3	1.0062	0.0180	1.79
		Mean lipid percentage	1.86

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

Table 5

BAF Values

Mean Measured Test Concentration (mg/Kg)	BAF[1]	BAFK[1]	k_1	k_2
20.8	4.27	4.29	0.830	0.193
336	0.46	0.46	0.092	0.201

[1] Equations for BAF, BAFK, k_1 and k_2:

BAF = (Day 28 tissue conc) ÷ (mean sediment conc)

BAFK = (k_1) ÷ (k_2)

k_1 = (k_2 x Day 28 uptake tissue concentration) ÷ (mean sediment conc × $(1 - e(-k_2 t))$)

k_2 = (ln(Day 28 uptake tissue conc) − ln(Day 14 depuration tissue conc)) ÷ (14 days of depuration)

Estimated Time to Reach 50 and 90% of Steady State

Mean Measured Test Concentration (mg/Kg)	$t_{1/2}$ (days)	t_{90} (days)
20.8	3.6	11.9
336	3.4	11.4

Table 6

Temperature of Overlying Water in the Test Chambers During the Uptake Phase

Nominal Test Concentration (mg a.i./Kg)	Temperature (°C)														
Day	0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	F	G	A
Negative Control	22.7	22.7	22.7	22.9	22.7	22.8	22.6	23.0	23.1	22.8	22.9	23.0	23.0	22.8	22.8
100	22.8	22.0	22.0	22.4	22.7	22.8	22.8	22.9	22.8	22.5	22.6	22.7	22.9	22.9	22.7
1000	22.4	22.4	22.1	22.6	22.4	22.3	22.4	22.9	23.0	22.5	22.6	22.8	22.8	22.7	22.8

[1] Temperature measured continuously in a beaker of water adjacent to the test chambers ranged from approximately 22.5 to 23.5°C during the test.

Nominal Test Concentration (mg a.i./Kg)	Temperature (°C)													
Day	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Replicate:	B	C	D	F	A	B	C	D	F	A	B	C	D	F
Negative Control	22.8	22.6	22.9	22.6	23.1	22.2	22.3	22.7	23.4	23.4	23.4	23.4	23.3	22.5
100	22.8	22.5	22.7	22.3	23.0	22.1	22.1	22.4	23.2	23.3	23.2	23.3	23.0	23.5
1000	22.7	22.6	22.7	22.6	22.9	22.2	22.4	22.3	23.4	23.3	23.3	23.2	23.1	22.6

Table 7

Temperature of Overlying Water in the Test Chambers During the Depuration Phase

Nominal Test Concentration (mg a.i./Kg)	Day	Temperature (°C)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control		23.4	23.3	23.3	23.1	22.4	23.0	23.1	22.8	23.0	23.2	23.3	23.3	23.4	23.4
100		23.2	23.3	23.3	23.1	22.5	23.1	22.9	22.8	23.0	23.1	23.2	23.2	23.4	23.4
1000		23.1	23.4	23.3	23.2	22.7	23.1	23.0	22.9	23.1	23.2	23.3	23.2	23.3	23.4

Nominal Test Concentration (mg a.i./Kg)	Day	Temperature (°C)							
		15	16	17	18	19	20	21	22
	Replicate:	C	A	B	A	B	A	B	A
Negative Control		23.3	23.2	23.1	23.2	23.2	23.2	23.3	23.2
100		23.3	23.2	23.1	23.2	23.4	23.2	23.2	23.0
1000		23.3	23.2	23.2	23.2	23.1	23.1	23.0	23.1

Table 8

Dissolved Oxygen in Overlying Water in the Test Chambers During the Uptake Phase

Nominal Test Concentration (mg a.i./Kg)	Day	0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	F	G	A
		Dissolved Oxygen (mg/L)														
Negative Control		7.7	7.1	7.1	7.1	7.4	7.3	7.3	7.4	6.9	7.5	7.3	7.5	7.6	7.5	7.7
100		7.7	7.3	7.1	7.0	7.3	7.2	7.3	7.5	7.1	7.4	7.7	7.6	7.6	7.4	7.7
1000		7.6	7.4	7.2	7.1	7.3	7.3	7.6	7.5	7.2	7.5	7.7	7.6	7.6	7.6	7.8

[1] A dissolved oxygen concentration of 5.1mg/L represents 60% saturation at 23°C.

Nominal Test Concentration (mg a.i./Kg)	Day	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	B	C	D	F	A	B	C	D	F	A	B	C	D	F
		Dissolved Oxygen (mg/L)													
Negative Control		7.1	7.5	8.0	7.5	8.0	7.8	7.9	7.8	7.6	7.6	7.6	7.4	7.6	7.7
100		7.3	7.2	8.2	7.6	8.0	7.7	7.6	7.8	7.7	7.5	7.7	7.5	7.6	7.7
1000		7.5	7.8	8.2	7.5	8.1	7.7	7.9	7.8	7.5	7.7	7.7	7.4	7.6	7.6

Table 9

Dissolved Oxygen in the Overlying Water of the Test Chambers During the Depuration Phase

Nominal Test Concentration (mg a.i./Kg)	Day	Dissolved Oxygen (mg/L)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control		7.4	6.4	6.6	7.2	7.3	7.3	7.5	7.6	7.8	7.8	7.7	7.8	8.0	8.5
100		7.5	7.0	7.1	7.4	7.4	7.3	7.4	7.5	7.5	7.6	7.5	7.8	7.9	8.4
1000		7.6	7.1	6.8	7.6	7.5	7.3	7.6	7.5	7.7	7.7	7.5	7.7	8.0	8.4

Nominal Test Concentration (mg a.i./Kg)	Day	Dissolved Oxygen (mg/L)							
		15	16	17	18	19	20	21	22
	Replicate:	C	A	B	A	B	A	B	A
Negative Control		7.7	7.9	7.6	7.5	7.7	7.6	7.7	7.7
100		7.6	7.8	7.6	7.4	7.8	7.5	7.7	7.6
1000		7.5	7.8	7.5	7.5	7.9	7.6	7.7	7.8

Table 10

pH of Overlying Water in the Test Chambers During the Uptake Phase

Nominal Test Concentration (mg a.i./Kg)	Replicate:	pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
		A	B	C	D	F
Negative Control		8.1	8.1	8.1	8.1	8.0
100		8.1	8.1	8.1	8.1	8.0
1000		8.1	8.1	8.1	8.1	8.0

Table 11

pH of Overlying Water in the Test Chambers During the Depuration Phase

Nominal Test Concentration (mg a.i./Kg)		pH			
		Day 7	Day 14	Day 21	Day 22
	Replicate:	A	B	A	B
Negative Control		8.1	8.1	8.1	8.1
100		8.1	8.1	8.1	8.1
1000		8.1	8.1	8.1	8.1

Table 12

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Chambers During the Test

Nominal Test Concentration (mg a.i./Kg)	Day 0 (Uptake)				Day 28 (Uptake)			
	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)
Negative Control	122	176	305	<0.17[1]	132	186	330	<0.17
100	140	184	310	<0.17	136	190	340	<0.17
1000	140	182	315	<0.17	136	188	340	<0.17
Replicate	A				F			

[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Nominal Test Concentration (mg a.i./Kg)	Day 22 (Depuration)			
	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)
Negative Control	138	184	320	<0.17[1]
100	140	182	330	<0.17
1000	140	184	330	<0.17
Replicate	A			

[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Figure 1.

Concentrations of D5 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 20.8 mg/Kg

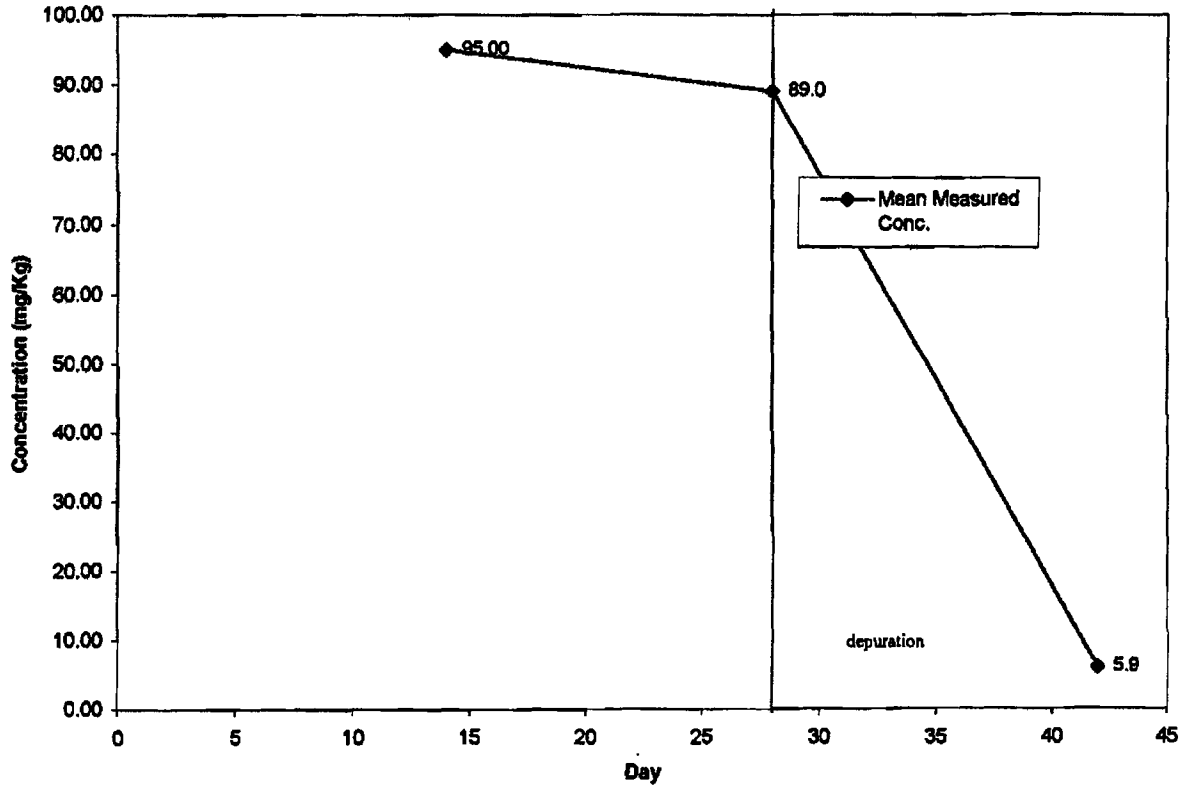
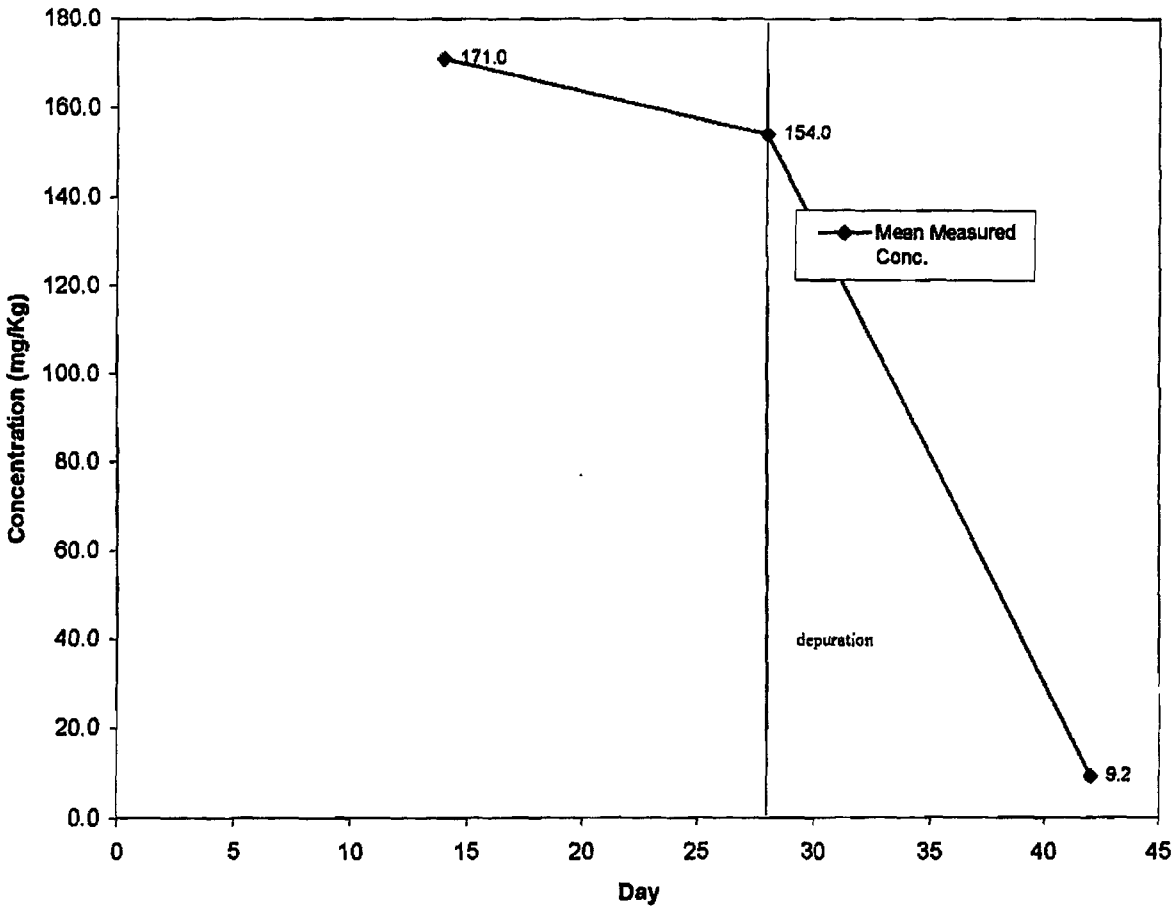


Figure 2.

Concentrations of D5 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 336 mg/Kg



Appendix 1

Study Protocol, Amendments and Deviation

PROTOCOL

D5: A BIOACCUMULATION TEST
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

ASTM Standard E 1706-00

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
583A-110

SPONSOR

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European Chemical Industry Council (Cefic)
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PERFORMING LABORATORY

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January 10, 2007

Wildlife International, Ltd.

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D5: A BIOACCUMULATION TEST
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

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LABORATORY MANAGEMENT: Willard B. Nixon, Ph. D.
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 4/12/07

LABORATORY MANAGEMENT — DATE 4/12/07

SPONSOR'S REPRESENTATIVE — DATE 1/12/07

Wildlife International, Ltd.

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FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: _____ Experimental Termination Date: _____

Project No.: 583A-110

Test Concentrations: SC, 100, and 1000 mg a.i./kg

Test Substance No.: 7556 Reference Substance No. (if applicable): 7566

INTRODUCTION

Wildlife International, Ltd. will conduct a bioaccumulation test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1); the U.S. EPA 600/R-99/064 *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.) (2), the ASTM Standard E 1706-00: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (5), and the Federal Environment Agency: Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (6). Raw data for all work performed at Wildlife International, Ltd. and 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. A copy of the final report will be archived at Dow Corning.

OBJECTIVE

The objective of this study is to determine the bioaccumulation potential of a sediment-incorporated test substance in the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions followed by a depuration period of at least 14 days in untreated sediment. The sediment used in the test will have an organic content of approximately 5%. A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after the sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding organisms. The measured endpoints of the test are tissue concentrations after 14 and 28 days of uptake and during depuration

EXPERIMENTAL DESIGN

Oligochaetes will be exposed to two test concentrations (100 and 1000 mg a.i./kg) and a negative control. Each group will consist of eight replicate test chambers. Seven replicates will contain up to approximately one gram of tissue (approximately 300 organisms) in each chamber. One

replicate will be used for analytical sampling of sediment on Day 0. The test will be divided into two phases: uptake and depuration. During the uptake phase, oligochaetes in the treatment groups will be exposed to sublethal concentrations of sediment incorporated D-5, while oligochaetes in the control group will be exposed only to untreated sediment. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Days 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Each test chamber will contain a quantity of sediment and overlying water. Only sediment will be analyzed in test systems, since the water solubility of D5 is 17.0 µg/L (7). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of D5 in water. Therefore, only sediment samples from the analytical sampling test chambers will be collected from each control and treatment group at specified intervals for analysis of the test substance. No oligochaetes will be placed in "analytical" replicate(s) sampled at the beginning of the test. However, the "analytical" replicate(s) sampled after the beginning of the test (e.g., on Day 14 and 28) will contain oligochaetes. Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be 100 and 1000 mg a.i./kg. Results of the analyses will be used to verify the exposure over time.

To control bias, oligochaetes will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to

GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	decamethylcyclopentasiloxane, supplied as Dow Corning ® 1693 Fluid
Batch No.:	0000341832
Expiration Date:	7 Sept 2008, extended as part of this study
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	541-02-6
Physical Description:	Colorless liquid, refer to PDM
Chemical Stability:	Stable, refer to MSDS
Purity:	99.19 ± 0.005 area % purity, as determined by this study
Solubility:	Ether, refer to PDM, and/or maybe soluble in other common organic solvents
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1.5 kg batch of sediment to 5%. The peat and test substance will be mixed overnight on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data. Approximately 1-2 L of the dry mix will then be transferred to each of eight test chambers. The same volume of sediment will be added to each chamber. The transfer time will be kept to a minimum (approximately 10-20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance.

After all 8 chambers have been filled with sediment, a 28 day ration of food (approximately 200 mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 5 L of well water will be added to the chambers. Test chambers will be maintained in an environmentally controlled room. Dilution water will be delivered to each test chamber at a rate that will result in two turnovers of dilution water per day After approximately 48 hours of acclimation and settling, organisms will be added to the chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study. Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd. Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Organisms will be fed YCT during holding. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test. Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (8) will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The

percent organic carbon is based solely on peat as the source of organic carbon. Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon.

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report.

Test Apparatus

The study will be conducted in a temperature-controlled room. Each test chamber will be a 9-L aquaria (approximately 15 X 30 X 20 cm high) holding approximately 5 liters of water. Each aquaria will be filled with the same amount of sediment (1 to 2 L). Aquaria will be set out in groups of four.

A continuous-flow diluter will be used to provide dilution water (well water) to each aquaria so that there are two volume additions per day. The flow of dilution water will be controlled using rotameters. The rotameters will be calibrated prior to test initiation and at approximately weekly intervals thereafter. The diluter system delivers dilution water to a mixing chamber from which one

line is split into four separate supply lines that deliver water to each aquaria in a group. The proportion of water split to each replicate will be checked prior to the test and at approximately weekly intervals thereafter to ensure that these flow rates vary by no more than ±10% of the mean flow rate of the replicates. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured daily using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured daily in the overlying water from one alternating replicate of each experimental group. Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week. Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a sample of the overlying water from one replicate of each experimental group at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (9). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, approximately 1 g (approximately 300) adult oligochaetes will be impartially distributed to transfer containers (e.g., glass beakers) until each container holds its complement of approximately 1 g. The organisms in each transfer container then will be transferred below the air/water interface to the test chambers (glass aquaria). An additional batch of individuals (~1 g) will be impartially selected at the beginning of the test and will be measured for dry weight. The weight of the subset of organisms will be used to determine the ration of organic carbon in the sediment to the dry weight of organisms. After completing the transfer of organisms, the test chambers will be maintained in a temperature-controlled room. Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming). At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group will be measured.

The test will be divided into two phases: uptake and depuration. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Day 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from one test chamber of each concentration at the beginning of the test (no more than one hour after completing

Wildlife International, Ltd.

the addition of organisms to the test system), at 14 days and at the end of the uptake phase (Day 28). The overlying water will be removed from the test chamber. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below:

Phase	Sample Interval	Sediment[1]	Oligochaetes[2]	Action
Uptake	0 hours	X	X[3]	Organisms added
	14 days	X	X	
	28 days	X	X	Organisms removed And purged
Depuration[4]	14 days		X	

[1] There will be two concentration tested (100 and 1000 mg a.i./kg dry weight). Sufficient sediment will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[2] Approximately 1g of oligochaetes will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[3] One sample of approximately 1g of oligochaetes from the culture will be used to determine background levels in oligochaetes.

[4] Additional depuration samples will be added if necessary pending the outcome of the Day 14 samples.

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

The kinetic uptake rate (k_1) and depuration rate (k_2) will be calculated for oligochaete tissue using the calculations described in draft OPPTS 850.1730 Guidance Document (10). These rate constants will be used to calculate a kinetic bioconcentration factor (BCFK = k_1/k_2) and also to calculate the half-life for clearance in tissue ($t_{1/2}$), time to reach 90% of steady-state (t_{90}), and BCFK values using the equations and graphical methods outlined in the draft OPPTS 850.1730 Guidance Document.

The BCFK of D5 can be defined as the ratio of the uptake rate constant, k_1 to the elimination (depuration) rate constant, k_2. That is,

$$BCFK = \frac{k_1}{k_2}. \quad \text{(Eq. 1).}$$

This kinetic definition of the BCFK is valid regardless of whether a steady-state concentration of a chemical is achieved during uptake as long as first-order kinetics can be assumed for uptake and elimination processes. Both the uptake rate, k_1, and elimination rate, k_2, of the chemical can be estimated by measuring concentrations of the chemical in water and fish tissues at several points in time.

If elimination follows first order kinetics, the decline in log concentration of the chemical in tissues over time is a straight line. The slope of this line is the elimination rate constant, k_2. Thus, if measurements of chemical concentration in tissues are made at only two time points, the estimate can be obtained using the simple algebraic relationship

$$k_2 = \frac{(\ln C_m - \ln C_t)}{t_e} \quad \text{(Eq. 2).}$$

where C_m is the concentration in fish tissue at the end of the uptake period and C_t is the concentration in the fish tissue after an elimination period of length t_e. Alternatively, k_2 can be estimated as the

slope calculated by linear regression if measurements are made at more than two time points.

Finally, the uptake rate is calculated as

$$k_1 = \frac{k_2 C_m}{C_w(1 - e^{-k_2 t})} \quad \text{(Eq. 3).}$$

where t is the length of the uptake (exposure) period and C_w is the concentration of D5 in the water during the exposure period.

The half-life for clearance in tissue during the depuration phase was determined by the following equation:

$$t_{1/2} = (1/k_2)\ln 2$$

It is assumed that the two chamber, two-parameter model applies. This means that the time to reach 90% steady-state in the uptake phase is about equal to the time it takes to reach 90% of depuration in the depuration phase. Therefore, k_2, the depuration rate constant, was used in the following equation to determine the time to reach 90% steady-state for uptake:

$$0.9 = 1 - e^{-k_2 t}$$

or

$$t_{90} = 2.3/k_2$$

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Lumbriculus variegatus* culture records.
5. Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations.
6. Biological observations.
7. Test conditions (light intensity, photoperiod, etc.).

8. Water chemistry results (e.g., hardness and alkalinity).
9. Statistical calculations, if applicable.
10. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.

14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

Wildlife International, Ltd.

REFERENCES

1 **Phipps, G.L., G.T. Ankley, D.A. Benoit, and V.R. Mattson.** 1993. Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants. Environ. Toxicol. Chem. 12:269-279.

2 **U.S. Environmental Protection Agency.** 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.). EPA 600/R-99/064

3 **ASTM Standard E 1706-00.** 2000. *Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates.* American Society for Testing and Materials.

4 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater.*

5 **European Chemicals Bureau.** 2003. *Technical Guidance Document on Risk Assessment, Part II.* Institute for Health and Consumer Protection.

6 **Federal Environment Agency.** 2001. *Validation of the Preliminary EU- Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances.* Institute of Hydrobiology Dresden University of Technology.

7 **Varaprath, S., C.L. Frye and J. Hamelink.** 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263 – 1265.

8 **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Organization for Economic Cooperation and Development. Adopted 13 April 2004.

9 **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.

10 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1730: *Fish BCF.*

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: D5: A Bioaccumulation Test with *Lumbriculus variegates* Using Spiked Sediment

PROTOCOL NO.: 583/011007/LUM-BIO/SUB583 **AMENDMENT NO.:** 1

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 583A-110

EFFECTIVE DATE: April 13, 2007

AMENDMENT: Page 3:

ADD: Proposed Dates: Experimental Start Date: April 16, 2007
Experimental Termination Date: May 28, 2007

REASON: This information was not available at the time the protocol was signed by the Study Director.

AMENDMENT: Page 3:

CHANGE: Test Concentrations: SC

TO: Test Concentrations: Negative control

REASON: Typographical error. This test will have a negative control, not a solvent control.

AMENDMENT: Preparation of Test Concentrations, Page 6:

Sediment will be prepared in 1 kg batches instead of 1.5 kg batches.

REASON: Smaller batches will be prepared for ease of handling and to reduce the amount of sediment left over after preparation of test chambers.

Wildlife International, Ltd.

AMENDMENT: Test Organism, Page 7:

CHANGE: Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test.

TO: Prior to the test, the organisms will be held in a container with ground up paper towels and overlying water from the same source and at approximately the same temperature as will be used in the test.

REASON: Ground up paper towels have been found to be an easier substrate to [illegible] culturing oligochaetes.

STUDY DIRECTOR — DATE 4/25/07

LABORATORY MANAGEMENT — DATE 4/25/07

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: D5: A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 583/011007/LUM-BIO/SUB583 **AMENDMENT NO.:** 2

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic)

PROJECT NO.: 583A-110

EFFECTIVE DATE: April 12, 2007

AMENDMENT: Data Analysis, Page 12:

CHANGE: The kinetic bioconcentration factor (BCFK).

TO: The kinetic bioaccumulation factor (BAFK).

REASON: While they are essentially the same thing, the kinetic bioaccumulation factor is the more appropriate term to describe the endpoint for a bioaccumulation study.

AMENDMENT: Data Analysis, Page 12:

ADD: A bioaccumulation factor (BAF) will be calculated using the following formula; (Day 28 tissue concentration) / (mean sediment concentration).

REASON: The sponsor requested the additional calculation to determine the bioaccumulation factor.

AMENDMENT: Sampling for Analytical Measurements, Page 10:

ADD: Approximately 1g of oligochaete tissue will be collected from each treatment group and control at the end of the test and used for lipid analysis.

REASON: To add the additional measurement of lipids in the oligochaete tissue.

Wildlife International, Ltd. Project Number 583A-110
Page 2 of 2

AMENDMENT: Sampling for Analytical Measurements, Page 10:

CHANGE: The overlying water will be removed from the test chamber.

TO: A layer of overlying water will be maintained over the sediment during sampling to prevent loss of material due to volatility.

REASON: The procedure will be changed to reduce the potential loss of material due to volatility, and will be based on the methodology developed by the sponsor.

STUDY DIRECTOR — DATE 12/12/07

LABORATORY MANAGEMENT — DATE 12/12/07

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: D5: A Bioaccumulation Test with Lumbriculus variegates Using Spiked Sediment

PROTOCOL NO.: 583/011007/LUM-BIO/SUB583 **DEVIATION NO.:** 1

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 583A-110

DATE OF DEVIATION: April 14, 2007

DEVIATION: The percent organic carbon of the sediment was 3%, not 5.0 ± 1.0% as indicated in the protocol.

REASON: The sediment was prepared using the recipe stated in the protocol, which typically results in a percent organic carbon value of 5.0 ± 1.0% This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DATE OF DEVIATION: May 14, 2007

DEVIATION: Hardness, Alkalinity, Conductivity and Ammonia were measured at the beginning and end of the Uptake phase and at the end of the depuration phase, instead of just at the beginning and end of the test.

REASON: Biologist oversight. The extra water quality measurements had no adverse impact upon the results or interpretation of the study.

Wildlife International, Ltd. Project Number 583A-110

DATE OF DEVIATION: May 29, 2007

DEVIATION: Sampling during the depuration phase occurred on Day 15 instead of Day 14.

REASON: The change was made to accommodate analytical schedules over a holiday. The change had no adverse impact upon the results or interpretation of the study.

STUDY DIRECTOR — DATE 12/12/07

LABORATORY MANAGEMENT — DATE 12/12/07

Appendix 2

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000[2]

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used.

[2] The amount of peat stated above is a theoretical value, based on 10% of the target final weight of the recipe. The peat was added to individual batches, based on the same percentage, and all ingredients were mixed on a rotary mixer.

Appendix 3

Analyses of Pesticides, Organics and Metals in
Wildlife International, Ltd. Formulated Sediment

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.85	Heptachlor	< 0.85
Alpha BHC	< 1.0	Heptachlor Epoxide	< 0.85
Alpha Chlordane	< 0.85	Kepone	< 7.1
Beta BHC	< 2.0	Malathion	< 68
Bolstar	< 68	Merphos	< 68
Chlordane	< 17	Methoxychlor	< 8.5
Coumaphos	< 68	Methyl Parathion	< 68
Delta BHC	< 0.85	Mevinphos	< 68
Demeton-O	< 68	Mirex	< 1.7
Demeton-S	< 68	Naled	< 68
Diazinon	< 68	o,p-DDD	< 1.7
Dichlorvos	< 68	o,p-DDE	< 1.7
Dieldrin	< 1.7	o,p-DDT	< 1.7
Disulfoton	< 77	p,p-DDD	< 1.7
Dursban (Chlorpyrifos)	< 68	p,p-DDE	< 1.7
Endosulfan I	< 0.85	p,p-DDT	< 1.7
Endosulfan II	< 1.7	PCB-1016	< 17
Endosulfan Sulfate	< 1.7	PCB-1221	< 43
Endrin	< 1.7	PCB-1232	< 17
Endrin Aldehyde	< 1.7	PCB-1242	< 23
Endrin Ketone	< 1.7	PCB-1248	< 17
EPN	< 68	PCB-1254	< 17
Ethion	< 68	PCB-1260	< 17
Ethoprop	< 68	Phorate	< 68
Ethyl Parathion	< 68	Ronnel	< 68
Famphur	< 68	Stirophos	< 68
Fensulfothion	< 68	Telodrin	< 1.2
Fenthion	< 68	Tokuthion	< 68
Gamma BHC – Lindane	< 0.85	Toxaphene	< 34
Gamma Chlordane	< 3.1	Trichloronate	< 68
Guthion (Azinphos-methyl)	< 68	Trithion	< 68
HCB	< 0.85		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 3 (Continued)

Analyses of Pesticides, Organics and Metals in
Wildlife International, Ltd. Formulated Sediment

Metals			
Component	Measured Concentration (mg/kg)	Component	Measured Concentration (mg/kg)
Aluminum	9200	Magnesium	2710
Antimony	< 2.00	Manganese	4.05
Arsenic	< 2.00	Mercury	< 0.0968
Barium	61.0	Nickel	2.81
Beryllium	< 0.500	Nitrate by IC (solid)	< 1.5
Bromide by IC (solid)	< 5.1	Nitrite by IC (solid)	< 1.0
Cadmium	< 0.500	Potassium	89.7
Calcium	5000	Selenium	< 2.00
Chloride by IC (solid)	20.1	Silver	< 0.500
Chromium	8.25	Sodium	< 100
Cobalt	< 0.500	Sulfate by IC (solid)	91.9
Copper	1.33	Thallium	< 2.00
Fluoride by IC (solid)	4.3	Vanadium	4.97
Iron	422	Zinc	3.96
Lead	11.7		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 4

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

	Mean	Range
Specific Conductance (µmhos/cm)	296 (N = 4)	290 - 305
Hardness (mg/L as $CaCO_3$)	136 (N = 4)	132-140
Alkalinity (mg/L as $CaCO_3$)	180 (N = 4)	178-180
pH	8.1 (N = 4)	8.1-8.1

Appendix 5

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.020	Heptachlor	< 0.010
Alpha BHC	< 0.010	Heptachlor Epoxide	< 0.024
Alpha Chlordane	< 0.010	Kepone	< 0.20
Beta BHC	< 0.024	Malathion	< 3.0
Bolstar	< 2.0	Merphos	< 6.0
Chlordane	< 0.50	Methoxychlor	< 0.10
Coumaphos	< 3.0	Methyl Parathion	< 2.0
Delta BHC	< 0.024	Mevinphos	< 4.0
Demeton-O	< 2.0	Mirex	< 0.11
Demeton-S	< 3.0	Naled	< 3.0
Diazinon	< 4.0	o,p-DDD	< 0.020
Dichlorvos	< 3.0	o,p-DDE	< 0.020
Dieldrin	< 0.020	o,p-DDT	< 0.020
Disulfoton	< 2.0	p,p-DDD	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDE	< 0.020
Endosulfan I	< 0.010	p,p-DDT	< 0.020
Endosulfan II	< 0.020	PCB-1016	< 0.50
Endosulfan Sulfate	< 0.040	PCB-1221	< 0.50
Endrin	< 0.020	PCB-1232	< 0.50
Endrin Aldehyde	< 0.10	PCB-1242	< 0.50
Endrin Ketone	< 0.040	PCB-1248	< 0.50
EPN	< 4.0	PCB-1254	< 0.50
Ethion	< 2.0	PCB-1260	< 0.50
Ethoprop	< 3.0	Phorate	< 2.0
Ethyl Parathion	< 2.0	Ronnel	< 2.0
Famphur	< 3.0	Stirophos	< 2.0
Fensulfothion	< 15	Telodrin	< 0.010
Fenthion	< 2.0	Tokuthion	< 2.0
Gamma BHC – Lindane	< 0.010	Toxaphene	< 1.0
Gamma Chlordane	< 0.010	Trichloronate	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Trithion	< 2.0
HCB	< 0.10		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 5

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	12.8
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	7.55
Calcium	34.9	Selenium	< 0.0200
Chloride	4.8	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.0
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	1.2	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 6

The Analysis of D5 in Sediment and Tissue

Appendix 6.1

Analytical Method Flowchart for the Analysis of D5 in Sediment

FLOWCHART FOR THE ANALYSIS OF D5 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample the sediment from the aquaria using a ladle through the overlying water. Stir the sediment-pore water to homogenize. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.

↓

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

↓

Add 10.0 mL HPLC-grade bottled water and 5 µL of internal standard to each sample and QC.

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately five minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50.0 mL volumetric flask containing 2.5 g of sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Transfer 1.00 mL of extract into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit for analysis.

Appendix 6.2

Analytical Method Flowchart for the Analysis of D5 in Tissue

FLOWCHART FOR THE ANALYSIS OF D5 IN TISSUE

Rinse all glassware with tetrahydrofuran (THF).

↓

Prepare samples by weighing 0.100 g of tissue into graduated centrifuge tubes. Fortify quality Control samples with appropriate stock solution. Fortify all samples and QC samples with internal standard (250 µg a.i./L at instrument), with the exception of the 10.0 mg/Kg QC sample, which was fortified at 1000 µg a.i./L. Homogenize tissues with a spatula.

↓

Add 975 µL THF to each sample and matrix blank. Add 875 µL THF to the low QC. Add 800 µL to high QC. Vortex for approximately 30 seconds. Sonicate for 15 seconds and vortex again briefly to wash the sides of the centrifuge tubes.

↓

Transfer an aliquot to a microcentrifuge tube and ultra-centrifuge each sample for approximately five minutes at 14,000 rpm.

↓

Dilute QC sample in THF as per sample preparation sheet.

↓

Transfer an aliquot of each extract to a 500 µL insert in an autosampler vial. Submit samples for analysis.

Appendix 6.3

Analytical Method Flowchart for the Analysis of Lipids in Tissue

METHOD OUTLINE FOR THE ANALYSIS OF LIPIDS IN TISSUES

Remove vials to be analyzed from freezer. Allow samples to thaw.

↓

To each sample, add 10 mL of HPLC-grade bottled water and homogenize for approximately one minute using a hand-held tissue homogenizer. Rinse the homogenizer with the appropriate solvent(s) in between samples.

↓

Transfer each homogenate to a 250-mL separatory funnel that contains 25 mL of chloroform and 50 mL of methanol.

↓

Rinse each vial with an additional 10 mL of HPLC-grade bottled water and pour rinse into respective separatory funnel.

↓

Shake each separatory funnel for approximately one minute with venting.

↓

Add 50 mL of chloroform followed by 50 mL of saturated sodium chloride to each separatory funnel.

↓

Briefly swirl each separatory funnel with venting.

↓

Allow the phases to separate. For each sample, drain the chloroform layer through a powder funnel packed with glass wool and anhydrous sodium sulfate into a 250-mL roundbottom flask.

↓

Add an additional 50-mL aliquot of chloroform to each separatory funnel and repeat the extraction and draining procedures.

↓

Rotary evaporate the extracts in a waterbath maintained at approximately 40°C to near dryness.

↓

Transfer each extract to a pre-weighed, labeled scintillation vial.

↓

Rinse each 250-mL roundbottom flask with a small volume of chloroform and transfer rinse to respective scintillation vial.

↓

Evaporate the remaining solvent in each vial under a gentle stream of nitrogen or clean dry air.

↓

Reweigh each vial and record weight.

Appendix 6.4

Typical Gas Chromatograph (GC) Operational Parameters for Sediment/Tissue Analysis

INSTRUMENT:	Agilent Model 5890 Gas Chromatograph (GC)	
DETECTOR:	Hewlett-Packard Flame Ionized Detector (FID)	
ANALYTICAL COLUMN:	J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness)	
INJECTOR TEMPERATURE:	280°C	
OVEN:	Initial temperature:	50°C
	Initial hold time:	1.00 minute
	Ramp:	8.00°C/minute
	Final temperature:	150°C
	Final hold time:	0.00 minute
DETECTOR TEMPERATURE:	310°C	
INJECTION VOLUME:	1.0 µL	
CARRIER GAS:	Helium	
HEAD PRESSURE:	~12 psi	
APPROXIMATE D5 PEAK RETENTION TIME:	11.9 minutes	

Appendix 6.5

Typical Gas Chromatograph with Mass Selective Detection (GC/MS) Operational Parameters for Tissue Analysis

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)	
DETECTOR:	Agilent Model 5973 Mass Selective Detector operated in SIM mode	
ANALYTICAL COLUMN:	ZB-5 HT Column (0.25 mm x 30 m ID, 1.0 µm)	
INJECTOR TEMPERATURE:	280°C	
OVEN:	Initial temperature:	50°C
	Initial hold time:	1.00 minute
	Ramp:	8.00°C/minute
	Final temperature:	150°C
	Final hold time:	0.00 minute
	Ramp:	40.00°C/minute
	Final temperature:	220°C
	Final hold time:	0.00 minute
DETECTOR TEMPERATURE:	300°C	
INJECTION VOLUME:	1.0 µL	
CARRIER GAS:	Helium	
HEAD PRESSURE:	~12 psi	
IONS MONITORED:	D5: 355.00; Internal standard: 281.00	
APPROXIMATE PEAK RETENTION TIMES:	D5: 7.4 minutes; Internal standard: 8.0 minutes	

Appendix 6.6

Analytical Stocks and Standards Preparation

For sediment, a stock solution of D5 was prepared by weighing 0.1000 g on an analytical balance. The test substance was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% (DMF) : 20% toluene. This primary stock solution (1.00 mg/mL) was used to prepare the calibration standards in ethyl acetate. Analytical standards were not corrected for the active ingredient of the test article (99.19%), therefore, sample results were presented as mg/Kg. 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)
1.00	10.0	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D5 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% (DMF) : 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% (DMF) : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to all sediment samples and calibration standards (5 µL and 50 µL, respectively).

For tissue, a stock solution of D5 was prepared by weighing 0.1000 g on an analytical balance. The test substance was transferred to a 100-mL Class A volumetric flask and brought to volume using THF. This primary stock solution (1.00 mg/mL) was serially diluted in THF to produce 0.100, 0.0100 and 0.00100 mg/mL stock solutions. The 0.0100 and 0.00100 mg/mL stock solutions were used to fortify the matrix fortifications. For GC/MS analysis, calibration standards were prepared in THF using the 0.0100 mg/mL stock solution. For GC/FID analysis, calibration standards were prepared in THF using the 1.00 mg/mL stock solution. The following shows the dilution scheme for the sets of calibration standards:

Appendix 6.6 (Continued)

Analytical Stocks and Standards Preparation

Stock Concentration (mg/mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg/L)
0.0100	50.0	10.0	0.0500
0.0100	150	10.0	0.150
0.0100	250	10.0	0.250
0.0100	350	10.0	0.350
0.0100	500	10.0	0.500
1.00	40.0	10.0	4.00
1.00	100	10.0	10.0
1.00	200	10.0	20.0
1.00	300	10.0	30.0
1.00	400	10.0	40.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D5 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using THF. This primary stock solution (1.00 mg a.i./mL) was serially diluted in THF to prepare a 0.100 and 0.0100 mg a.i./mL stock solution. The 0.0100 mg a.i./mL stock solution was added to all tissue samples and calibration standards analyzed by GC/MS (25 µL and 250 µL, respectively). For tissue samples analyzed by GC/FID, there was no internal standard added to samples.

Appendix 6.7

Example Calculations for a Representative Sample

The analytical result and percent recovery for sediment sample number 583A-110-S6, with a nominal concentration of 1000 mg a.i./Kg, were calculated using the following equations:

$$\text{Concentration of D5 in sample (mg/Kg)} = \frac{\text{peak area - (y-intercept)}}{\text{slope}} \times \text{dilution factor}$$

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

Peak Area = 4463.29590
Y-Intercept = 25.14844
Slope = 108.81
Dilution Factor = 8.38

$$\text{Concentration of D5 in sample (mg/Kg)} = \frac{4463.29590 - 25.14844}{108.81} \times 8.38$$

Concentration of D5 in sample (mg/Kg) = 342

$$\text{Percent of nominal concentration} = \frac{342 \text{ mg/Kg}}{1000 \text{ mg a.i./Kg}} \times 100$$

Percent of nominal concentration = 34.2%

Appendix 6.8

Quality Control Samples of D5 in Sediment

Sample Number (583A-110-)	Sample Type	Concentration of D5		Percent Recovery
		Fortified[1] (mg/Kg)	Measured[2] (mg/Kg)	
SMAS-1	Matrix Fortification	100	91.0	91.0
SMAS-2	Matrix Fortification	500	383	76.7
SMAS-3	Matrix Fortification	100	100	100
SMAS-4	Matrix Fortification	500	423	84.5
SMAS-5	Matrix Fortification	100	95.0	95.0
SMAS-6	Matrix Fortification	500	437	87.5
			Mean =	89.1
			Standard Deviation =	8.18
			CV =	9.18%

1 Matrix fortification samples were prepared from 5 mL of sediment, which was equivalent to 5 grams of sediment.

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

Appendix 6.9

Quality Control Samples of D5 in Tissue Samples

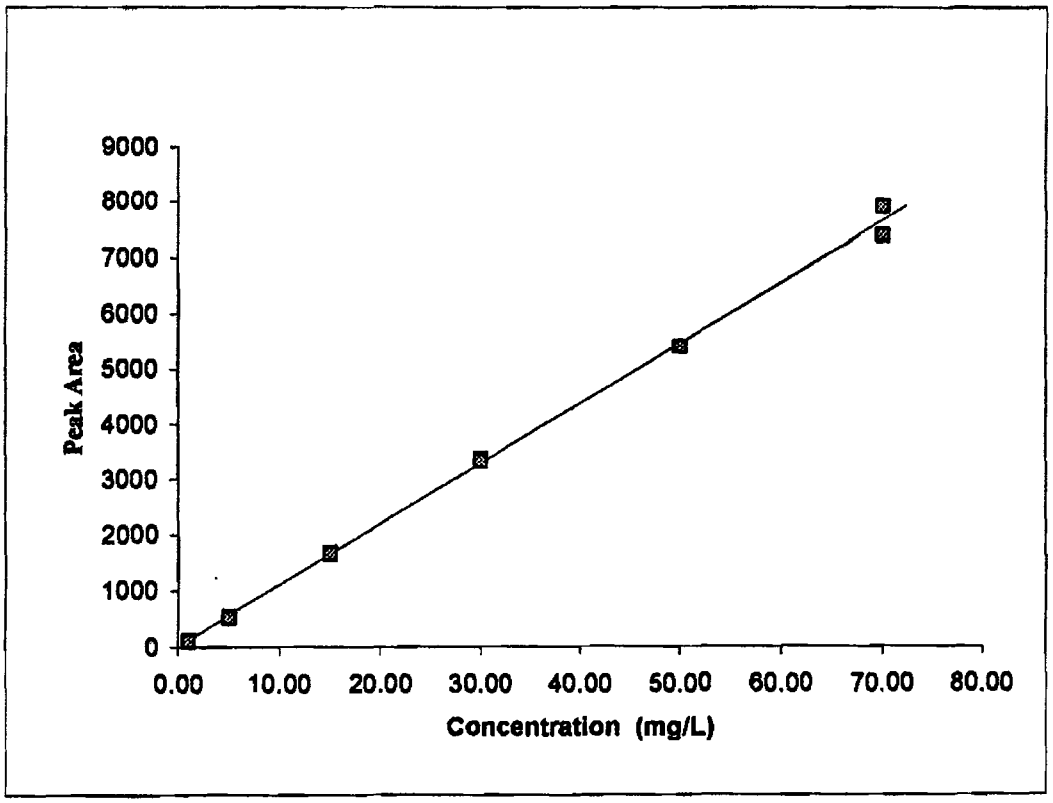
Sample Number (583A-110-)	Sample Type	Concentration of D5		Percent Recovery
		Fortified (mg/Kg)	Measured[1] (mg/Kg)	
TMAB-1	Matrix Blank	< LOQ	--	--
TMAB-2	Matrix Blank	< LOQ	--	--
TMAB-3	Matrix Blank	< LOQ	--	--
TMAB-4	Matrix Blank	< LOQ	--	--
TMAS-1	Matrix Fortification	1.00	0.974	97.4
TMAS-2	Matrix Fortification	10.0	9.83	98.3
TMAS-3	Matrix Fortification	1.00	1.33	133
TMAS-4	Matrix Fortification	10.0	10.3	103
TMAS-5	Matrix Fortification	1.00	1.22	122
TMAS-6	Matrix Fortification	10.0	9.86	98.6
TMAS-7	Matrix Fortification	1.00	1.14	114
TMAS-8	Matrix Fortification	10.0	11.0	110
			Mean =	110
			Standard Deviation =	12.9
			CV =	11.7%

[1] The limit of quantitation (LOQ) was 0.500 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.0500 mg/Kg) and the dilution factor of the matrix blank samples (10.0).

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

Appendix 6.10

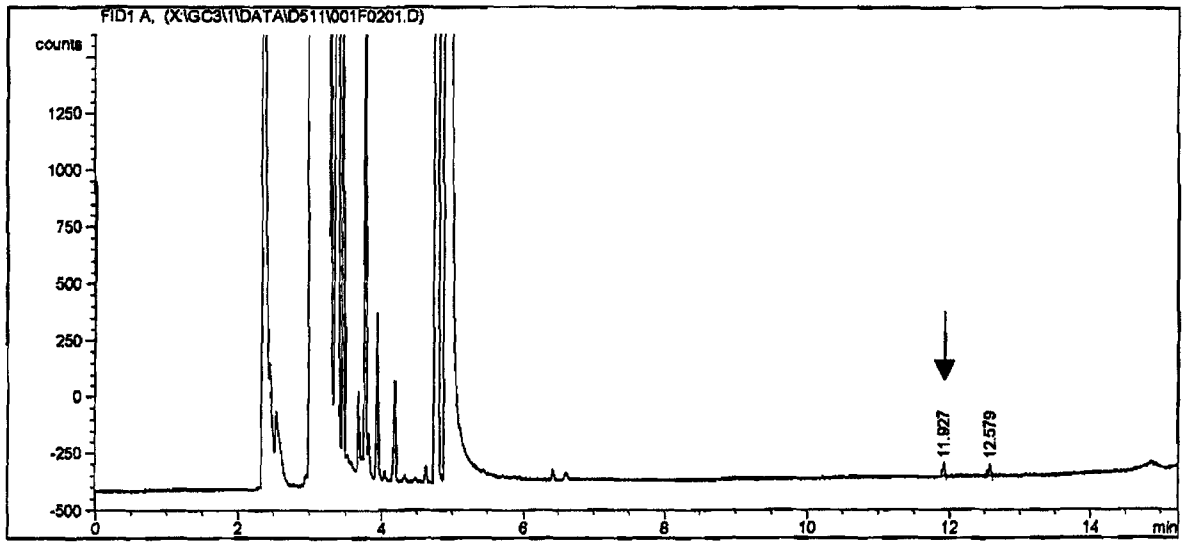
Representative Calibration Curve for D5 in Sediment Samples



Slope = 108.81; Y-Intercept = 25.14844; R^2 = 0.9984

Appendix 6.11

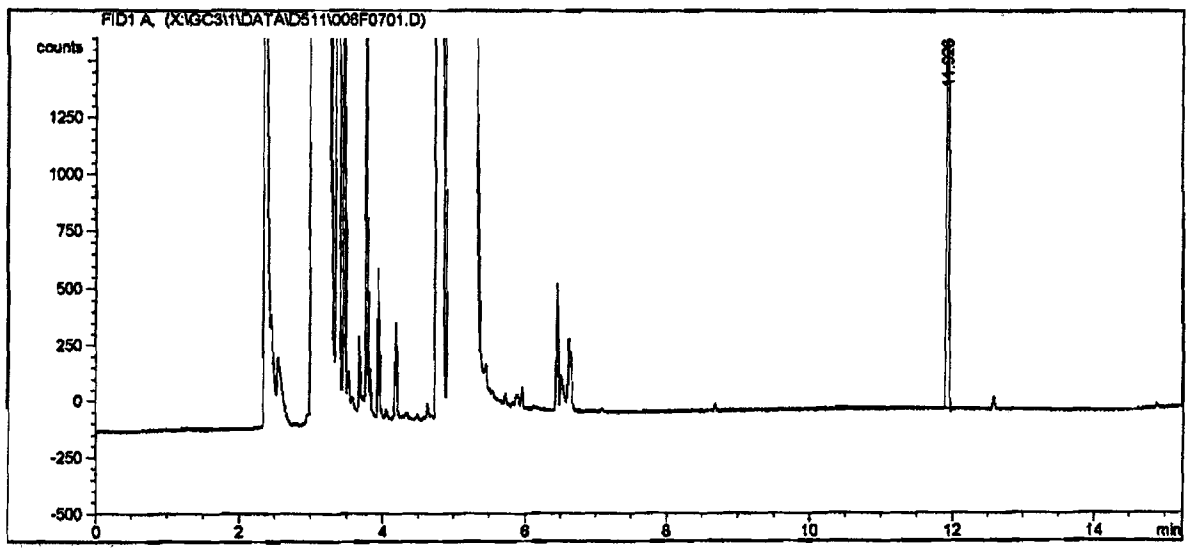
Representative Chromatogram of a Low-level D5 Sediment Calibration Standard



Nominal concentration: 1.00 mg/L. The arrow indicates the approximate retention time of D5.

Appendix 6.12

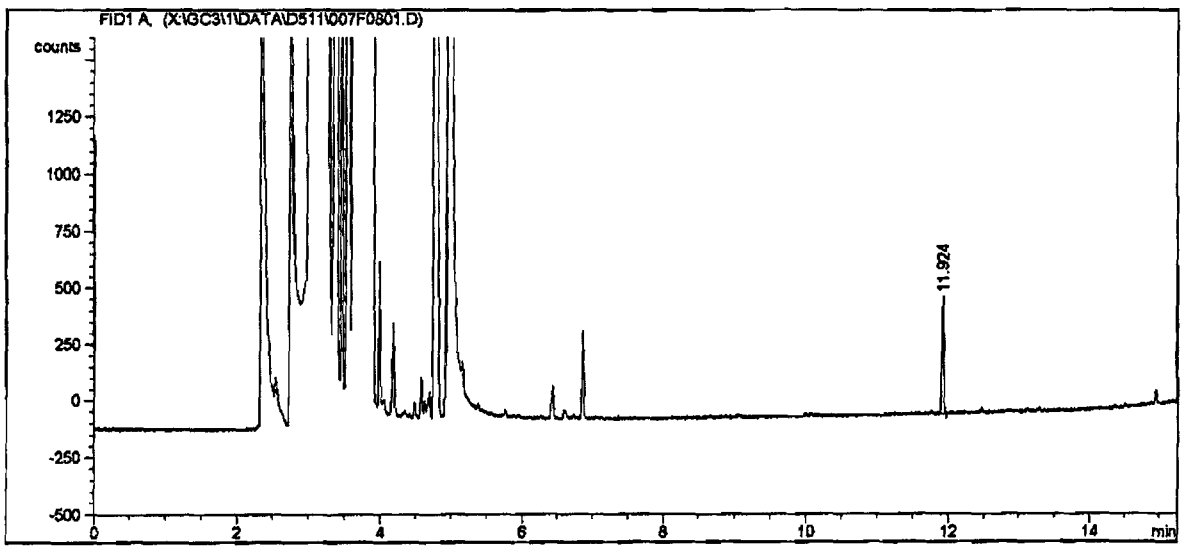
Representative Chromatogram of a High-level D5 Sediment Calibration Standard



Nominal concentration: 70.0 mg/L

Appendix 6.13

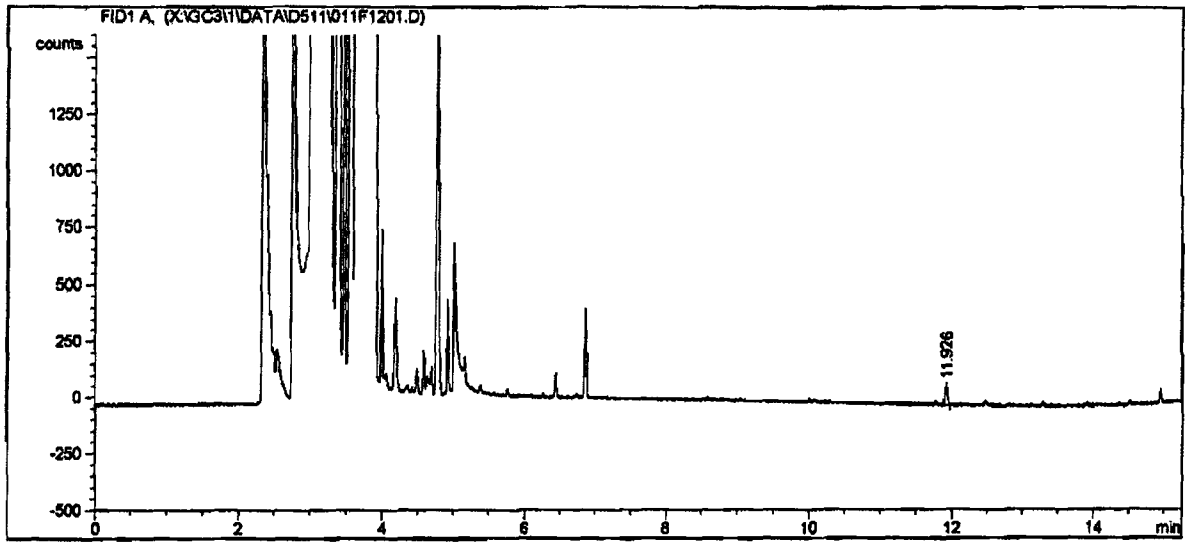
Representative Chromatogram of a Sediment Matrix Fortification Sample



Sample number 583A-110-SMAS-1; nominal concentration 100 mg/Kg.

Appendix 6.14

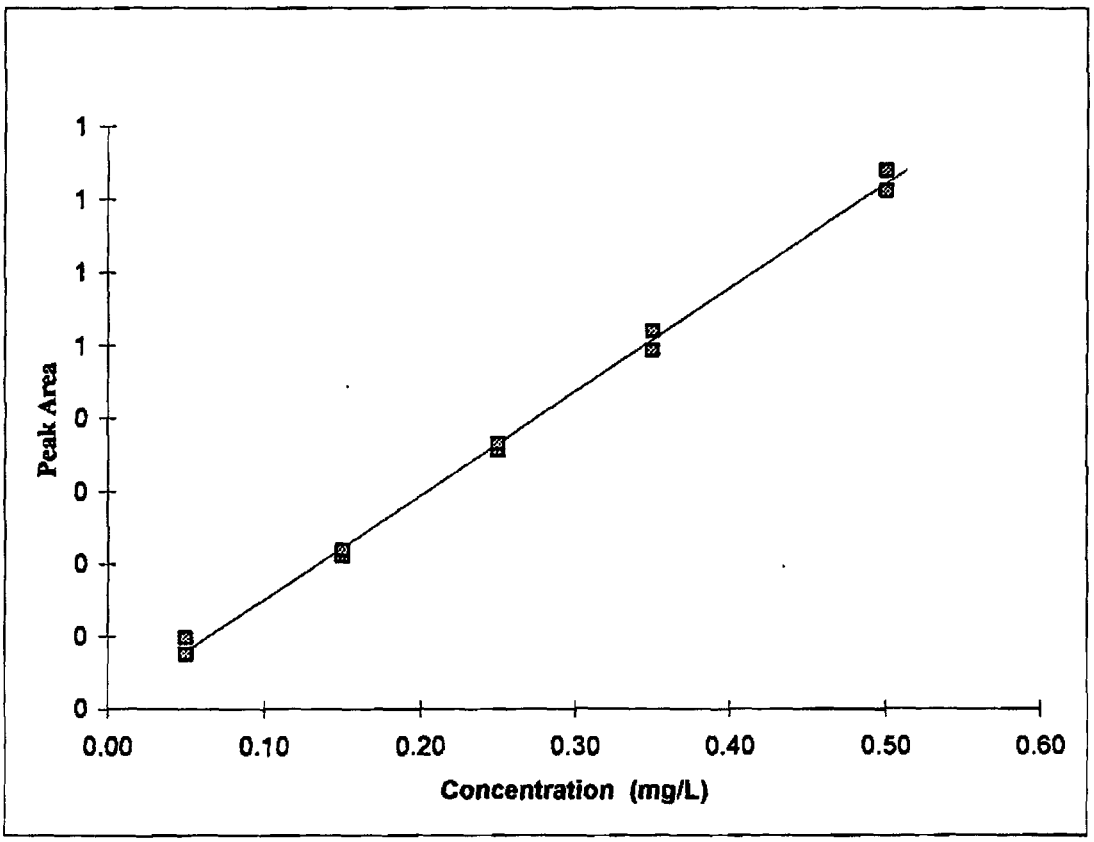
Representative Chromatogram of a Sediment Test Sample



Sample number 583A-110-S3; Day 0; 100 mg a.i./Kg nominal concentration

Appendix 6.15

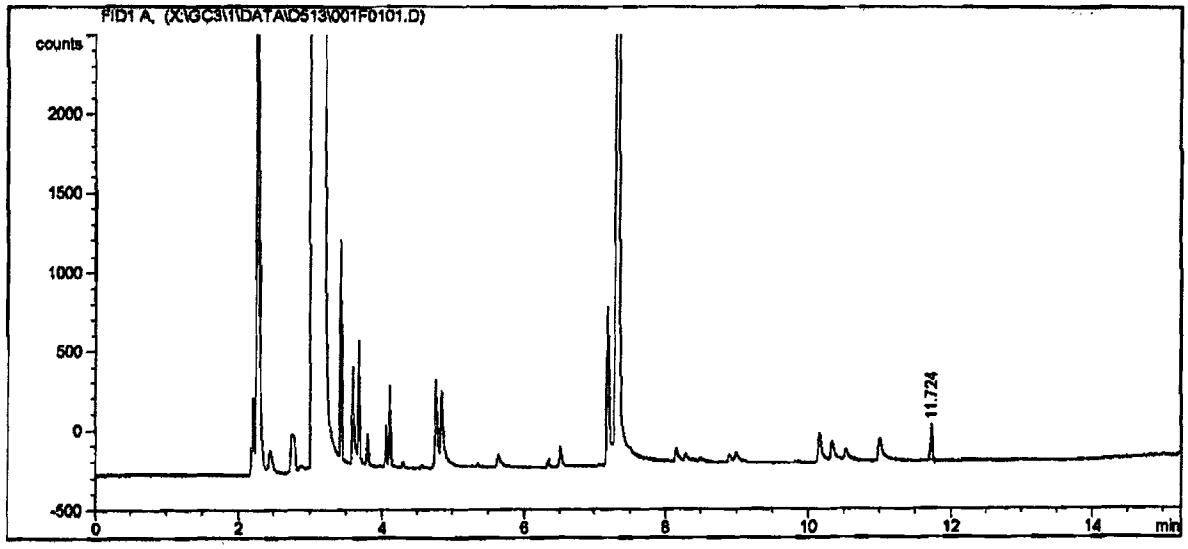
Representative Calibration Curve for D5 in Tissue Samples



Slope = 1.43; Y-Intercept = 0.01; R^2 = 0.9973

Appendix 6.16

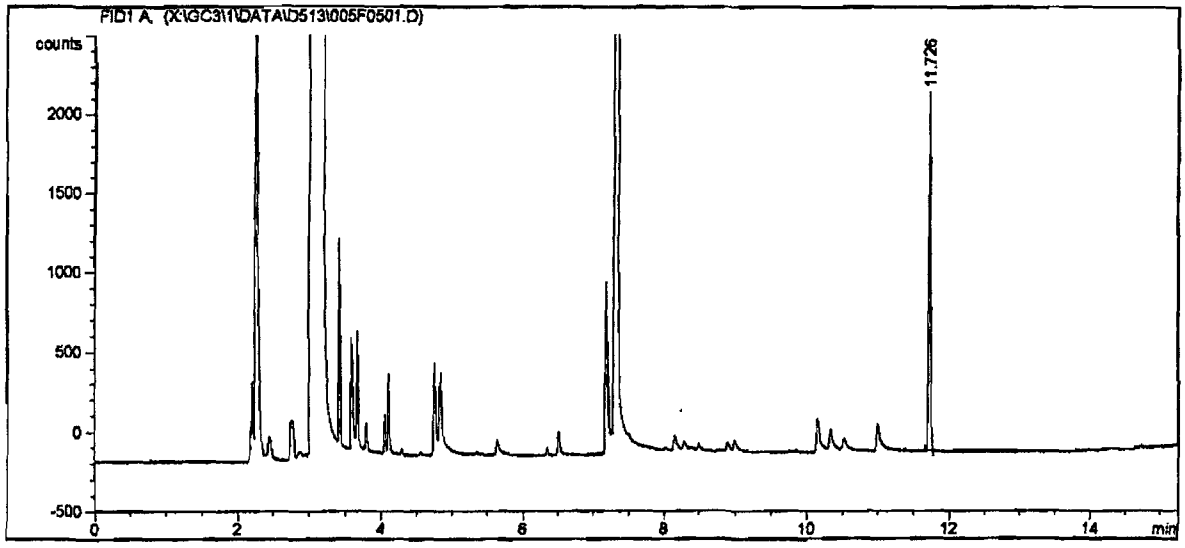
Representative Chromatogram of a Low-level D5 Calibration Standard for Tissue Analysis



Nominal concentration: 4.00 mg/L

Appendix 6.17

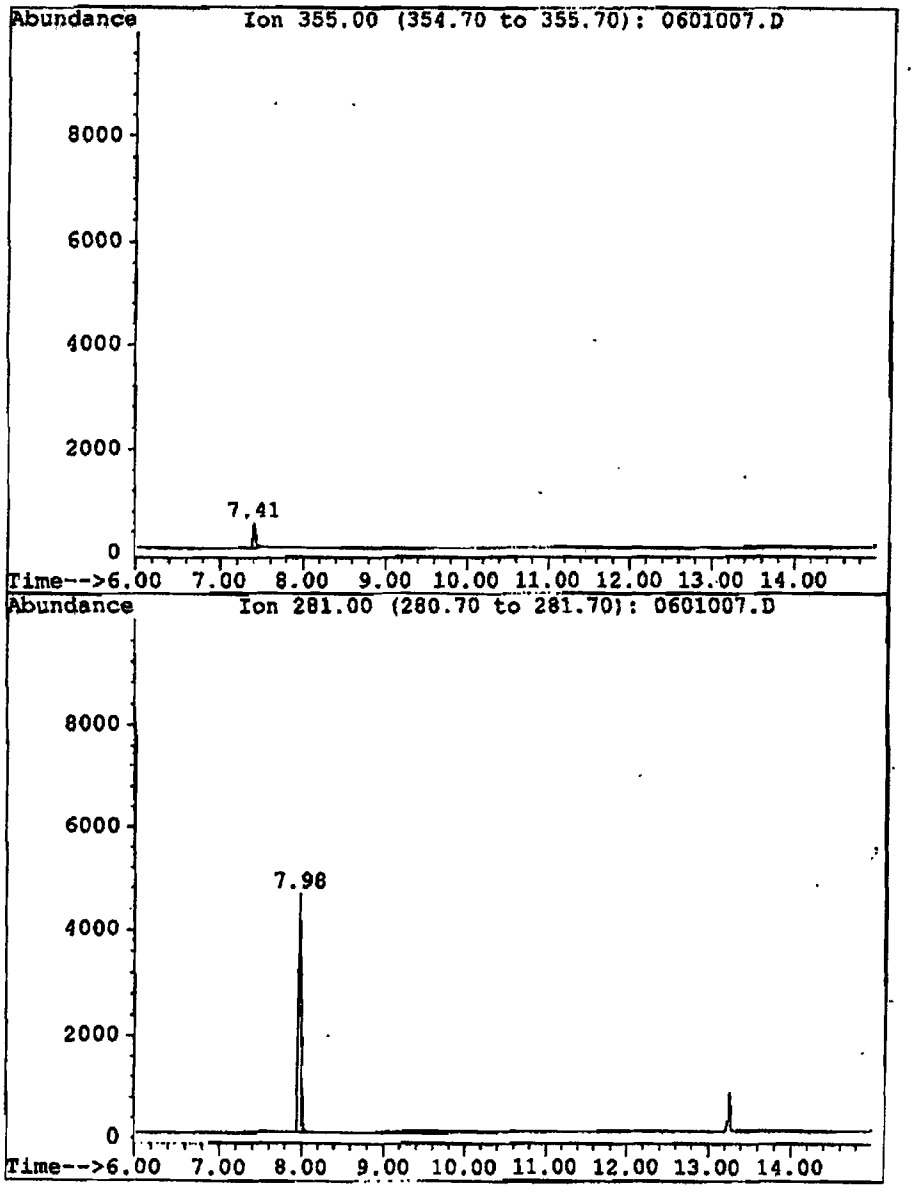
Representative Chromatogram of a High-level D5 Calibration Standard for Tissue Analysis



Nominal concentration: 40.0 mg/L

Appendix 6.18

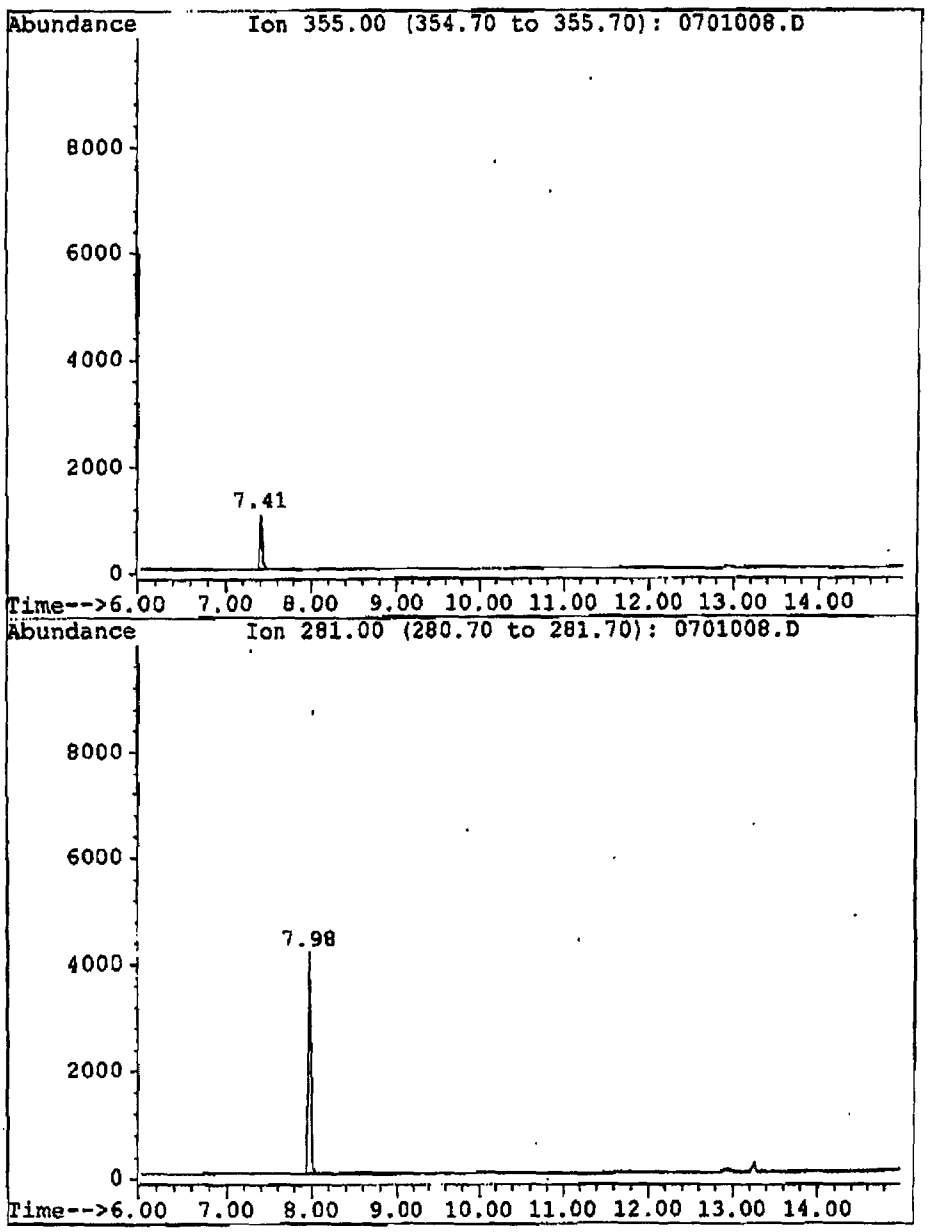
Representative Chromatogram of a Matrix Blank Sample for Tissue Analysis



Sample number 583A-110-TMAB-1; nominal concentration 1.00 mg/Kg.

Appendix 6.19

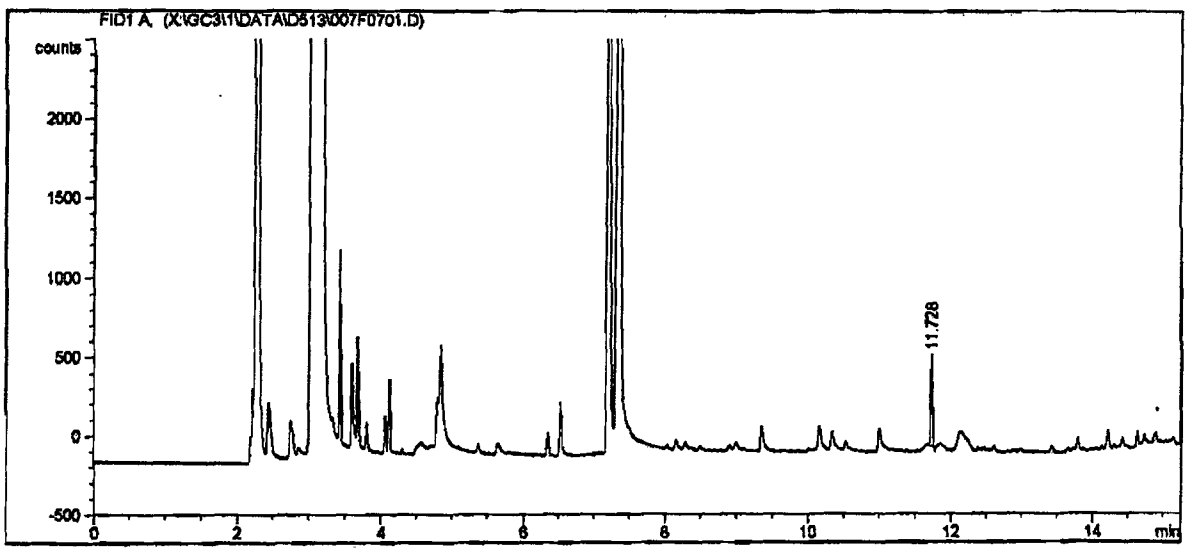
Representative Chromatogram of a Matrix Fortification Sample for Tissue Analysis



Sample number 583A-110-MAS-1; nominal concentration 10.0 mg/Kg.

Appendix 6.20

Representative Chromatogram of a Tissue Test Sample



Sample number 583A-110-T3B; Day 14; 100 mg a.i./Kg nominal concentration

Appendix 7

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	1G; rest AN	0	0	1G; rest AN	0	0
4	1G; rest AN	0	0	1G; rest AN	0	0
5	AN	0	0	1G; rest AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	AN	0	0	AN	0	0
8	1G; rest AN	0	0	1G; rest AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	2G; rest AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	AN	0	0	1G; rest AN	0	0
14	AN	0	0	1G; rest AN	0	0
15	AN	0	0	1G; rest AN	0	0
16	AN	0	0	1G; rest AN	0	0
17	AN	0	0	1G; rest AN	0	0
18	1G; rest AN	0	0	1G; rest AN	0	0
19	AN	0	0	2G; rest AN	0	0
20	AN	0	0	3G; rest AN	0	0
21	AN	0	0	1G; rest AN	0	0
22	AN	0	0	1G; rest AN	0	0
23	AN	0	0	1G; rest AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	1G; rest AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 241 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 259 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	2G; rest AN	0	0	1G; rest AN	0	0
3	2G; rest AN	0	0	2G; rest AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	1G; rest AN	0	0	AN	0	0
8	1G; rest AN	0	0	1G; rest AN	0	0
9	1G; rest AN	0	0	2G; rest AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	1G; rest AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	AN	0	0	1G; rest AN	0	0
14	AN	0	0	1G; rest AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	1G; rest AN	0	0	AN	0	0
21	1G; rest AN	0	0	AN	0	0
22	1G; rest AN	0	0	AN	0	0
23	1G; rest AN	0	0	AN	0	0
24	1G; rest AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	1G; rest AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	0[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 260 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 0.9612g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	1G; rest AN	0	0
5	AN	0	0	1G; rest AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	AN	0	0	1G; rest AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	[2]	AN	0	0
15	--	--	--	AN	0	0
16	--	--	--	AN	0	0
17	--	--	--	AN	0	0
18	--	--	--	AN	0	0
19	--	--	--	AN	0	0
20	--	--	--	AN	0	0
21	--	--	--	AN	0	0
22	--	--	--	AN	0	0
23	--	--	--	AN	0	0
24	--	--	--	AN	0	0
25	--	--	--	AN	0	0
26	--	--	--	AN	0	0
27	--	--	--	1G; rest AN	0	0
28	--	--	--	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 1.4453g (wet weight of organism tissue) were collected for analytical measurement.
[3] Replicate was used for analytical confirmation in the sediment on Day 28.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	2G; rest AN	0	0
4	2G; rest AN	0	0
5	1G; rest AN	0	0
6	1G; rest AN	0	0
7	1G; rest AN	0	0
8	2G; rest AN	0	0
9	1G; rest AN	0	0
10	AN	0	0
11	1G; rest AN	0	0
12	2G; rest AN	0	0
13	1G; rest AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

100 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	1G; rest AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	1G; rest AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	1G; rest AN	0	0	AN	0	0
7	1G; rest AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0
9	1G; rest AN	0	0	2G; rest AN	0	0
10	1G; rest AN	0	0	2G; rest AN	0	0
11	1G; rest AN	0	0	1G; rest AN	0	0
12	1G; rest AN	0	0	AN	0	0
13	1G; rest AN	0	0	1G; rest AN	0	0
14	1G; rest AN	0	0	AN	0	0
15	1G; rest AN	0	0	AN	0	0
16	1G; rest AN	0	0	AN	0	0
17	1G; rest AN	0	0	AN	0	0
18	1G; rest AN	0	0	AN	0	0
19	1G; rest AN	0	0	AN	0	0
20	2G; rest AN	0	0	AN	0	0
21	1G; rest AN	0	0	AN	0	0
22	1G; rest AN	0	0	AN	0	0
23	1G; rest AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 260 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 205 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

100 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	1G; rest AN	0	0
8	1G; rest AN	0	0	1G; rest AN	0	0
9	AN	0	0	2G; rest AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	1G; rest AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	0[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 258 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 0.9605g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

100 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	1G; rest AN	0	0
4	AN	0	0	1G; rest AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	1G; rest AN	0	0
7	1G; rest AN	0	0	AN	0	0
8	1G; rest AN	0	0	1G; rest AN	0	0
9	1G; rest AN	0	0	1G; rest AN	0	0
10	AN	0	0	1G; rest AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	[2]	1G; rest AN	0	0
15	--	--	--	AN	0	0
16	--	--	--	AN	0	0
17	--	--	--	AN	0	0
18	--	--	--	AN	0	0
19	--	--	--	AN	0	0
20	--	--	--	AN	0	0
21	--	--	--	AN	0	0
22	--	--	--	1G; rest AN	0	0
23	--	--	--	AN	0	0
24	--	--	--	AN	0	0
25	--	--	--	AN	0	0
26	--	--	--	AN	0	0
27	--	--	--	1G; rest AN	0	0
28	--	--	--	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 1.7065g (wet weight of organism tissue) were collected for analytical measurement.
[3] Replicate was used for analytical confirmation in the sediment on Day 28.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

100 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	2G; rest AN	0	0
5	AN	0	0
6	1G; rest AN	0	0
7	1G; rest AN	0	0
8	1G; rest AN	0	0
9	1G; rest AN	0	0
10	1G; rest AN	0	0
11	AN	0	0
12	1G; rest AN	0	0
13	1G; rest AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

1000 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	2G; rest AN	0	0	1G; rest AN	0	0
3	1G; rest AN	0	0	AN	0	0
4	1G; rest AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	2G; rest AN	0	0	AN	0	0
8	AN	0	0	1G; rest AN	0	0
9	2G; rest AN	0	0	2G; rest AN	0	0
10	3G; rest AN	0	0	AN	0	0
11	2G; rest AN	1	0	AN	0	0
12	1G; rest AN	1	0	AN	0	0
13	2G; rest AN	1	0	AN	0	0
14	3G; rest AN	1	0	AN	0	0
15	2G; rest AN	1	0	AN	0	0
16	2G; rest AN	1	0	AN	0	0
17	2G; rest AN	1	0	AN	0	0
18	2G; rest AN	1	0	AN	0	0
19	AN	1	0	AN	0	0
20	AN	1	0	AN	0	0
21	AN	1	0	AN	0	0
22	1G; rest AN	1	0	AN	0	0
23	AN	1	0	AN	0	0
24	AN	1	0	AN	0	0
25	1G; restAN	1	0	AN	0	0
26	AN	1	0	AN	0	0
27	1G; rest AN	1	0	AN	0	0
28	AN	1	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 229 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 248 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

1000 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	1G; rest AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	2G; rest AN	0	0	AN	0	0
10	1G; rest AN	0	0	1G; rest AN	0	0
11	1G; rest AN	0	0	1G; rest AN	0	0
12	1G; rest AN	0	0	1G; rest AN	0	0
13	1G; rest AN	0	0	1G; rest AN	0	0
14	1G; rest AN	0	0	1G; rest AN	0	0
15	1G; rest AN	0	0	1G; rest AN	0	0
16	AN	0	0	AN	0	0
17	1G; rest AN	0	0	AN	0	0
18	1G; rest AN	0	0	AN	0	0
19	1G; rest AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	1G; rest AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	1G; rest AN	0	0	AN	0	0
27	1G; rest AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	0[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 256 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved using a 425 µm sieve. 0.9960g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

1000 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	1G; rest AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	1G; rest AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	1G; rest AN	0	0
10	1G; rest AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	2G; rest AN	0	0
13	AN	0	0	1G; rest AN	0	0
14	AN	0	[2]	AN	0	0
15	--	--	--	AN	0	0
16	--	--	--	AN	0	0
17	--	--	--	AN	0	0
18	--	--	--	AN	0	0
19	--	--	--	AN	0	0
20	--	--	--	AN	0	0
21	--	--	--	AN	0	0
22	--	--	--	AN	0	0
23	--	--	--	AN	0	0
24	--	--	--	AN	0	0
25	--	--	--	AN	0	0
26	--	--	--	AN	0	0
27	--	--	--	AN	0	0
28	--	--	--	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 1.5060g (wet weight of organism tissue) were collected for analytical measurement.
[3] Replicate was used for analytical confirmation in the sediment on Day 28.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

1000 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	2G; 1H; rest AN	0	0
8	AN	0	0
9	AN	0	0
10	AN	0	0
11	AN	0	0
12	AN	0	0
13	AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment; H = organisms observed climbing the walls of the test chamber.

[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment-Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Depuration Phase

	Replicate A			Replicate B			Replicate C		
Day	Observations[1]	Cumulative Number Dead	Number Sampled	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	1G; rest AN	0	0	AN	0	0	AN	0	0
4	1G; rest AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0	1G; rest AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0	AN	0	[3]
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 254 organisms were found and used for lipid analysis on Depuration Day 22 (0.9391g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 1.2654g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

100 mg a.i./Kg – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	1G; rest AN	0	0
6	AN	0	0	AN	0	0	1G; rest AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0	2G; rest AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	1G; rest AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0	AN	0	[3]
16	1G; rest AN	0	0	AN	0	0	–	–	–
17	AN	0	0	AN	0	0	–	–	–
18	AN	0	0	AN	0	0	–	–	–
19	AN	0	0	AN	0	0	–	–	–
20	AN	0	0	AN	0	0	–	–	–
21	AN	0	0	AN	0	0	–	–	–
22	AN	0	[2]	AN	0	0	–	–	–

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 297 organisms were found and used for lipid analysis on Depuration Day 22 (1.1225g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 0.9834 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

1000 mg a.i./Kg – Depuration Phase

	Replicate A			Replicate B			Replicate C		
Day	Observations[1]	Cumulative Number Dead	Number Sampled	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	1G; rest AN	0	0	AN	0	0
5	1G; rest AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0	1G; rest AN	0	0
9	AN	0	0	1G; rest AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	1G; rest AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0	AN	0	[3]
16	AN	0	0	AN	0	0	–	–	–
17	AN	0	0	1G; rest AN	0	0	–	–	–
18	AN	0	0	AN	0	0	–	–	–
19	AN	0	0	AN	0	0	–	–	–
20	AN	0	0	AN	0	0	–	–	–
21	AN	0	0	AN	0	0	–	–	–
22	AN	0	[2]	AN	0	0	–	–	–

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved using a 425 µm sieve. 253 organisms were found and used for lipid analysis on Depuration Day 22 (1.0062g wet weight of organism tissue).
[3] The sediment was sieved using a 425 µm sieve. 1.4737g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 8

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. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

Appendix 9

Report Amendment

1.	Original Report:	Pages 1 – 4 and 7
	Amendment:	The pages were changed to include the amended report date, revised page numbers, and/or new signatures and dates due to the addition of the report amendment as Appendix 9.
	Reason:	To reflect the issuing of an amended report.
2.	Original Report:	Summary, Page 8 and Table 5, Page 27
	Amendment:	The k_1 value for the 336 mg/Kg treatment group was changed to 0.092 and the k_2 value for the 20.8 mg/Kg treatment group was changed to 0.193.
	Reason:	Typographical error. The k_1 value for the 336 mg/Kg treatment group and the k_2 value for the 20.8 mg/Kg treatment group were inadvertently reversed in the original report. This had no effect on the reported BAF and BAFK values.

AMENDMENT SIGNATURES:

STUDY DIRECTOR:

Henry O. Krueger, Ph.D.
Director of Aquatic, Plant and Insect Toxicology

Date 2/23/10

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

Willard B. Nixon, Ph.D.
Director of Chemistry

Date 2/23/10

REVIEWED BY:

Marshall T. Hynson, B.A.
Quality Assurance Program Supervisor

Date 2/23/2010

AMENDED

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10 MAY -2 AM 10: 16

D5:
A PROLONGED SEDIMENT TOXICITY TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-108

ASTM Standard E 1706-00

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

AUTHORS:

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SUBMITTED TO

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Centre Europeen des Silicones
European Chemical Indusrty Council (Cefic)

TITLE: D5: A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-108

STUDY COMPLETION: January 11, 2007

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exception:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods.

STUDY DIRECTOR:

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SPONSOR APPROVAL:

Sponsor Representative — 1/17/07 Date

Applicant/Submitter — Date

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM(98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: STUDY DIRECTOR	MANAGEMENT:
Test Substance Preparation	August 21, 2006	August 22, 2006	October 16, 2006
Observations	September 12, 2006	September 12, 2006	September 28, 2006
Calibration Standard Preparation	September 21, 2006	September 21, 2006	September 27, 2006
Dry Weights	September 22, 2006	September 22, 2006	September 27, 2006
Protocol	October 3, 2006	October 3, 2006	October 13, 2006
Analytical Data and Draft Report	October 3, 2006	October 3, 2006	October 5, 2006
Biological Data and Draft Report	October 4 and 5, 2006	October 5, 2006	October 13, 2006
Final Report	December 21, 2006	December 21, 2006	January 11, 2007

All inspections were study-based unless otherwise noted.

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REPORT APPROVAL

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WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-108

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SUMMARY

SPONSORS: Centre Europeen des Silicones
European Chemical Indusrty Council (Cefic)

TITLE: D5: A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 583A-108

TEST DATES:		
	Study Initiation:	August 21, 2006
	Experimental Start (OECD):	August 6, 2006
	Experimental Start (EPA):	August 24, 2006
	Biological Termination:	September 21, 2006
	Experimental Termination:	September 22, 2006

LENGTH OF EXPOSURE: 28 Days

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
Superior, Wisconsin 54880
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
63 mg/Kg	24 mg/kg
125 mg/Kg	46 mg/Kg
250 mg/Kg	94 mg/Kg
500 mg/Kg	226 mg/Kg
1000 mg/Kg	495 mg/Kg
2000 mg/Kg	1272 mg/Kg

RESULTS: 28 Day EC50: >1272 mg/Kg, the highest test concentration.
NOEC: 1272 mg/Kg
LOEC: >1272 mg/Kg

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Centre Europeen des Silicones, European Chemical Industry Council (Cefic) at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from August 24, 2006 to September 21, 2006 and dry-weight measurements were completed on September 22, 2006. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 583A-108 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated D5, on the oligochaete, *Lumbriculus variegatus* during a 28-day exposure period under flow-through test conditions. The measured endpoints of the test were survivorship (original organisms and/or offspring) and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of oligochaetes were exposed to a geometric series of six test concentrations and a negative control for 28 days under flow-through test conditions. Eight replicate test compartments were maintained in each treatment and control group, with 10 oligochaetes in each test compartment, for a total of 80 oligochaetes per test concentration. Each test compartment contained sediment and overlying water. An additional five replicates were added in each treatment and control group for analytical sampling of sediment. Organisms were added at test initiation to two analytical replicates for the Day 7 and Day 28 analyses. The remaining three analytical replicates for Days 0, 7 and 28 were "abiotic" and did not contain organisms.

Nominal test concentrations selected in consultation with the sponsor were 63, 125, 250, 500, 1000 and 2000 mg/Kg of sediment based on the dry weight of the sediment. The results of the study are based on mean measured test concentrations. Due to the low solubility of D5 in water (17.0 µg/L) only sediment samples were collected and analyzed from the "analytical replicates" of each treatment group and control (1). Overlying water and pore water samples were not analyzed Sediment samples were collected immediately after the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test Results of the analyses were used to calculate mean measured exposure concentrations.

Test compartments were indiscriminately and slowly positioned in a diluter unit according to treatment group approximately 48 hours prior to test initiation to condition the sediment prior to introduction of organisms. Oligochaetes were impartially assigned to exposure compartments at test initiation. Observations of mortality and abnormal behavior were made daily during the test. Survivorship/reproduction and growth (dry weights) were determined at the end of the 28-day test period. The percent reduction in the numbers of organisms present in the treatment groups at test termination in comparison to the control group was used to determine the 28-day EC50 value. The lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) were determined by the concentration-response pattern and statistical analysis of the survival/reproduction and dry weight data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "D5: A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et. Al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (2); the ASTM E Guideline 1706-00: *Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the OPPTS Guideline 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (5), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (6).

Test Substance

The test substance was received from Dow Corning on March 24, 2006 and assigned Wildlife International identification number 7556. The test substance, a liquid, was identified as: Dow Corning 1693 Fluid: CAS Number 541-02-6, Batch No. 0000341832. The reported purity was 99.19%, with an expiration date of September 7, 2008 (Appendix 2). The test substance was stored under ambient conditions.

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and assigned Wildlife International identification number 7566. The analytical standard, a liquid, was identified as: Tetrakis (trimethylsilyloxy) silane, 97%; Batch Number: 13222MB; CAS Number: 3555-47-3. The internal standard had a reported purity of 97%, expiration date of March 30, 2011 and was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (7) was used as the test sediment. The sediment was composed of approximately 13% peat, 10% kaolin clay, and 77% industrial quartz sand (Appendix 3). The dry constituents of the sediment were mixed in a mixer for approximately 20 minutes without the peat. The peat was mixed with the test material and added to the rest of the ingredients on a later date. The pH of the final mixture of sediment was found to be 7.1. The percent organic carbon of the sediment was found to be 3.7%. The sediment was stored under ambient conditions until used.

A 28-day ration of food (200 mg salmon starter) was dry mixed into the test compartments after the sediment was added, but immediately before adding the water to the test compartments, and 48 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. The food added to the test system during the test was not included in the measurement of organic carbon [7].

Preparation of Test Concentrations

Nominal test concentrations (63, 125, 250, 500, 1000 and 2000 mg/Kg) were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer. Second, a predetermined amount of sand and clay were added to the peat and test material in the nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 30 minutes).

After mixing, approximately 100 mL of dosed formulated sediment was transferred to 13 replicate test beakers (300 mL glass beakers with stainless steel mesh covered holes on opposite sides).

The transfer time was kept to a minimum (approximately 10-20 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 13 beakers had approximately 100 mL of sediment added, a 28-day ration of food (approximately 200 mg of Salmon Starter) was added and gently stirred into the dry sediment. Finally, approximately 150 mL of well water was added to the beakers. After the water was added to the beakers, the beakers were placed in the water bath that was used for the study. The overlying water in the test beakers was exchanged two times daily.

Of the thirteen replicate test compartments prepared for each treatment and control group, eight test compartments were used for the evaluation of survival and growth and five compartments for analytical purposes. The sediment/water mixtures were allowed to acclimate for approximately 48 hours prior to the introduction of the test organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 14-day holding period immediately preceding the test, water temperature ranged from 22.6 to 23.5°C, while the pH ranged from 8.2 to 8.6 and dissolved oxygen ranged from 7.4 to 8.3 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, adult oligochaetes were collected from the culture and impartially added one and two at a time into transfer chambers until each chamber contained 10 organisms. Each group of ten organisms was impartially assigned and transferred to a test compartment. All transfers were made below the water surface using wide-bore pipettes. Ten replicate test compartments in each treatment and control group contained organisms (eight test compartments for the evaluation of effects plus two compartments for analytical purposes), while three test compartment per group were maintained without organisms for analytical purposes.

Oligochaetes were fed salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the holding period and during the test. A 200 mg aliquot of food (enough food for the entire study) was weighed for each test compartment that contained organisms during the test and was added to the test compartments after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. No additional food supplementation was made during the study.

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 measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The test apparatus consisted of test compartments placed in stainless steel diluter tanks. The tanks were placed in a temperature-controlled water bath in a Wildlife International, Ltd. diluter unit. One tank was assigned to each treatment or control group. Standpipes were placed in each tank to maintain the water height. Dilution water was delivered on a timer to a splitting chamber two times per day. Each splitting chamber contained 14 syringes. Each syringe delivered dilution water to individual test compartments which were indiscriminately and carefully positioned in diluter tanks according to treatment group.

Test compartments were 300-mL glass beakers with two stainless steel mesh-covered holes on opposite sides to allow for the flow of water through the test compartment. Each beaker contained approximately 100 mL of sediment (3.8 cm in a representative test chamber) and approximately 150 mL of overlying water. The depth of overlying water in each test compartment was maintained by the water

levels in the diluter tanks (e.g. 5.4 cm in a representative test compartment). The diluter was adjusted so that approximately 783 ml of water was delivered per minute for three minutes to each splitting chamber two times per day resulting in approximately two volume additions in each test compartment per day. Test compartments were labeled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 164 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test compartment at the beginning of the test, three times per week during the test, and at the end of the test using a liquid-in-glass thermometer. Temperature also was measured continuously in a beaker beside the test chambers in the negative control diluter tank using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, three times per week during the test, and at test termination. Measurements of pH were made on samples of overlying water collected from one alternating replicate test compartment of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a composite of the overlying water from the control group replicates and the highest concentration treatment group (2000 mg/Kg) replicates at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (8). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test compartments at the beginning of the test, an additional 20 organisms were impartially selected at the beginning of the test and measured for dry weight. The test compartments were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). On Day 28 of the test, oligochaetes were removed from the sediment, and the numbers of live or dead worms were enumerated. Since *Lumbriculus variegatus* generally reproduces over a 28-day period, an increase in the number or size of worms in each test compartment is not unexpected. However, it is not possible to differentiate between adults and young. Therefore, survival and reproduction were considered one endpoint, i.e. the total number of organisms present at test termination.

Dry Weight Measurements

At test termination, each surviving worm was rinsed of excess sediment, placed in a pre-weighed labeled crucible and dried for approximately 24.5 hours at approximately 60°C. The worms were weighed after allowing the crucibles to cool at room temperature for approximately 40 minutes.

Statistical Analyses

The results of the test were based on the mean measured sediment concentrations. Since the percent reduction in the number of organisms present at test termination in comparison to the negative control group was less than 50% in all treatment groups, the 28-day EC50 value was estimated to be greater than the highest sediment concentration tested.

The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival/reproduction and dry weight data. Using the statistical program TOXSTAT Version 3.5 the survival/reproduction and dry weight (growth) data were evaluated for normality (Chi-Square Test) and homogeneity of variances (Bartlett's Test or Hartley's Test). After the data was deemed normal with

homogeneous variance, the survival/reproduction and dry weight data were analyzed using Dunnett's test to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group (9,10).

Analytical Sampling

Analyses of sediment were conducted using separate test compartments collected from the control group and from each concentration treatment groups just after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28. The replicate used for sample collection on Day 0 did not contain oligochaetes. On Day 7 and Day 28 biotic and abiotic replicates were sampled. The sediment and overlying water were mixed to create a slurry and the slurry was used for analysis. The samples were analyzed on the day of collection.

Analytical Method

The method used for the analysis of the D5 samples was based on methodology developed by Wildlife International, Ltd. The analytical method consisted of extracting sediment with ethyl acetate (two times) and transferring to appropriate vessel containing sodium sulfate Ethyl acetate (1 mL) was added to an autosampler vial containing 50 µL of MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to remove interferences Samples were analyzed by gas chromatography equipped with flame ionization detection.

Concentrations of D5 in the samples were determined using an Agilent 6890 Gas Chromatograph (GC) equipped with Flame Ionization Capture Detector (FID). Chromatographic separations were achieved using a J&W DB-5MS Column (0 25 mm x 30 m, 0.25 µm film thickness) A flowchart for the analysis of D5 is provided in Appendix 6.1 and the instrumental parameters for the analyses are provided in Appendix 6 2

Calibration standards of D5, ranging in concentration from 1.00 to 70.0 mg/L, were prepared in ethyl acetate using stock solutions of D5 test substance in 80% dimethylformamide (DMF) 20% toluene (Appendix 6.3). Fresh standards were prepared and analyzed with each sample set Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of D5 in the samples was determined by substituting 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 6.4.

The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg/L) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample. The control samples resulted in reported values of less than 12.5 mg/Kg (Day 0), less than 13.8 and 13.1 mg/Kg (Day 7) and less than 14.5 and 14.7 mg/Kg (Day 28). A reasonable approximation of the LOQ would therefore be calculated as the mean value of all control samples and reported as less than 13.7 mg/Kg (Appendix 6.5).

Sediment samples were fortified at 60 and 200 mg/Kg, using stock solutions of D5 in 80% (DMF) : 20% toluene (Appendix 6.3). The measured concentrations for the matrix fortification samples were 90.4 to 107% (Appendix 6.5).

A representative calibration curve is presented in Appendix 6 6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 6.7 and 6.8, respectively. Representative chromatograms of a sediment matrix blank and a matrix fortification sample are presented in Appendices 6.9 and 6.10, respectively. A representative chromatogram of a test sample is presented in Appendix 6.11.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of D5 in samples collected during the definitive test are presented in Table 1 and Appendix 6. Samples of sediment were collected from the negative control and each of the test concentrations on Day 0, Day 7 and Day 28. The purpose of these samples was to verify the concentrations in sediment at test initiation and to monitor the test substance over the

course of the study. Negative control samples on Days 0, 7, and 28 were all below the limit of quantitation (LOQ).

Nominal sediment concentrations of D5 were 63, 125, 250, 500, 1000 and 2000 mg/kg. Measured concentrations on Day 0 were 19.0, 33.8, 90.6, 204, 522 and 1325 mg/Kg. These concentrations represented 30, 27, 36, 41, 52 and 66% of nominal, respectively. Measured concentrations of biotic samples on Days 7 and Day 28 were 20.4, 39.6, 79.6, 262, 631, and 1350 mg/Kg and 33.0, 64.2, 111, 211, 331 and 1141 mg/Kg, respectively. When measured concentrations for samples collected at test initiation, Day 7 (biotic) and Day 28 (biotic) were averaged, the mean measured concentrations were 24, 46, 94, 226, 495 and 1272 mg/Kg which represented 38, 37, 37, 45, 49 and 64% of nominal, respectively. Low recoveries on Day 0 were thought to be due to the volatile nature of D5 and the mixing process. However after mixing and addition of water to the test compartments, the test concentrations remained stable throughout the 28 day exposure period. Mean measured concentrations of Day 0 and biotic samples were used in the estimation of the test results.

Measured sediment concentrations of D5 in the abiotic replicates on Day 7 and 28 were similar to measured concentrations in the biotic replicates. Consequently, the presence of organisms did not effect the concentrations of D5 in the sediment.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4 respectively. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 76% (6.5 mg/L) of saturation throughout the test. Measurements of pH ranged from 7.9 to 8.3 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 5 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatment and control groups. At test termination, there was an increase in the numbers of oligochaetes present in some test compartments from the 10 worms originally placed in each replicate,

indicating that reproduction had occurred. The mean number of worms in the negative control group at test termination was 26 worms (Table 6). The mean number of worms in the 24, 46, 94, 226, 495, and 1272 mg/Kg treatment groups was 33, 30, 31, 21, 19 and 26, respectively, at test termination. The mean number of worms in the treatment groups was not significantly different ($p > 0.05$) from the negative control group, and was not concentration-dependent.

At the beginning of the test, a group of 20 organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the average individual dry weight of those twenty organisms was 0.51 mg (Appendix 8). The average individual dry weights for the surviving worms in each replicate at test termination are presented in Appendix 8. The average dry weight per worm in the negative control group was 0.92 mg (Table 6). The average dry weight per worm in the 24, 46, 94, 226, 495, and 1272 mg/Kg treatment groups was 0.94, 0.92, 0.99, 1.19, 1.03 and 1.12 mg, respectively. The dry weights of the treatment groups were not significantly different ($p > 0.05$) from the negative control weights, and were not concentration-dependent.

CONCLUSIONS

The 28-day EC50 value for oligochaetes (*Lumbriculus variegatus*) exposed to D5 in sediment was > 1272 mg/Kg, the highest mean measured concentration tested. Determination of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) was based on an evaluation of the survival/reproduction and dry weight data. Based on the results of this study, the LOEC was > 1272 mg/Kg dry weight of sediment and the NOEC was 1272 mg/Kg dry weight of sediment.

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Table 1

Measured Concentrations of D5 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (583A-108-)	Sampling Time (Day)	Measured Concentration (mg/Kg)[1]	Percent of Nominal[2]	Mean Measured Concentration (Percent of Nominal)
Negative Control (0.0)	1 (abiotic)	0	< 12.5	--	--
	8 (biotic)[3]	7	< 13.8	--	
	22 (biotic)	28	< 14.5	--	
	9 (abiotic)	7	< 13.1	--	--
	23 (abiotic)	28	< 14.7	--	
63	2 (abiotic)	0	19.0	30.2	24.1 (38%)
	10 (biotic)	7	20.4	32.4	
	24 (biotic)	28	33.0	52.3	
	11 (abiotic)	7	17.8	28.2	27.4 (43%)
	25 (abiotic)	28	36.9	58.6	
125	3 (abiotic)	0	33.8	27.0	45.9 (37%)
	12 (biotic)	7	39.6	31.6	
	26 (biotic)	28	64.2	51.3	
	13 (abiotic)	7	34.2	27.4	38.8 (31%)
	27 (abiotic)	28	43.3	34.6	
250	4 (abiotic)	0	90.6	36.3	93.7 (37%)
	14 (biotic)	7	79.6	31.8	
	28 (biotic)	28	111	44.3	
	15 (abiotic)	7	82.4	33.0	85.1 (34%)
	29 (abiotic)	28	87.7	35.1	
500	5 (abiotic)	0	204	40.9	226 (45%)
	16 (biotic)	7	262	52.5	
	30 (biotic)	28	211	42.3	
	17 (abiotic)	7	246	49.3	353 (71%)
	31 (abiotic)	28	459	91.9	

[1] The limit of quantitation (LOQ) was calculated as the product of the low standard (1.00 mg a.i./L) and the dilution factor of the negative control samples.
[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[3] Analytical "biotic" replicates contained organisms, while "abiotic" analytical replicates did not.

Table 1
(continued)
Measured Concentrations of D5 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (583A-108-)	Sampling Time (Day)	Measured Concentration (mg/Kg)[1]	Percent of Nominal[2]	Mean Measured Concentration (Percent of Nominal)
1000	6 (abiotic)	0	522	52.2	495 (49%)
	18 (biotic)	7	631	63.1	
	32 (biotic)	28	331	33.1	
	19 (abiotic)	7	548	54.8	563 (56%)
	33 (abiotic)	28	578	57.8	
2000	7 (abiotic)	0	1325	66.3	1272 (64%)
	20 (biotic)	7	1350	67.5	
	34 (biotic)	28	1141	57.1	
	21 (abiotic)	7	1392	69.6	1342 (67%)
	35 (abiotic)	28	1291	64.5	

[1] The limit of quantitation (LOQ) was calculated as the product of the low standard (1.00 mg a.i./L) and the dilution factor of the negative control samples.
[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[3] Analytical "biotic" replicates contained organisms. while "abiotic"analytical replicates did not. .

Table 2

Temperature of Overlying Water in the Test Compartments

Mean Measured Concentrations (mg Kg)	Temperature (°C)													
	Day 0[1]	Day 1	Day 4	Day 6	Day 8	Day 11	Day 13	Day 15	Day 18	Day 20	Day 22	Day 25	Day 27	Day 28
Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control	23.4	23.1	22.8	23.0	23.0	23.2	23.3	23.1	23.0	22.7	23.2	22.9	22.7	22.7
24	23.4	23.2	22.8	23.0	23.0	23.3	23.4	23.2	23.0	22.7	23.2	22.9	22.8	22.7
46	23.3	23.1	23.0	23.0	23.0	23.3	23.3	23.2	23.1	22.7	23.2	22.9	22.9	22.8
94	23.2	23.2	22.9	23.1	23.0	23.3	23.4	23.2	23.2	22.8	23.2	23.0	22.9	22.7
226	23.2	23.0	23.0	23.1	23.1	23.2	23.3	23.3	23.0	22.7	23.2	23.0	22.8	22.8
495	23.3	23.2	23.0	23.2	23.1	23.3	23.3	23.2	23.1	22.7	23.1	23.0	22.8	22.7
1272	23.3	23.2	23.0	23.2	23.0	23.4	23.4	23.2	23.2	22.7	23.3	23.0	22.9	22.7

[1] Temperature measured continuously in the negative control diluter tank ranged from approximately 22.5 to 23.5°C during the test.

Table 3

Dissolved Oxygen in Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)	Dissolved Oxygen (mg/L)													
	Day 0[1]	Day 1	Day 4	Day 6	Day 8	Day 11	Day 13	Day 15	Day 18	Day 20	Day 22	Day 25	Day 27	Day 28
Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control	7.5	7.1	7.3	6.5	7.1	6.8	7.6	7.3	7.2	7.3	7.5	7.6	7.3	7.5
24	7.1	6.9	6.9	6.9	6.8	6.9	7.3	7.1	7.1	7.1	7.3	7.2	7.0	7.4
46	7.3	7.0	7.1	7.2	7.5	6.9	7.6	6.9	7.4	7.2	7.2	7.4	7.1	7.5
94	7.5	7.3	7.0	7.2	7.1	7.1	7.3	7.3	7.0	7.1	7.2	7.2	7.1	7.3
226	6.8	7.2	7.3	6.7	6.7	7.1	7.5	6.9	7.4	7.1	7.4	7.0	7.2	7.3
495	6.7	7.0	7.5	7.1	7.0	8.1	7.4	7.0	7.1	7.1	7.2	7.0	7.2	7.4
1272	7.5	7.2	7.3	6.6	6.9	7.3	7.4	7.1	7.3	6.9	7.4	7.3	7.1	7.4

[1] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

Table 4

pH of Overlying Water in the Test Compartments

Mean Measured Concentrations (mg/Kg)		pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
	Replicate:	A	E	H	C	G
Negative Control		8.1	8.0	7.9	8.0	8.3
24		8.0	8.0	7.9	8.0	8.3
46		8.0	8.0	7.9	8.0	8.3
94		8.1	8.0	7.9	8.0	8.3
226		8.0	8.1	7.9	8.0	8.3
495		8.0	8.1	7.9	8.0	8.3
1272		8.1	8.1	7.9	8.0	8.3

Table 5

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/cm)	Day 0 Ammonia (mg/L)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)	Day 28 Ammonia (mg/L)
Negative Control	132	176	330	0.243	116	160	290	<0.17[1]
1272	124	178	330	0.175	116	162	300	<0.17

Measurements were taken in a composite made from each of the replicates of the respective treatment groups.
[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Table 6

Mean Survival/Reproduction and Growth of Oligochaetes
(*Lumbriculus variegatus*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Mean Number of Surviving Worms (± Stdev)[1,3]	Percent Reduction[2] (%)	Mean Individual Dry Weight[3] (mg)	Percent Reduction[2] (%)
Negative Control	25.6 (± 5 2)	-	0.918	--
24	32.8 (± 11.6)	-28	0.945	-2.9
46	30 1 (± 6.9)	-18	0.918	0
94	31.3 (± 12.2)	-22	0.988	-7.6
226	20.5 (± 13.7)	20	1.191	-30
495	18.6 (± 5.2)	27	1.035	-13
1272	26 3 (± 14.6)	-2.7	1.121	-22

[1] Each replicate contained 10 oligochaetes at test initiation. The mean number of surviving worms is based on the total number of living worms at test termination since offspring cannot be differentiated from adults.
[2] Percent reduction was calculated in relation to the mean of the negative control
[3] There were no statistically significant differences ($p<0.05$) in the mean number of surviving worms and the mean individual dry weight in comparison to the negative control group using Dunnett's test.

Appendix 1

Protocol and Deviations

PROTOCOL

D5: A PROLONGED SEDIMENT TOXICITY TEST
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

ASTM Standard E 1706-00

U S Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER
583A-108

SPONSOR

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European Chemical Industry Council (Cefic)
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B-1160 Brussels
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PERFORMING LABORATORY

Wildlife International, Ltd.

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August 2, 2006

Wildlife International, Ltd.

D5: A PROLONGED SEDIMENT TOXICITY
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic)
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LABORATORY MANAGEMENT: Willard B. Nixon, Ph. D.
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 21 Aug 06

For Will Nixon — LABORATORY MANAGEMENT — DATE 21 Aug 06

SPONSOR'S REPRESENTATIVE — DATE 8/21/06

Wildlife International, Ltd.

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FOR LABORATORY USE ONLY

Proposed Dates.

Experimental Start Date: August 24, 2006

Experimental Termination Date: September 21, 2006

Project No.. 583A-108

Test Concentrations. Negative Control, 63, 125, 250, 500, 1000, 2000 mg a.i./Kg

Test Substance No 7556 Reference Substance No (if applicable): 7566

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd will conduct a prolonged sediment toxicity test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland The study will be based upon a study design proposed by Phipps et al (1993) Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1), the ASTM Standard E 1706-00 *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (2), the U.S EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850 1735. *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (3), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (4), and the Federal Environment Agency Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (5). Raw data for all work performed at Wildlife International, Ltd. and 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. A copy of the final report will be archived at Dow Corning

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions. The sediment used in the test will have an organic content of approximately 5% A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after the sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding organisms The measured endpoints of the test are survivorship and growth as determined by dry weight measurements

EXPERIMENTAL DESIGN

Groups of oligochaetes will be exposed to a geometric series of at least five test concentrations and a negative (untreated sediment) control for approximately 28 days Eight replicate test compartments will be maintained in each treatment and control group, with 10 oligochaetes in each compartment for a total of 80 individuals per test concentration. Each test compartment will contain a quantity of sediment and overlying water Additional replicate test compartments will be included for analytical sampling of sediment No oligochaetes will be placed in "analytical"

replicate(s) sampled at the beginning of the test. However, the "analytical" replicate(s) sampled after the beginning of the test (e g , on Day 7 and at the end of the test) will contain oligochaetes. An additional two " abiotic replicates" (analytical replicates without oligochaetes) will be added to each treatment group for analysis on Day 7 and Day 28 of the test.

Test concentrations in the sediment will be prepared on a dry weight basis (i e., mg/Kg dry sediment) Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate.

The water solubility of D5 is 17.0 µg/L (6) Preliminary trials have shown that the test material is stable in sediment Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of D5 in water. Therefore, only sediment samples from the analytical sampling test compartments will be collected from each control and treatment group at specified intervals for analysis of the test substance. Results of the analyses will be used to verify the exposure over time. The results of the study will be based on the nominal test concentrations in sediment, when appropriate

To control bias, oligochaetes will be impartially assigned to exposure compartments at test initiation. No other potential sources of bias are expected to affect the results of the study Survivorship and growth (dry weight) will be determined at the end of the test period (maximum of 28 days). Mortality in the treatment groups will be used to calculate, when possible, the EC50 value. The dose-response pattern and appropriate statistical analyses of survivorship and growth will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC)

Wildlife International, Ltd.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd verification that the test substance has been characterized according to GLPs prior to its use in the study GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning

Identification	decamethylcyclopentasiloxane, supplied as Dow Corning ® 1693 Fluid
Batch No	0000341832
Expiration Date	7 Sept 2008, extended as part of this study
Source	Dow Corning Corporation, Auburn, Michigan 48611
CAS No	541-02-6
Physical Description	Colorless liquid, refer to PDM
Chemical Stability	Stable, refer to MSDS
Purity	99 19 ± 0.005 area % purity, as determined by this study
Solubility	Ether, refer to PDM, and/or maybe soluble in other common organic solvents
Storage Conditions	Room Temperature
Archive Requirements	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms

Neat test substance will first be incorporated into peat The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1 5 kg batch of sediment to 5% The peat and test substance will be mixed overnight (approximately 12 hours) on a rotary mixer The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance The mixing time will be as short as possible (approximately 30 minutes) or until the mixture appears to be uniformly mixed into the sediment The exact mixing

time will be recorded in the data Approximately 100 mL of the dry mix will then be transferred to 13 replicate test beakers. The test replicates will be 300-mL glass beakers with stainless steel mesh-covered holes on opposite sides of the beaker The transfer time will be kept to a minimum (approximately 10-20 minutes) and one batch of sediment will be completely processes before the other to prevent the potential for volatilization of the test substance

After all 13 beakers have been filled with sediment, a 28 day ration of food (approximately 200mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 150mL of well water will be added to the beakers. After the beakers have been filled with water, they will be transferred to a water bath that will be used for the test. In the water bath the overlying water will be exchanged twice per day by adding one volume addition every 12 hours to each beaker and allowing water to passively flow out the holes in the sides of the beakers After approximately 48 hours of acclimation and settling, organisms will be added to the beakers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (7) will be used The sediment will be composed of approximately 10% sphagnum peat moss, 20%silt and clay (kaolin clay) and 70% industrial quartz sand The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later Calcium carbonate will be added as needed to adjust the pH to 7 0 ± 0.5. The organic carbon

content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding the organisms The amount of food added will be documented in the raw data The percent organic carbon is based solely on peat as the source of organic carbon Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles Prior to use the water will be filtered to 0.45 µm in order to remove fine particles Water used for holding and testing is characterized as moderately hard Typical values for hardness, alkalinity, pH and specific conductance are approximately.

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report

Test Apparatus

The test apparatus will consist of a Wildlife International, Ltd. diluter unit that is designed to hold up to 14 tanks in a temperature controlled water bath Each tank can receive renewal rates that can range from static to greater than 10 tank volume additions per day

Test compartments will consist of 300 mL glass beakers with stainless-steel mesh-covered holes on opposite sides of the beaker. The compartments will be labeled with the project number, test concentration and replicate All replicate beakers in each treatment group will be indiscriminately positioned in one or more diluter tanks. Each beaker will contain approximately 100 ml of sediment and approximately 150 ml of overlying water Dilution water will be delivered directly into each beaker, passively forcing water out through the holes in the sides of the beakers and exchanging the water overlying the sediment. Each replicate beaker will receive approximately two-volume additions of water per day Dilution water delivery will be verified by calibration prior to initiation of the test Proper system operation will be verified at least one time daily during the test

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e g , Colorione® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity Light intensity will be measured at test initiation with a SPER Scientific Ltd light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test compartment of each experimental group at the beginning and end of the test and at least three times per week during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test compartments or in the negative control diluter tank Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation

Dissolved oxygen will be measured in the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least three times per week during the test Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a composite sample of overlying water from the control group replicates and the highest concentration treatment group replicates at the beginning and end of the test Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (8) Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two adult oligochaetes will be impartially distributed to transfer containers (e g., glass beakers) until each container holds its complement of 10 individuals. The organisms in each transfer container then will be transferred below the air/water interface to the test compartments An additional batch of 20 individuals will be impartially selected at the beginning of the test and will be measured for dry weight After completing the transfer of organisms, the test compartments will be maintained in temperature-controlled diluter tanks Test compartments will be observed daily to make visual assessments of any abnormal behavior (e g leaving sediment, unusual swimming).

Observations will be made at test termination to determine the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior On Day 28, oligochaetes will be segregated from the sediment and live and dead organisms will be enumerated. When the total numbers of individuals found in each replicate at test termination are fewer than the number initially placed in each replicate, then those missing will be considered dead. Dry weights of surviving oligochaetes in each replicate will be measured at the end of the test to evaluate effects on growth

Wildlife International, Ltd.

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test compartments will be collected from each concentration at the beginning of the test (no more than one hour after completing the addition of organisms to the test system), on Day 7, and at the end of the test. There will be two additional abiotic replicates in each test group so that additional samples can be collected on Day 7 and Day 28. The overlying water will be removed from the test compartment. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e g.. glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below

PROPOSED NUMBERS OF VERIFICATION SAMPLES

	Day 0	Day 7		Termination (28)	
Experimental Group	Sed	Sed	Abiotic	Sed	Abiotic
Control	1	1	1	1	1
Solvent Control (if needed)	1	1	1	1	1
Level 1-Low Concentration	1	1	1	1	1
Level 2	1	1	1	1	1
Level 3	1	1	1	1	1
Level 4	1	1	1	1	1
Level 5-High Concentration	1	1	1	1	1
	7	7	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test compartments and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor

and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the mortality data will be analyzed using the computer software of C E Stephan (9) The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (10, 11, 12). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test

Growth (dry weight) data will be evaluated for normality and homogeneity of variances using TOXSTAT Version 3 5 (13) If the data are deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe) will be used (12,13) If the data fail the tests for normality or homogeneity, then data transformations will be tried in an attempt to correct the condition When the data transformations fail to correct for non-normality or heterogeneity of variances, nonparametric procedures will be used to identify statistically significant differences among the experimental groups Additional analysis of data may be conducted if deemed appropriate by the Study Director The results of the analysis will be documented in the raw data and summarized in the final report

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to

1 A copy of the signed protocol
2 Identification and characterization of the test substance, if provided by the Sponsor
3 Dates of initiation and termination of the test
4 *Lumbriculus variegatus* culture records
5 Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations
6 Biological observations
7 Test conditions (light intensity, photoperiod, etc)
8 Water chemistry results (e g , hardness and alkalinity)

9 Statistical calculations, if applicable

10 The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable

11 Copy of final report.

FINAL REPORT

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

1 Name and address of the facility performing the study.

2 Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.

3 A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards

4 Objectives and procedures, as stated in the approved protocol, including all changes to the protocol

5 The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.

6 Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.

7 A description of the methods used to conduct the test

8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod

9 A description of the preparation of the test solutions

10 The methods used to allocate organisms to test chambers and begin the test, the number of organisms and compartments per chamber per treatment, and the duration of the test.

11 A description of circumstances that may have affected the quality or integrity of the data

12 The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.

13 A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses

14 Statistical methods used to evaluate the data

Wildlife International, Ltd.

15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17), and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999) Each study conducted by Wildlife International, Ltd is routinely examined by the Wildlife International, Ltd Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g, residue analyses or pathology) Raw data for all work performed at Wildlife International, Ltd and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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DEVIATION TO STUDY PROTOCOL

STUDY TITLE: D5: A Prolonged Sediment Toxicity Test with Lumbriculus variegates Using Spiked Sediment

PROTOCOL NO.: 583/080206/OLIG-SED/SUB583 **DEVIATION NO.:** 1

SPONSOR: Centre Europeen des Siliconcs
European Chemical Industry Council (Cefic) **PROJECT NO.:** 583A-108

DATE OF DEVIATION: August 18, 2006

DEVIATION: There was no adjustment for purity of the test substance in this study.

REASON: The sponsor had indicated that test concentrations should not be corrected for purity prior to study initiation, however, the protocol and data sheets were inadvertently prepared using mg a.i./Kg. This deviation had no impact on the study.

DEVIATION: The neat test material was mixed with the peat for approximately 15 hours instead of approximately 12 hours.

REASON: A slightly longer mixing time was used to accommodate work schedules This deviation had no adverse impact on the results of the study.

STUDY DIRECTOR DATE 10/6/06

LABORATORY MANAGEMENT DATE 10/6/06

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: D5. A Prolonged Sediment Toxicity Test with Lumbriculus variegates Using Spiked Sediment

PROTOCOL NO.: 583/080206/OLIG-SED/SUB583 **DEVIATION NO.:** 2

SPONSOR: Centre Europeen des Silicones
European Chemical Industry Council (Cefic) **PROJECT NO.:** 583A-108

DATE OF DEVIATION: August 18, 2006

DEVIATION: The organic content of the sediment was 3.7% instead of 5%.

REASON: The carbon content can vary dependent upon the organic content of the peat. The slightly lower value did not impact the study and was in acceptable ranges for risk assessment.

STUDY DIRECTOR DATE 12/12/06

LABORATORY MANAGEMENT DATE 12/12/06

Appendix 2

Chemical Characterization of D5

Test Article / Substance / Item

Identification: decamethylcyclopentasiloxane, supplied as Dow Corning® 1693 Fluid
Batch Number: 0000341832
Expiration Date: 7 Sept 2008, extended as part of this study
Source: Dow Corning Corporation, Auburn, Michigan 48611
CAS Number: 541-02-6
Physical Description: Colorless liquid, refer to PDM
Stability: Stable, refer to MSDS
Purity: 99.19 ± 0.005 area % purity, as determined by this study
Solubility: Ether, refer to PDM, and/or maybe soluble in other common organic solvents
Storage Conditions: Room Temperature
Archive: A reserve sample was not retained for this study

The test article, ZMAT Number or Chemical Name (batch Number 0000341832) was characterized in accordance with EPA (TSCA) Good Laboratory Practices 40 CFR Part 792 The physical appearance of the test article was observed to be a colorless liquid. The average area percent purity for decamethylcyclopentasiloxane (D_5), was 99.19 ± 0.005 as determined by GC-FID. The GC-MS data verified the major component in the test article to be D_5 and the minor components as D_4 and D_6. This batch of test article meets the specifications for physical description, purity and composition as defined in the MDMS/PDM. The expiration date of the test article was extended to 7 September 2008.

Appendix 3

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	30,800
Kaolin Clay	4,000
Peat Moss	5,200

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used. The peat was mixed with neat test material and added at a later date. The pH of the final sediment mixture was 7.1.

Appendix 4

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

	Mean	Range
Specific Conductance (µmhos/cm)	314 (N = 4)	310-320
Hardness (mg/L as $CaCO_3$)	136 (N = 4)	136 – 136
Alkalinity (mg/L as $CaCO_3$)	182 (N = 4)	180-184
pH	8.1 (N = 4)	8.0-8.2

Appendix 5

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

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 5

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water[1]
Page 2

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	27	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 6

The Analysis of D5 in Sediment

Appendix 6.1

Analytical Method Flowchart for the Analysis of D5 in Sediment

FLOWCHART FOR THE ANALYSIS OF D5 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Slowly decant the overlying water from the sample beakers to a glass container. Stir the remaining sediment-pore water to homogenize. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.

↓

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

↓

Add 10.0 mL HPLC-grade bottled water and 5 µL of internal standard to each sample and QC.

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately five minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50.0 mL volumetric flask containing 2.5 g of sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Transfer 1.00 mL of extract into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit for analysis.

Appendix 6.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)	
DETECTOR:	Hewlett-Packard Flame Ionized Detector (FID)	
ANALYTICAL COLUMN:	J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness)	
INJECTOR TEMPERATURE:	280°C	
OVEN:	Initial temperature:	50°C
	Initial hold time:	1.00 minute
	Ramp:	8.00°C/minute
	Final temperature:	150°C
	Final hold time:	0.00 minute
DETECTOR TEMPERATURE:	310°C	
INJECTION VOLUME:	1.0 µL	
CARRIER GAS:	Helium	
HEAD PRESSURE:	~12 psi	
APPROXIMATE D5 PEAK RETENTION TIME:	11.9 minutes	

Appendix 6.3

Analytical Stocks and Standards Preparation

A stock solution of D5 was prepared by weighing 0.1004 g on an analytical balance. The test substance was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% (DMF) : 20% toluene. This primary stock solution (1.00 mg/mL) was used to prepare the calibration standards in ethyl acetate. 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)
1.00	10.0	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D5 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% (DMF) : 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% (DMF) : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to all samples and calibration standards (5 µL and 50 µL, respectively).

Appendix 6.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 583A-108-4, with a nominal concentration of 250 mg/Kg, were calculated using the following equations:

$$\text{Concentration of D5 in sample (mg/Kg)} = \frac{\text{peak area - (y-intercept)}}{\text{slope}} \times \text{dilution factor}$$

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

Peak Area = 683.20709
Y-Intercept = 24.42368
Slope = 81.95
Dilution Factor = 11.3

$$\text{Concentration of D5 in sample (mg/Kg)} = \frac{683.20709 - 24.42368}{81.95} \times 11.3$$

Concentration of D5 in sample (mg/Kg) = 90.6*

$$\text{Percent of nominal concentration} = \frac{90.6 \text{ mg/Kg}}{250 \text{ mg/Kg}} \times 100$$

Percent of nominal concentration = 36.3%*

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

Appendix 6.5

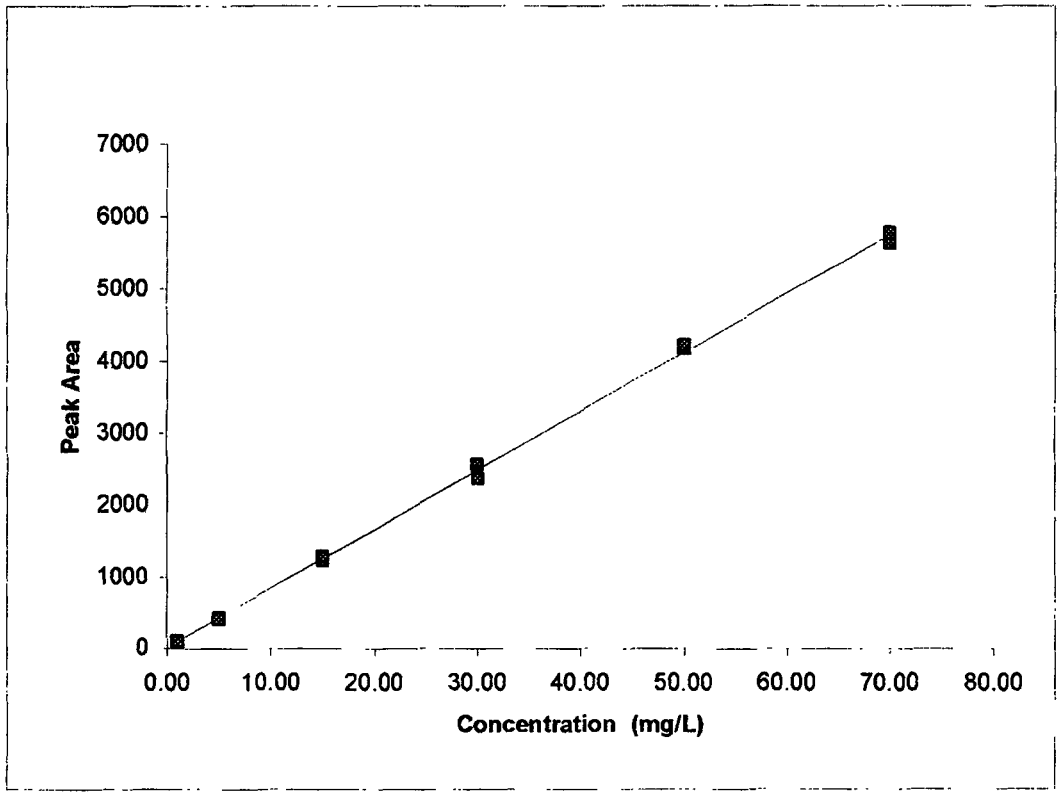
Quality Control Samples of D5 in Sediment

Sample Number (583A-108-)	Sample Type	Concentration of D5 Fortified (mg/Kg)	Concentration of D5 Measured[1] (mg/Kg)	Percent Recovery[2]
MAB-1	Matrix Blank	0.0	ND	--
MAB-2	Matrix Blank	0.0	ND	--
MAB-3	Matrix Blank	0.0	ND	--
MAS-1	Matrix Fortification	60	58.3	97.2
MAS-2	Matrix Fortification	200	183	91.4
MAS-3	Matrix Fortification	60	63.1	105
MAS-4	Matrix Fortification	200	197	98.3
MAS-5	Matrix Fortification	60	64 3	107
MAS-6	Matrix Fortification	200	181	90.4
			Mean =	98.2
			Standard Deviation =	6.81
			CV =	6.93%

[1] Not detected.
[2] Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Appendix 6.6

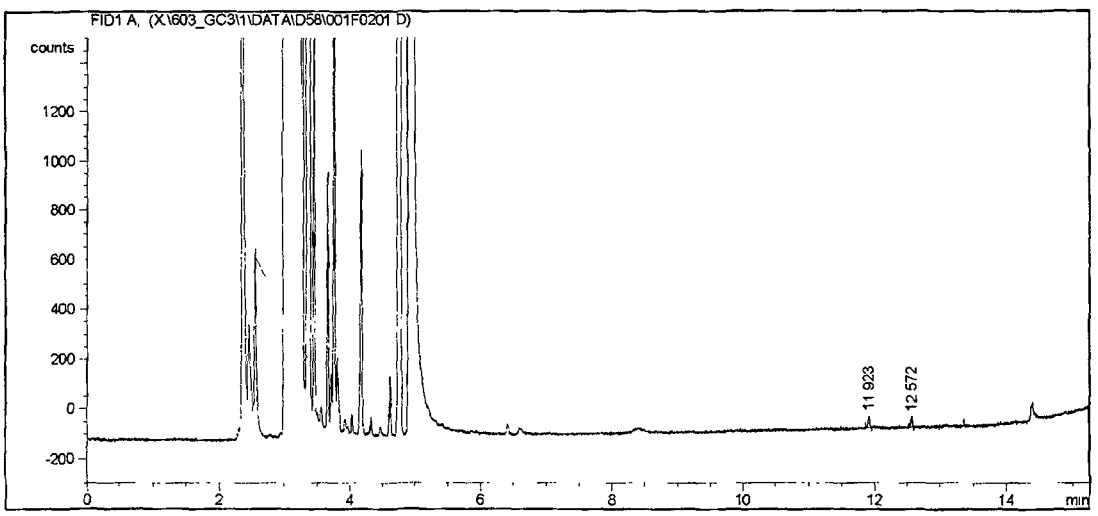
Representative Calibration Curve for D5



Slope = 81.95; Y-Intercept = 24.42368; R^2 = 0.9990

Appendix 6.7

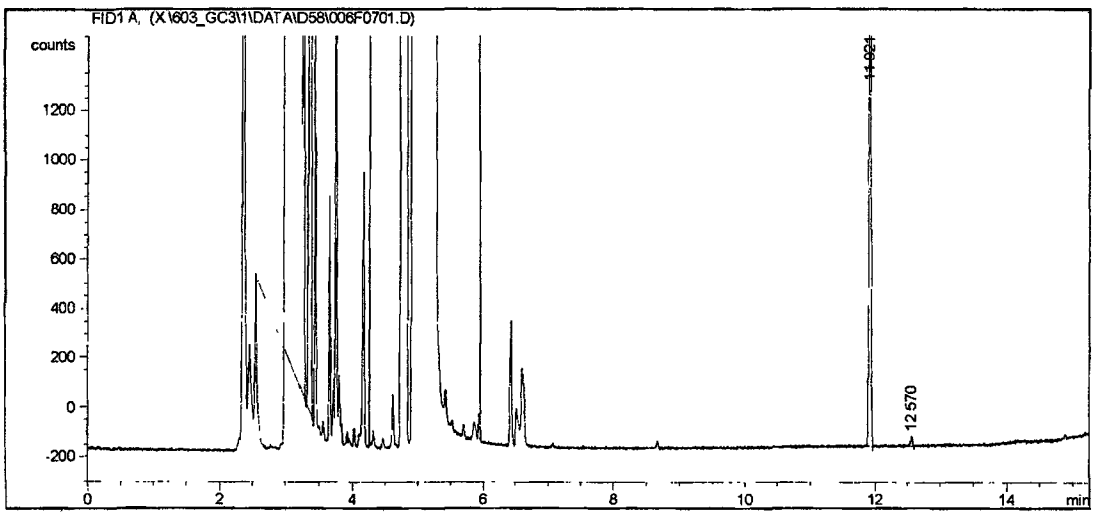
Representative Chromatogram of a Low-level D5 Calibration Standard



Nominal concentration: 1 00 mg/L

Appendix 6.8

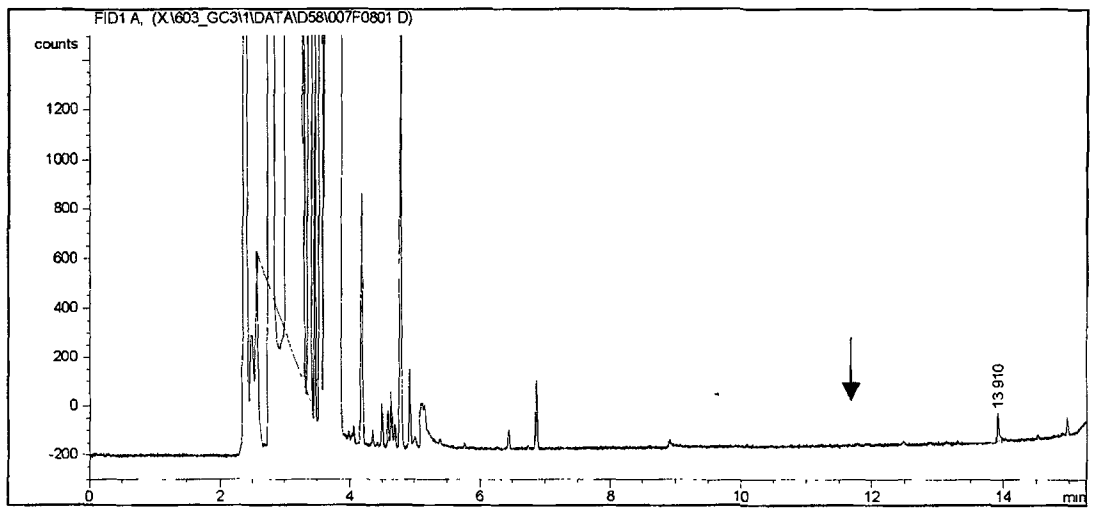
Representative Chromatogram of a High-level D5 Calibration Standard



Nominal concentration: 70.0 mg/L

Appendix 6.9

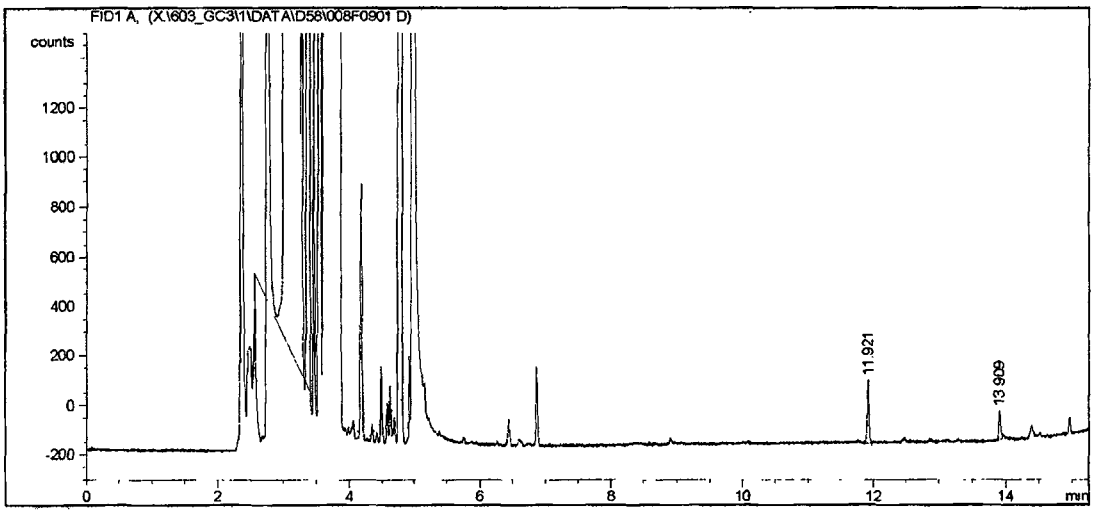
Representative Chromatogram of a Matrix Blank Sample



Sample number 583A-108-MAB-1. The arrow indicates the approximate retention time of D5.

Appendix 6.10

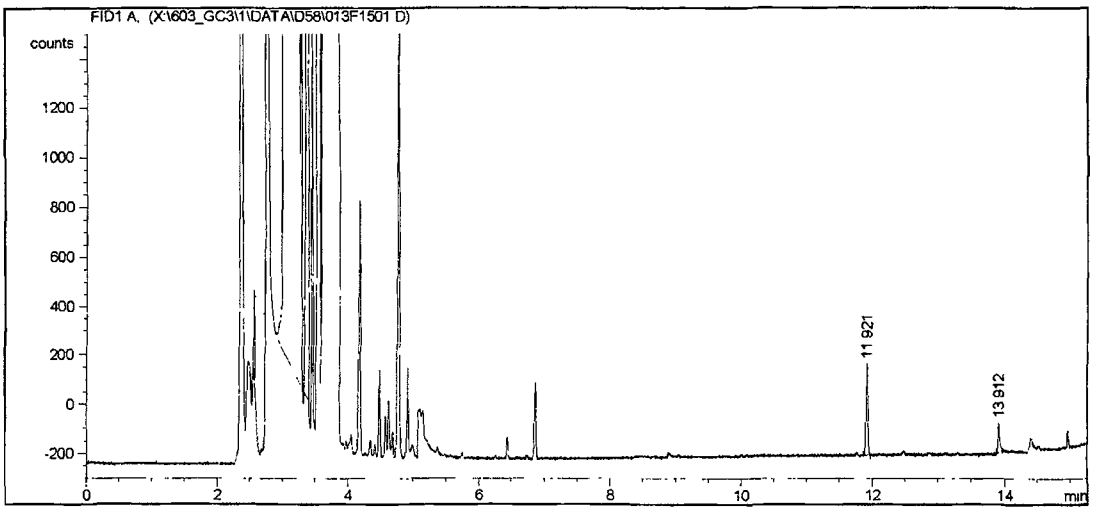
Representative Chromatogram of a Matrix Fortification Sample



Sample number 583A-108-MAS-1; nominal concentration 60.0 mg/Kg.

Appendix 6.11

Representative Chromatogram of a Test Sample



Sample number 583A-108-4; Day 0; 250 mg/Kg nominal concentration

Appendix 7

Observations[1] of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Negative Control	A	10	AN	AN	AN	AN	2H; 3G; AN	2G; AN	2H; 3G; AN	1J; 1G; AN	4G; AN	1H; 3G; AN	1G; AN	AN	2G; AN	3G; AN	AN
	B	10	AN	AN	AN	AN	6G; AN	8G; AN	1H; 4G; AN	1J; AN	4G; AN	3G; AN	AN	AN	1G; AN	1H; 2G; AN	1G; AN
	C	10	AN	AN	AN	AN	4H; 2G; AN	1H; 4G; AN	8G; AN	AN	AN	AN	AN	AN	1G; AN	AN	1H; AN
	D	10	AN	AN	AN	AN	1H; AN	2H; 3G; AN	4G; AN	1G; AN	3G; AN	5G; AN	AN	AN	3G; AN	2G; AN	AN
	E	10	AN	AN	AN	AN	2G; AN	2H; 4G; AN	2H; 2G; AN	AN	1G; AN	1H; 3G; AN	1G; AN	1G; AN	5G; AN	1G; AN	1G; AN
	F	10	AN	AN	AN	AN	4H; 1G; AN	4H; 1G; AN	2H; 3G; AN	3G; AN	1H; 1G; AN	1H; AN	AN	AN	3G; AN	1G; AN	AN
	G	10	AN	AN	AN	AN	3H; AN	3H; 3G; AN	3H; AN	2G; AN	AN	5G; AN	2G; AN	1G; AN	3G; AN	2G; AN	2G; AN
	H	10	AN	AN	AN	AN	1H; 3G; AN	3G; 1J; AN	2H; 5G; AN	AN	AN	1H; AN	1G; AN	AN	1G; AN	1G; AN	AN

[1]Observations: AN - appear normal. G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber.

Appendix 7
Page 2
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	AN	AN	4H; 1G; AN	7H; AN	2H; 4G; 1J; AN	3G; AN	2J; 2G; AN	3H; AN	1G; AN	AN	2G; AN	1H; AN	2G; AN
	B	10	AN	AN	AN	AN	1H; 6G; AN	7H; 1J; AN	2H; 7G; AN	AN	2G; AN	1J; 3G; AN	AN	1G; AN	1G; AN	3G; AN	1G; AN
	C	10	AN	AN	AN	AN	2G; AN	4G; 1J; AN	3H; 2G; AN	1G; AN	1G; AN	1G; AN	1H; AN	AN	2G; 1J; AN	2H; AN	1G; AN
	D	10	AN	AN	AN	AN	2G; AN	1H; 1J; AN	3H; 3G; AN	2G; AN	1J; AN	2G; AN	1G; AN	AN	AN	2G; AN	AN
24																	
	E	10	AN	AN	AN	AN	3H; 1G; AN	4H; AN	1H; 1J; AN	4G; AN	1G; AN	1H; 1G; AN	1G; AN	AN	2H; 3G; AN	1G; AN	AN
	F	10	AN	AN	AN	AN	AN	1J; AN	2G; 1H; AN	AN	1G; AN	AN	1J; 1G; AN	AN	1H; 1J; AN	AN	1H; AN
	G	10	AN	AN	AN	AN	1H; 2G; AN	2G; 1J; AN	2G; AN	AN	AN	3G; AN	2J; 3G; AN	AN	3G; AN	2G; AN	1H; 1G; AN
	H	10	AN	AN	AN	AN	1H; AN	1H; AN	AN	AN	1J; 1G; AN	AN	1J; AN	AN	AN	1J; AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

Appendix 7
Page 3
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	1H; AN	AN	2G; AN	2H; 1G; AN	3G; 1J; AN	3G; AN	1G; AN	1G; AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	3H; 4G; AN	3G; 2H; AN	2H; 7G; AN	AN	1J; 3G; AN	1J; AN	3G; AN	AN	AN	1G; 1J; AN	1H; 1G; AN
	C	10	AN	AN	AN	AN	3G; AN	1G; AN	2G; AN	2G; AN	1J; 1G; AN	1G; AN	1G; AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	2H; 3G; AN	4G; AN	3G; AN	3G; AN	1G; AN	3G; AN	1G; AN	AN	1G; AN	AN	1G; AN
46	E	10	AN	AN	AN	AN	1H; 1G; AN	6H; 3G; AN	2H; 3G; AN	1G; AN	AN	3G; AN	2G; AN	AN	1H; 1G; AN	AN	3G; AN
	F	10	AN	AN	AN	AN	2G; AN	4H; 2G; AN	4G; AN	2G; AN	3G; AN	2H; AN	AN	AN	1H; 3G; AN	1G; AN	2G; AN
	G	10	AN	AN	AN	AN	1G; AN	2H; 2G; AN	1H; 5G; AN	AN	1G; AN	3G; AN	AN	AN	AN	AN	1G; AN
	H	10	AN	AN	AN	1G; AN	3H; AN	3H; AN	1H; AN	AN	AN	3G; AN	3G; AN	AN	2G; AN	1G; AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	AN	AN	1G; AN	1H; 1J; AN	1H; 3G; AN	AN	3J; AN	1H; AN	1G; AN	AN	2G; 1J; AN	1H; 1G; AN	AN
	B	10	AN	AN	AN	AN	2G; AN	1H; 2G; 1J; AN	1H; 1G; AN	AN	1G; AN	1G; AN	AN	AN	2G; AN	AN	1G; AN
	C	10	AN	AN	AN	AN	1H; 1G; AN	1H; 2G; AN	8G; AN	AN	1J; 1G; AN	1G; 1H; AN	AN	1G; AN	AN	AN	3G; AN
	D	10	AN	AN	AN	AN	1H; 4G; AN	3H; 2G; 2J; AN	8G; AN	1J; AN	1G; AN	3H; AN	1J; AN	AN	1H; 2G; AN	4G; 1H; AN	1G; 1J; AN
94																	
	E	10	AN	AN	AN	1G; AN	AN	2J; AN	2G; 3J; AN	AN	AN	1H; AN	AN	AN	AN	3G; 1J; AN	1G; AN
	F	10	AN	AN	2H; AN	2G; AN	1G; AN	1H; 2J; AN	2G; 1J; AN	1G; AN	1J; AN	1H; AN	AN	AN	1J; AN	AN	1G; 1H; AN
	G	10	AN	AN	1H; AN	AN	3G; 1H; AN	1H; 4G; AN	3G; 2J; AN	1G; AN	AN	2G; AN	AN	1G; AN	2G; 1J; AN	2G; AN	2G; AN
	H	10	AN	AN	AN	1G; AN	1G; AN	AN	3J; AN	1G; AN	AN	1H; 1G; AN	2G; AN	AN	1G; AN	1G; AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	AN	1G; AN	1H; 3G; AN	3J; 2H; AN	2G; AN	1J; AN	1G; AN	AN	AN	AN	2H; AN	1H; AN	1G; AN
	B	10	AN	AN	AN	AN	AN	2J; AN	2G; AN	1J; AN	AN	AN	AN	AN	AN	AN	1H; AN
	C	10	AN	AN	AN	AN	AN	3J; AN	2G; 4J; AN	2J; AN	1J; AN	AN	AN	AN	AN	AN	1H; AN
	D	10	AN	AN	1G; AN	AN	AN	AN	5J; AN	1G; AN	2J; AN	AN	1H; AN	AN	2H; AN	AN	AN
226	E	10	AN	AN	AN	1G; AN	3G; AN	2H; 5G; AN	2H; 2G; 4J; AN	1G; AN	AN	4G; AN	2G; AN	AN	3H. 1G; AN	AN	1G; AN
	F	10	AN	AN	AN	AN	4G; AN	1G; 3J; AN	1G; 3J; AN	1J; AN	1J; AN	AN	AN	AN	1J; AN	AN	AN
	G	10	AN	AN	AN	AN	3G; AN	1G; 4J; AN	5G; 1J; AN	2J; AN	AN	AN	2J; AN	AN	AN	AN	2H; AN
	H	10	AN	AN	AN	AN	3G; AN	3J. AN	4G; AN	1J; AN	3G; AN	1H; 1G; AN	3G; AN	AN	3G; AN	AN	1H; AN

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	1H; AN	AN	3H, 7G	3H; 1J, AN	1H; 2J; AN	AN	AN	2H; AN	AN	AN	1H; 1G; AN	1J; AN	1H; AN
	B	10	AN	AN	AN	AN	4G; AN	3H; 1J; AN	1G; 1H; 4J; AN	2G; AN	1H; AN	1H; AN	1G; AN	AN	2H; 1G; AN	AN	1H; 1J; AN
	C	10	AN	AN	AN	AN	1J; 4G; AN	5J; AN	2G; 4J; AN	2G; AN	AN	1J, 2H; 3G; AN	AN	1J; AN	AN	1G; AN	AN
495	D	10	AN	AN	AN	AN	1H, 3J; AN	1G, 4J; AN	1G, 1J; AN	1G; AN	1J; AN	2H; AN	1G; AN	2J; AN	AN	AN	AN
	E	10	AN	AN	AN	AN	1G; 1J; AN	2J; AN	4J; AN	3J; AN	AN	AN	AN	AN	3J; AN	AN	AN
	F	10	AN	AN	AN	AN	1H; 1J; AN	3G; AN	2J; AN	1J; AN	AN	AN	AN	AN	AN	1J; AN	AN
	G	10	AN	AN	AN	AN	1H; 4J, AN	1H, 1J; AN	2J; AN	1G; AN	AN	3H; AN	AN	AN	AN	AN	AN
	H	10	AN	AN	1H; AN	AN	2G; 3J; AN	3G; AN	1H, AN	AN	AN	2H; AN	2G; AN	AN	1G; AN	AN	AN

[1]Observations AN = appear normal; G observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A	10	AN	AN	AN	AN	4G; 1J; 1H; AN	1H; 1G; 2J; AN	AN	1G; AN	1G; AN	2G; AN	AN	AN	1G; AN	3G; AN	AN
	B	10	AN	AN	AN	AN	6H; 1J; AN	3H; 4G; 1J; AN	3J; 1G; AN	1J; 7G; AN	5G; AN	5G; AN	AN	1J; AN	4H; 3G; AN	4H; AN	3G; AN
	C	10	AN	AN	AN	1G; AN	6H; 4G	7G; AN	3G; 1J; AN	3J; AN	1G; AN	2G; AN	AN	AN	3H; 1J; AN	1H; 4G; AN	2H; 1G; AN
1272	D	10	AN	AN	AN	2G; AN	2H; 4J; AN	4G; AN	6G; 1J; AN	1H; 6G; AN	3G; AN	5H; AN	AN	AN	3H; AN	3G; AN	1G; AN
	E	10	AN	AN	AN	AN	1G; 1H; 1J; AN	3G; 1H; AN	AN	1J; AN	AN	AN	AN	AN	1G; 1J; AN	1H; AN	1J; AN
	F	10	AN	AN	AN	AN	3H; 3J; AN	2G; 3J; AN	1J; AN	AN	AN	AN	AN	1J; AN	AN	1H; 1J; AN	AN
	G	10	AN	AN	AN	AN	1H; 2G; 4J; AN	2J; AN	1G; 1J; AN	AN	1J; AN	2H; AN	AN	1H; AN	3J; AN	AN	AN
	H	10	AN	AN	AN	AN	1H; 2G; 3J; AN	1H; 2J; AN	AN	1G; AN	1H; AN	AN	AN	AN	1G; AN	AN	AN

[1]Observations. AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
Negative Control	A	10	2G; AN	3G, AN	2G; AN	AN	1H; AN	AN	1G; AN	AN	AN	AN	AN	AN	AN
	B	10	1G; AN	1H; 2G; AN	1H; AN	AN	AN	AN	1G; AN	1G; AN	AN	AN	AN	AN	AN
	C	10	1G; AN	1G, AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	1G, AN	AN	AN
	D	10	3G; AN	3G; AN	3G; AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN
	E	10	2G; AN	1G; AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	1G; AN	AN	AN
	F	10	1G; AN	2G; AN	AN	AN	AN	AN	1G, AN	AN	AN	AN	AN	AN	AN
	G	10	1G; AN	2G; AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	6G; AN	2G; AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
	A	10	2G: AN	4H: 3G: AN	2G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	3G: AN	2H: 4G: AN	3G: AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	3G: AN	1H: AN	1G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	2G: AN	2H: 2G: AN	AN	1G: AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN
24															
	E	10	1G: AN	2G: AN	AN	AN	AN	AN	AN	AN	1G: AN	AN	AN	AN	AN
	F	10	2G: AN	1H: 3G: AN	AN	AN	AN	1G: AN	AN	AN	AN	AN	AN	AN	1G. AN
	G	10	AN	3G: AN	1G. AN	AN	1G: AN	AN	AN	1G; AN	AN	AN	1G: AN	AN	1G; AN
	H	10	1G: AN	AN	1H: 2G: AN	AN	AN	AN	1J. AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
	A	10	AN	AN	AN	AN	AN	AN	AN	AN	1G: AN	AN	AN	AN	AN
	B	10	AN	3G: AN	3G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	1H: 2G: AN	2G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
46	D	10	AN	1G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	2G. AN	2G: AN	2G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	2H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	2G: AN	AN	AN	AN	AN	AN	1G: AN	AN	AN	AN	AN	AN
	H	10	AN	5G: AN	3G. AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
	A	10	1H: 2G: AN	2H: 1G: AN	AN	1G: AN	2H: AN	AN	AN	AN	1G: AN	AN	AN	AN	AN
	B	10	1G; AN	1H: 4G: AN	2H: 1G: AN	AN	AN	AN	1G: AN	1G: AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	1J: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
94	D	10	AN	2H: 1G: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	1G. AN	AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	1H: AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	1J: AN
	G	10	AN	1H: AN	1H. AN	1J: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	1J: AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
226	A	10	AN	1H: 3G: AN	AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	1G: AN	1H: AN	1G, AN	AN	AN	AN	AN	AN	AN	AN	1J: AN	AN	AN
	C	10	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	1G; AN
	D	10	AN	AN	1H: AN	AN	AN	1J: AN	AN	AN	AN	AN	1J; AN	AN	AN
	F	10	2H, AN	3G: AN	AN	AN	AN	AN	1G: AN	AN	AN	AN	AN	AN	AN
	F	10	1J: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	2H: AN	2H: 3G; AN	1G, AN	1H: AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	1J; AN	AN	AN	AN	AN	AN	1G: AN	AN	AN	AN	1J; AN	AN	AN

[1]Observations. AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 15	16	17	18	19	20	21	22	23	24	25	26	27
	A	10	1H; AN	2G; AN	AN	AN	4J; AN	AN	1J; AN	1G; 2J; AN	1J; AN	AN	AN	1G; AN	AN
	B	10	1H; AN	1G; AN	AN	2J; AN	2J; AN	AN	1J; AN	AN	1J; AN	AN	1G; AN	1G; 1J; AN	1J. AN
	C	10	1J; AN	AN	1H; AN	1J; AN	AN	AN	AN	AN	2J; AN	1J; AN	AN	1J; AN	AN
495	D	10	AN	AN	AN	AN	1J; AN	AN	1J; AN	AN	AN	AN	1G; AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	1J; AN	AN	1J; AN
	F	10	1H; AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	1J; AN	1H; AN	3G; AN	3J; AN	AN	AN	AN	AN	AN	1J; AN	AN	1J; AN	AN

[1]Observations. AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens; J = observed outside the test chamber, placed back in the nearest test chamber

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Number Exposed	Day												
			15	16	17	18	19	20	21	22	23	24	25	26	27
	A	10	1H; AN	2G; AN	AN	1G, AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	3G; AN	2H; 3G; AN	2H; 1G; AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	1G; AN
	C	10	1H; AN	3H; AN	2H; AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
1272	D	10	1G, AN	2G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	1H; AN	1H; AN	1G; AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test chamber or on the screens.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
Negative Control	A	0	AN	29
	B	0	AN	33
	C	0	AN	17
	D	0	AN	20
	E	0	AN	29
	F	0	AN	27
	G	0	AN	24
	H	0	AN	26
			Mean (± Stdev)	25.6 (± 5.2)
24	A	0	AN	40
	B	0	AN	45
	C	0	AN	42
	D	0	AN	33
	E	0	AN	30
	F	0	AN	23
	G	0	AN	39
	H	0	AN	10
			Mean (± Stdev)	32.8 (± 11.6)

[1]Observations AN - appear normal.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Day 28		
		No Dead	Obs	No. Survivors
46	A	0	AN	14
	B	0	AN	35
	C	0	AN	32
	D	0	AN	29
	E	0	AN	31
	F	0	AN	36
	G	0	AN	33
	H	0	AN	31
			Mean (± Stdev)	30.1 (± 6.9)
94	A	0	AN	43
	B	0	AN	38
	C	0	AN	34
	D	0	AN	43
	E	0	AN	15
	F	0	AN	18
	G	0	AN	41
	H	0	AN	18
			Mean (± Stdev)	31.3 (± 12.2)

[1]Observations: AN = appear normal.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
226	A	0	AN	12
	B	0	AN	14
	C	0	AN	10
	D	0	AN	23
	E	0	AN	53
	F	0	AN	16
	G	0	AN	18
	H	0	AN	18
			Mean (± Stdev)	20.5 (± 13.7)
495	A	0	AN	21
	B	0	AN	21
	C	0	AN	23
	D	0	AN	16
	E	0	AN	20
	F	0	AN	13
	G	0	AN	10
	H	0	AN	25
			Mean (± Stdev)	18.6 (± 5.2)

[1]Observations: AN = appear normal.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
1272	A	0	1G; AN	23
	B	0	AN	51
	C	0	AN	35
	D	0	AN	41
	E	0	AN	20
	F	0	AN	10
	G	0	AN	18
	H	0	AN	12
			Mean (± Stdev)	26.3 (± 14.6)

[1]Observations: AN = appear normal; G = observed on the surface of the sediment.

Appendix 8

Worm Dry Weights by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

	Average Individual Dry Weight (mg) by Mean Measured Concentration						
Replicate	Negative Control	24 mg/Kg	46 mg/Kg	94 mg/Kg	226 mg/Kg	495 mg/Kg	1272 mg/Kg
A	0.928	0.910	1.050	0.923	1.142	1.071	1.374
B	0.797	0.864	0.946	1.076	1.514	1.262	0.894
C	1.006	0.933	0.838	0.941	1.600	1.122	0.980
D	0.865	0.873	0.855	0.853	0.883	1.344	1.124
E	0.969	0.907	0.842	1.187	0.947	1.090	1.075
F	0.885	1.044	0.947	1.061	0.950	0.869	1.190
G	0.958	0.977	0.852	0.912	1.117	0.740	0.878
H	0.939	1.050	1.013	0.950	1.372	0.780	1.450
Mean ± Std. Dev	0.918 ± 0.067	0.945 ± 0.072	0.918 ± 0.083	0.988 ± 0.11	1.191 ± 0.27	1.035 ± 0.22	1.121 ± 0.21

On Day 0, a subset of 20 organisms were impartially removed from the culture and used to determine dry weight. The average individual dry weight was determined to be 0.51 mg.

Appendix 9

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. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S , Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

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D5: A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-108

OECD Guideline 218

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: D5: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-108

STUDY COMPLETION: February 27, 2008

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999), with the following exceptions:

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

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

STUDY DIRECTOR:

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

27 Feb 08
Date

SPONSOR APPROVAL:

Sponsor Representative

3/3/08
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 Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Laboratory Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR:	MANAGEMENT:
Test Substance Preparation	July 13, 2007	July 13, 2007	July 16, 2007
Protocol	July 23, 2007	July 23, 2007	July 27, 2007
Matrix Fortification	August 13, 2007	August 13, 2007	August 20, 2007
Dissolved Oxygen Measurements	August 13, 2007	August 13, 2007	August 20, 2007
Analytical Data and Draft Report	November 26-28, 2007	November 28, 2007	December 3, 2007
Biological Data and Draft Report	November 28-30, 2007	November 30, 2007	December 13, 2007
Final Audit	February 27, 2008	February 27, 2008	February 27, 2008

Linda R. Mitchell

Linda R. Mitchell
Director of Regulatory and Ecotox Operations

27 FEBRUARY 2008
Date

REPORT APPROVAL

SPONSOR: Silicones Environmental, Health and Safety Council

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WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER. 570A-108

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SUMMARY

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: D5. A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER. 570A-108

TEST SUBSTANCE: Decamethylcyclopentasiloxane (D5), supplied as Dow Corning® 1693 Fluid (Batch No. 0000341832; 99.19%)

TEST DATES:

Experimental Start (OECD):	July 13, 2007
Experimental Start (EPA):	July 16. 2007
Biological Termination:	August 13, 2007
Experimental Termination:	August 13, 2007

LENGTH OF EXPOSURE: 28 Days

TEST ORGANISM: Midge (*Chironomus riparius*)

AGE OF TEST ORGANISMS: 1 - 4 Days

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
1423 N. 8th St. Suite 118
Superior. WI 54880

TEST CONCENTRATIONS.

Nominal	Mean Measured
Negative Control	<LOQ
156 mg/Kg	35 mg /Kg
259 mg/Kg	70 mg/Kg
432 mg/Kg	160 mg/Kg
720 mg/Kg	248 mg/Kg
1200 mg/Kg	390 mg/Kg
2000 mg/Kg	759 mg/Kg

RESULTS: Based on mean measured test concentrations

28-Day LC50:	257 mg/Kg with 95% confidence limits of 213 and 318 mg/Kg
NOEC:	70 mg/Kg
LOEC:	160 mg/Kg, based on development rate

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Silicones Environmental, Health and Safety Council at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from July 16, 2007 to August 13, 2007. Raw data generated by Wildlife International, Ltd., and a copy of the final report are filed under Project Number 570A-108 in archives located on the Wildlife International, Ltd. site. The original final report is archived at Silicones Environmental, Health and Safety Council.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated test substance on the midge, *Chironomus riparius*, during a 28-day exposure period under semi-static test conditions. The measured endpoints of the test were total number of adults emerged and the development time.

EXPERIMENTAL DESIGN

Groups of midges (first instar chironomid larvae) were exposed to a series of six nominal test concentrations in sediment (156, 259, 432, 720, 1200 and 2000 mg/Kg, dry weight) and a negative control for 28 days under semi-static test conditions. Four replicate test chambers were maintained in each treatment and control group, with 20 midges in each test chamber, for a total of 80 midges per test concentration. Each test chamber contained a quantity of sediment and overlying water. Three additional replicates were added in each treatment and control group for analytical sampling of sediment and one additional replicate was added to each treatment and control group for Day 0 water quality measurements. The "analytical" and "water quality" replicates sampled on Day 0 contained no midges, while midges were added at test initiation to the "analytical" replicates sampled on Days 7 and 28. Biological observations were not made for Day 7 and 28 "analytical" replicates.

The results of the study are based on the mean measured test concentrations in sediment. Sediment samples were collected and analyzed from the "analytical replicates" of each concentration. The collection and analyses were done after the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test.

Test chambers were arranged in a randomized pattern in a temperature controlled environmental chamber approximately 48.5 hours prior to test initiation to condition the sediment prior to introduction of organisms. Midges were impartially assigned to exposure chambers at test initiation. Observations of mortality and abnormal behavior were made daily during the test. The total number of adults emerged at the end of the test period was recorded.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "D5: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment" (Appendix 1). The protocol was based on the OECD Guideline 218: *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment* (1).

Test Substance

The test substance used to prepare the dosed sediment, matrix fortifications and analytical calibration standards was received from Dow Corning on March 24, 2006. It was assigned Wildlife International, Ltd. identification number 7556 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as Dow Corning 1693 Fluid; Lot number 0000341832; Chemical Abstract (CAS) number 541-02-6. The test substance contained 99.19% active ingredient by analysis and had an expiration date of September 7, 2008.

Internal Standard

The internal standard was received on March 30, 2006 and was identified as: Tetrakis (trimethylsilyloxy) silane, 97%, Lot number: 13222MB; CAS Number: 3555-47-3. The internal standard, a liquid, had a reported purity of 97%. Upon receipt, the internal standard was assigned Wildlife International, Ltd. Identification Number 7566 and stored under ambient conditions.

Test Sediment

The sediment used in the study was a formulated sediment based on the recommendations of OECD Guideline 218 (1). The sediment was composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand (Appendix 3). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the peat, since the peat was added later. The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry soil was stored under

ambient conditions until used. The final pH of the sediment was 6.6. The percent organic carbon of the sediment was found to be 3.2 as determined by Agvise Laboratories (Northwood, ND) (Appendix 5). Analyses were performed to determine the concentrations of selected organic and inorganic constituents in a representative sample of sediment similar to that which was used in the study and the results of the analyses are summarized in Appendix 6.

A 28-day ration of food (280 mg Tetramin Flake food) was dry mixed into the test chambers after the sediment was added, but immediately before adding the water to the test chambers, and 48 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. The food added to the test system during the test was not included in the measurement of organic carbon.

Preparation of Test Concentrations

The test substance was administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment dwelling organisms.

Nominal test concentrations (156, 259, 432, 720, 1200 and 2000 mg/Kg) were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer. Second, a predetermined amount of formulated sediment was added to the peat and test material in the nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 30 - 40 minutes). The nominal test concentrations exceeded the recommendations of OECD Guideline 218 because preliminary evaluation of D5 mixed with artificial sediment yielded measured concentrations that were below nominal concentrations (2).

After mixing, dosed formulated sediment was transferred to eight replicate test beakers (2000 mL glass beakers) until reaching a depth of 2 cm. The transfer time was kept to a minimum (approximately 15 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 8 beakers had approximately 2 cm of sediment added, a 28-day ration of food (approximately 280 mg of Tetramin flake food) was added to each beaker that was to contain organisms and was gently stirred into the dry sediment. Finally,

approximately 8 cm of well water was added to the beakers. After the water was added to the beakers, the beakers were placed in the environmental test chamber that was used for the study.

Of the eight replicate test chambers prepared for each treatment and control group, four test chambers were used for the evaluation of survival and emergence, three chambers were used for analytical confirmation and one test chamber was used for water quality measurements on Day 0. The sediment/water mixtures were allowed to acclimate for approximately 48 hours prior to the introduction of the test organisms. At test initiation and termination, the overlying water in all test chambers appeared slightly cloudy tan

Test Organism

The midge, *Chironomus riparius*, was selected as the test species for this study. *Chironomus riparius* are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. Egg masses were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The supplier verified the identity of the species. The organisms were held for four days prior to the start of the test at approximately the same temperature used during the test and in water from the same source as the water used during the test.

At test initiation, the midges were collected from the culture and impartially added one and two at a time to test chambers. All transfers were made below the air/water interface using wide-bore pipettes. Six replicate test chambers in each treatment and control group contained organisms (four test chambers for the evaluation of effects plus two chambers for Day 7 and Day 28 analytical purposes), while two test chambers per group were prepared without organisms, one for Day 0 analytical purposes and one for Day 0 water quality measurements.

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 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 again was filtered (0.45 µm) and passed through an ultraviolet sterilizer to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 4. Hardness, alkalinity, pH and specific conductance were measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test are summarized in Appendix 2.

Test Apparatus

Test chambers consisted of 2000-mL glass beakers containing approximately 2 cm of sediment and 8 cm of overlying water Loose plastic covers were placed over each test chamber during the test. Aeration was applied to each test chamber through a glass pipette that did not extend to a depth closer than 2 cm from the surface of the sediment. Air was bubbled into the test chamber at a rate greater than 1 bubble per second but not so great as to disturb the sediment. Test chambers were arranged in a randomized block order in an environmental chamber. Test chambers were labeled with project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 340 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 20 ± 2°C. Temperature was measured using a liquid-in-glass thermometer in the overlying water of alternating replicate test chambers of each treatment and control group daily during the study. Temperature also was measured continuously in a beaker of water adjacent to the test chambers using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH and ammonia were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, weekly during the test and at test termination. Hardness, alkalinity and specific conductance were measured in a sample of overlying water from the control group and the highest concentration treatment group (2000 mg/Kg) at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (3). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion Model 720Aplus pH/ISE meter.

Observations

The test chambers were observed daily during the test to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. During the period of expected emergence, the sex and number of fully emerged midges was recorded on a daily basis. After identification, the midges were removed from the test chambers. If the total number of emerged midges at the end of the test were fewer than what was initially placed in each replicate, then the number of midges not accounted for were considered dead. The numbers of emerged midges by day are found in Appendix 8

Statistical Analyses

The 28-Day LC50 was calculated using the computer software of C.E. Stephan (5). The program is designed to calculate the LC50 value and 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (6,7,8). The LC50 value was calculated using the mortality data collected at the end of the test. The no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence ratios and development rates.

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, was calculated for each replicate of each treatment and control groups. The emergence ratio (rate) and development rate were calculated for each replicate of each control and treatment group using the SAS System for Windows Version 8.2 (4). The emergence ratio, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_e}{n_a}$$

where: ER = emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The emergence ratio data were arcsine transformed using the following formula to normalize the data prior to statistical analyses:

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_e}{n_a}}\right)$$

where: ERarc = arcsin-transformed emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day. Development rate was calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where: i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment ($= \sum f_i$)
x_i = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

where: day_i = inspection day (days since application).

All statistical procedures were performed using SAS version 8.2 (4) and Stephan version 1.1 (5). The data were analyzed using Dunnett's test, as appropriate, to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group. An ANOVA procedure looking at the interaction between sexes was used to evaluate sensitivity between sexes for development rates and total number emerged. There were no statistically significant interactions found between sex and treatments for development rate and therefore the data for each sex were pooled for this variable. There is no feasible way to determine the sex of one – four day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence ratio are not meaningful because calculations require knowing how many individuals of each sex are initially exposed. However, since there were no statistically significant interactions found between sex and treatment for the total number emerged, the emergence ratio data for both sexes were pooled for analysis.

Analytical Sampling

Sediment samples were collected from the analytical replicates from each test concentration and control shortly after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28.

Analytical Method

The analytical method used for the analysis of D5 was developed by Wildlife International, Ltd. Sediment was sampled through overlying water from the test beakers and approximately 5 mL of sediment was added to each centrifuge tube. To each sample, 10.0 mL of HPLC-grade bottled water and 5 µL of the internal standard were added. Samples were vortexed and shaken after the addition of approximately 20 mL of ethyl acetate, and the samples were centrifuged for approximately five minutes at 60% speed. The ethyl acetate (top layer) was transferred into a 50 mL volumetric flask containing 2.5 g of sodium sulfate. Samples were extracted with an additional 20 mL of ethyl acetate. The extracts (1.00 mL) were transferred into autosampler vials and 50.0 µL of N-methyl, N-

trimethylsilyl trifluoroacetamide (MSTFA) was added to each vial. Samples were then submitted for analysis.

Concentrations of D5 in the samples were determined using an Agilent Model 6890 Gas Chromatograph (GC) using Flame Ionization Detection (FID). Chromatographic separations were achieved using a J&W DB-5MS column (30 m x 0.25 mm, 0.25 µm film thickness). A method flowchart for the analysis of D5 is presented in Appendix 7.1 and typical instrumental parameters are summarized in Appendix 7.2.

Calibration standards of D5, ranging in concentration from 1.00 to 70.0 mg a.i./L were prepared using stock solutions of D5 in 80% DMF : 20% toluene (Appendix 7.3). Fresh calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of D5 in the samples was determined by substituting 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 7.4.

The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg a.i./L) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample mass; the initial mass was different for each sample.

Sediment samples were fortified at 150, 500 and 2000 mg/Kg using a stock solution of D5 in 80% DMF : 20% toluene (Appendix 7.3). and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 78.9 to 93.6% of nominal concentrations (Appendix 7.5).

A representative calibration curve is presented in Appendix 7.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 7.7 and 7.8, respectively. A representative chromatogram of a matrix fortification sample is presented in Appendix 7.9. A representative chromatogram of a test sample is presented in Appendix 7.10.

RESULTS AND DISCUSSION

Confirmation of Test Concentrations

Measured concentrations of D5 in the sediment samples collected during the definitive test are presented in Table 1. Measured concentrations of D5 in the sediment samples collected on Day 0 from the 156, 259, 432, 720, 1200 and 2000 mg/Kg treatment groups were 29.3, 72.8, 77.4, 239, 358 and 997 mg/Kg, respectively. Measured concentrations of D5 in the sediment samples collected on Day 7 from the 156, 259, 432, 720, 1200 and 2000 mg/Kg treatment groups were 52.1, 89.3, 172, 299, 531 and 611 mg/Kg, respectively. Measured concentrations of D5 in the sediment samples collected on Day 28 from the 156, 259, 432, 720, 1200 and 2000 mg/Kg treatment groups were 23.9, 48.1, 230, 206, 280 and 670 mg/Kg, respectively. Measured concentrations of the test substance in the negative control sediment samples collected on Days 0, 7 and 28 were below the limit of quantification (<10.0 mg/Kg wet sediment). Mean measured concentrations of D5 in the sediment, for the duration of the study, for the 156, 259, 432, 720, 1200 and 2000 mg/Kg treatment groups were determined to be 35, 70, 160, 248, 390 and 759 mg/Kg, respectively, which are equivalent to 22, 27, 37, 34, 33 and 38% of nominal, respectively.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4, respectively. Temperatures measured in the test chambers were within the 20 ± 2°C range established for the test. On Day 5 of the test, temperatures monitored continuously during the test using a Fulscope ER/C recorder, had gradually increased to 24.0°C over a 7-hour period due to a malfunction in the environmental chamber. The entire study was moved to a different environmental chamber on Day 7 so that repairs could be performed, then moved back to the original environmental chamber on Day 9 after repairs were complete. The slight increase in temperature had no adverse impact on the study, since the temperatures in the test chambers stayed within the desired range for the study. Dissolved oxygen concentrations remained ≥ 7.4 mg/L (82% of saturation) throughout the test, and measurements of pH ranged from 8.0 to 8.4. Measurements of ammonia in the overlying water are presented in Table 4. Ammonia levels exceeded 4.0 mg/L in the overlying water on Days 7, 14 and 21; therefore, the overlying water was partially renewed on those days to prevent toxicity caused by high levels of ammonia. Measurements of hardness, alkalinity and conductivity of the overlying water in the test chambers are presented in Table 5 and were consistent with typical water quality of Wildlife International, Ltd well water.

Observations of emergence in individual replicates at each observation interval are presented in Appendix 8. Adults that emerged appeared normal. There were a few observations of dead pupae or larvae, organisms swimming in the water column and organisms on the surface of the sediment or climbing the walls of the test chamber (Appendix 9). Although few in number, they were mostly seen in the higher treatment groups, and are therefore considered to be treatment-related. Emergence was first noted on Day 14 of the test. Percent mortality at test termination was 13, 15, 16, 29, 49, 69 and 75 in the negative control, 35, 70, 160, 248, 390 and 759 mg/Kg treatment groups, respectively (Table 6). The LC50 value based on mortality of midges (*Chironomus riparius*) exposed to sediment-incorporated D5 was 257 mg/Kg with 95% confidence limits of 213 and 318 mg/Kg.

Mean development time in the negative control group was 16.2 days (Table 7). Mean development times in the 35, 70, 160, 248, 390 and 759 mg/Kg treatment groups were 16.7, 17.5, 18.5, 21.4, 21.9 and 23.5 days, respectively. There were statistically significant differences ($p<0.05$) in development times between the negative control group and the 248, 390 and 759 mg/Kg treatment groups, using a two-tailed Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for development time was determined to be 160 mg/Kg and the LOEC for development time was determined to be 248 mg/Kg.

The mean emergence ratios in the negative control, 35, 70, 160, 248, 390 and 759 mg/Kg treatment groups were 0.88, 0.85, 0.88, 0.71, 0.53, 0.31 and 0.26, respectively (Table 8). There were statistically significant reductions ($p<0.05$) in emergence ratios between the negative control and the 248, 390 and 759 mg/Kg treatment groups, using a Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for emergence ratios was determined to be 160 mg/Kg and the LOEC for emergence ratio was determined to be 248 mg/Kg (Table 8).

Mean development rates (defined as the portion of larval development which takes place per day) in the negative control, 35, 70, 160, 248, 390 and 759 mg/Kg treatment groups were 0.0640, 0.0629, 0.0601, 0.0562, 0.0489, 0.0476 and 0.0441, respectively (Table 8). There were statistically significant reductions ($p<0.05$) in development rates between the negative control and the 160, 248, 390 and 759 mg/Kg treatment groups, using a Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for development rate was determined to be 70 mg/Kg and the LOEC for development rate was determined to be 160 mg/Kg (Table 8).

CONCLUSIONS

The 28-Day LC50 value based on mortality of midges (*Chironomus riparius*) exposed to sediment-incorporated D5 was 257 mg/Kg with 95% confidence limits of 213 and 318 mg/Kg. There were treatment-related effects observed on development times, mean emergence ratios and development rates in the 248, 390 and 759 mg/Kg treatment groups. There was also a treatment-related effect observed on development rate in the 160 mg/Kg treatment group, making it the most sensitive endpoint. Therefore, the 28-Day LOEC, based on development rate, was 160 mg/Kg and the 28-Day NOEC was 70 mg/Kg.

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Table 1

Measured Concentrations of D5 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Chironomus riparius*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-108-)	Sampling Time (Days)	Measured Concentration (mg/Kg)	Mean Measured Concentration (mg/Kg)	Percent of Nominal [1]
Negative Control	1	0	< 11.5	--	--
	8	7	< 10.2		
	15	28	< 8.79		
156	2	0	29.3	35	22.5
	9	7	52.1		
	16	28	23.9		
259	3	0	72.8	70	27.1
	10	7	89.3		
	17	28	48.1		
432	4	0	77.4	160	37.0
	11	7	172		
	18	28	230		
720	5	0	239	248	34.4
	12	7	299		
	19	28	206		
1200	6	0	358	390	32.5
	13	7	531		
	20	28	280		
2000	7	0	997	759	38.0
	14	7	611		
	21	28	670		

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

Table 2

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Temperature (°C) [1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep.	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Neg. Control	20.0	19.8	20.1	20.0	20.0	21.0	20.0	21.6	20.7	20.9	19.9	20.1	20.2	19.0
35	20.0	20.0	19.8	20.0	19.9	20.5	19.9	21.4	20.5	20.8	20.1	20.0	20.1	19.9
70	20.1	20.1	19.9	20.0	19.9	20.5	19.9	21.3	20.6	20.9	20.0	20.1	20.1	20.0
160	20.0	20.0	20.0	20.0	20.0	20.8	20.0	21.6	20.6	20.8	20.0	20.1	20.1	20.1
248	19.9	20.1	19.9	20.0	19.9	20.9	19.9	21.5	20.7	20.8	20.0	20.0	19.9	20.1
390	20.0	20.0	19.8	20.0	20.0	20.6	19.8	21.6	20.5	20.8	20.1	20.1	20.1	20.2
759	19.9	20.0	19.9	20.1	20.1	20.7	19.9	21.4	20.5	20.8	20.1	20.1	20.0	20.2

[1] Temperature measured continuously during the test ranged from approximately 19.0 to 24.0°C.

Table 2 (Continued)

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Rep.	Temperature (°C)														
		Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
		C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Neg. Control		20.2	20.1	20.1	20.0	20.1	20.1	20.1	19.9	20.1	20.0	20.1	20.0	20.1	20.1	20.2
35		20.0	20.0	20.0	20.0	20.0	20.1	20.0	20.0	20.0	20.0	19.9	20.0	20.1	20.1	20.2
70		20.1	20.1	20.1	20.2	20.1	20.0	20.0	20.0	20.0	20.0	19.9	20.1	20.1	20.2	20.2
160		20.0	20.1	20.0	20.1	20.0	20.0	19.9	20.0	20.0	20.0	19.9	20.1	20.1	20.2	20.2
248		20.0	20.0	19.9	20.1	20.0	20.1	19.9	20.1	20.0	19.9	19.9	20.0	20.1	20.1	20.0
390		19.9	20.1	20.1	20.0	19.9	20.0	20.0	20.0	19.9	20.0	20.0	20.0	20.0	20.2	20.2
759		19.8	20.1	20.1	20.1	19.8	20.0	20.0	20.0	19.9	20.0	19.9	20.0	20.0	20.2	20.2

Table 3

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Dissolved Oxygen (mg/L)[1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep.	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Neg. Control	8.2	8.8	8.0	8.5	8.0	7.8	8.0	8.3	8.7	8.3	7.8	8.6	8.4	8.7
35	8.1	8.6	8.0	8.0	7.9	8.0	8.0	7.7	8.2	7.8	7.9	8.7	8.3	8.0
70	7.8	8.2	7.7	8.3	7.7	7.7	7.6	8.2	7.7	7.7	7.8	8.5	8.3	8.6
160	8.1	8.2	7.8	7.9	7.6	7.7	8.0	8.0	8.0	7.8	8.3	8.2	8.2	8.4
248	8.3	8.5	7.8	8.1	8.3	8.0	7.9	7.9	8.3	8.3	7.9	8.4	8.2	8.3
390	7.9	8.4	8.0	8.0	7.7	7.9	8.2	7.9	8.0	8.1	8.1	8.4	8.0	8.4
759	8.2	7.9	8.4	7.4	8.3	7.8	8.5	8.2	8.4	7.9	8.4	8.5	8.1	8.3

[1] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20°C in freshwater.

Table 3 (Continued)

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Dissolved Oxygen (mg/L)														
	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
Rep.	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Neg. Control	7.8	8.8	8.6	8.6	8.4	8.5	8.9	8.7	8.1	8.7	8.7	8.7	8.4	8.7	8.6
35	8.0	8.6	8.4	8.3	8.4	8.5	8.8	8.1	8.1	8.6	8.1	8.4	8.3	8.7	8.3
70	7.8	8.4	8.6	8.4	8.2	8.4	8.7	8.4	8.2	8.4	8.6	8.5	7.9	8.7	8.4
160	8.2	8.1	7.9	8.1	8.3	8.2	8.6	8.0	8.4	8.1	8.3	8.3	8.4	8.3	7.8
248	8.1	8.3	8.4	8.2	8.4	8.2	8.7	8.4	8.4	8.1	8.6	8.3	8.5	8.4	8.4
390	8.0	8.2	8.0	8.2	8.3	8.2	8.4	8.1	8.0	8.2	8.3	8.4	8.4	8.5	8.1
759	8.3	8.2	8.4	8.1	8.4	8.3	8.3	8.2	8.4	7.9	8.4	7.8	8.3	8.5	8.3

Table 4

pH and Ammonia (mg/L) of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day									
	0		7		14		21		28	
	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia
Rep:	H		D		C		B		A	
Negative Control	8.1	<0.17[1]	8.2	3.37	8.1	4.30	8.2	0.210	8.3	<0.17
35	8.2	<0.17	8.1	4.17	8.2	5.35	8.1	2.60	8.3	<0.17
70	8.2	<0.17	8.3	4.19	8.2	5.97	8.2	4.32	8.4	<0 17
160	8.0	0.186	8.2	4.33	8.3	6.04	8.2	2.85	8.1	0.440
248	8.2	0.188	8.2	4 17	8.3	5.52	8.2	<0.17	8.4	<0.17
390	8.2	<0.17	8.2	3.93	8.3	5.55	8.2	2.71	8.2	<0.17
759	8.3	0.188	8.3	4.33	8.4	5.80	8.2	4.84	8.3	<0.17

[1] The lowest standard used for ammonia calibration was 0.17 mg/L.

Table 5

Hardness. Alkalinity and Conductivity of the Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/cm)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)
Replicate:		H			A	
Negative Control	144	176	345	136	152	320
759	148	170	330	160	180	340

Table 6

Percent Emergence of Midges (*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Number of Emerged Midges - Day 28			Percent Emergence	Percent Mortality[1,2]
			Males	Females	Total		
Negative Control	A	20	9	9	18	90	10
	B	20	7	10	17	85	15
	C	20	15	2	17	85	15
	D	20	13	5	18	90	10
	Group	80	44	26	70	88	13
35	A	20	14	5	19	95	5
	B	20	9	8	17	85	15
	C	20	7	9	16	80	20
	D	20	7	9	16	80	20
	Group	80	37	31	68	85	15
70	A	20	9	7	16	80	20
	B	20	7	12	19	95	20
	C	20	8	9	17	85	15
	D	20	7	11	18	90	10
	Group	80	31	39	70	88	16
160	A	20	8	8	16	80	20
	B	20	9	9	18	90	10
	C	20	3	7	10	50	50
	D	20	7	6	13	65	35
	Group	80	27	30	57	71	29
248	A	20	5	5	10	50	50
	B	20	6	6	12	60	40
	C	20	7	5	12	60	40
	D	20	4	4	8	40	65
	Group	80	22	20	42	53*	49
390	A	20	5	2	7	35	65
	B	20	1	1	2	10	90
	C	20	5	4	9	45	55
	D	20	3	4	7	35	65
	Group	80	14	11	25	31*	69
759	A	20	5	0	5	25	75
	B	20	3	2	5	25	75
	C	20	1	0	1	5	95
	D	20	10	0	10	50	55
	Group	80	19	2	21	26*	75

[1] Percent mortality includes organisms that died in the pupae or larval stages, organisms that emerged and died and organisms that did not emerge by the end of the study.

[2] The EC50 (calculated based on percent survival) was 257 mg/Kg with 95% confidence limits of 213 and 318 mg/Kg, using the Probit method.

* There was a statistically significant difference (p 0.05) from the negative control using Dunnett's t-test.

Table 7

Mean Development Time of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Mean Development Time	
		Replicate Mean (days)	Group Mean[1,2] (days)
Negative Control	A	15.6	16.2
	B	16.4	
	C	16.1	
	D	16.8	
35	A	15.2	16.7
	B	18.4	
	C	16.9	
	D	16.4	
70	A	17.0	17.5
	B	17.3	
	C	16.8	
	D	18.8	
160	A	18.4	18.5
	B	19.1	
	C	18.4	
	D	18.2	
248	A	22.6	21.4*
	B	20.8	
	C	19.4	
	D	22.6	
390	A	19.3	21.9*
	B	24.0	
	C	22.9	
	D	21.3	
759	A	24.8	23.5*
	B	24.6	
	C	23.0	
	D	21.6	

[1] Calculated using Excel 2000. Manual calculations may differ slightly
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
* There was a statistically significant difference from the negative control ($p < 0.05$) using Dunnett's t-test.

Table 8

Emergence Ratio and Development Rate of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Emergence Ratio		Development Rate[1]	
		Replicate	Mean[2]	Replicate	Mean[2]
Negative Control	A	0.90	0.88	0.0668	0 0640
	B	0.85		0.0630	
	C	0.85		0.0644	
	D	0.90		0.0618	
35	A	0.95	0.85	0.0684	0.0629
	B	0.85		0.0577	
	C	0.80		0.0620	
	D	0.80		0.0634	
70	A	0.80	0.88	0.0616	0.0601
	B	0.95		0.0601	
	C	0.85		0.0624	
	D	0.90		0.0564	
160	A	0 80	0.71	0.0568	0.0562*
	B	0.90		0.0545	
	C	0.50		0.0562	
	D	0.65		0.0572	
248	A	0.50	0.53*	0.0463	0.0489*
	B	0.60		0.0501	
	C	0.60		0.0537	
	D	0 40		0.0457	
390	A	0.35	0.31*	0.0536	0.0476*
	B	0.10		0.0426	
	C	0.45		0.0450	
	D	0.35		0.0491	
759	A	0 25	0.26*	0.0414	0.0441*
	B	0.25		0.0425	
	C	0.05		0.0444	
	D	0.50		0.0481	

[1] The development rate represents that portion of larval development which takes place per day.
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
[3] There was a statistically significant reduction from the negative control ($p < 0.05$), using Dunnett's t-test.

Appendix 1

Study Protocol, Amendment and Deviations

PROTOCOL

D5 A PROLONGED SEDIMENT TOXICITY TEST
WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

Guidelines

OECD Guideline 218

Submitted to

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

May 3, 2007

Wildlife International, Ltd.

D5: A PROLONGED SEDIMENT TOXICITY TEST
WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

SPONSOR: Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

SPONSOR'S REPRESENTATIVE: Kurt R. Drottar, M.S

TESTING FACILITY: Wildlife International, Ltd.
8598 Commerce Drive
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STUDY DIRECTOR: Henry O. Krueger Ph.D.
Director of Aquatic Toxicology/Terrestrial Plants and Insects
Wildlife International, Ltd

LABORATORY MANAGEMENT: Willard B. Nixon, Ph D
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 7/13/07

LABORATORY MANAGEMENT — DATE 7/13/07

SPONSOR'S REPRESENTATIVE — DATE 6/4/07

PROTOCOL NO: 570/050307/CHI-SEDb/SUB570

Wildlife International, Ltd.

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FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 16 July 07 Experimental Termination Date: 13 Aug 07

Project No.: 570A-108

Test Concentrations 156, 259, 432, 720, 1200 and 2000 mg/Kg

Test Substance No.: 7556 Reference Substance No. (if applicable). 7566

PROTOCOL NO. 570/050307/CHI-SEDb/SUB570

Wildlife International, Ltd.

INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the midge, *Chironomus riparius*, for the Sponsor at the Wildlife International, Ltd aquatic toxicology facility in Easton, Maryland The study will be based upon the OECD Guideline 218 Sediment-Water Chironomid Toxicity Test Using Spiked Sediment (1). Raw data for all work performed at Wildlife International, Ltd and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the midge, *Chironomus riparius*, during a 20 to 28 day exposure period under static or semistatic test conditions The measured endpoints of the test are emergence ratio (percent emergence), development rate, and the development time

EXPERIMENTAL DESIGN

Groups of midges will be exposed to a geometric series of at least five test concentrations, a negative (untreated sediment) control, and if necessary, a solvent control for approximately 20 to 28 days Four replicate test chambers will be maintained in each treatment and control group, with 20 midges in each chamber for a total of 80 individuals per test concentration Each test chamber will contain a quantity of sediment and overlying water Additional replicate test chambers may be included for analytical sampling of water and sediment and for water quality measurements No midges will be placed in "analytical" or "water quality" replicate(s) sampled at the beginning of the test However, replicate(s) sampled on Day 7 and at the end of the test will contain midges Test concentrations in the sediment will be prepared on a mg/kg dry weight basis.

The formulated sediment will use peat as its source of organic matter in order to achieve an organic content of approximately 4 to 5%. Test substance will first be mixed with peat and then dry components of the formulated sediment (sand and clay). After mixing, dry formulated sediment will be transferred to each test chamber and a 28-day ration of food (tetramin) will be dry mixed into each test chamber Water will then be added to each chamber approximately 48 hours before adding organisms Partial renewals of overlying water will occur if weekly ammonia concentrations approach 4 mg/L

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Nominal test concentrations will be selected in consultation with the Sponsor, and may be based upon the results of exploratory range-finding toxicity data. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate Sediment samples from the analytical sampling test chambers will be collected from each of the test concentrations and controls at test initiation, on Day 7, and at the end of the test Results of the analyses will be used to verify the exposure over time. Nominal or mean measured test concentrations will be used to calculate EC50 values, when appropriate

To control bias, midges will be impartially assigned to exposure chambers at test initiation (e g 4 groups of 20 larvae per chamber). No other potential sources of bias are expected to affect the results of the study. The total number of adults emerged at the end of the test period (approximately 28 days) will be recorded Percent emergence in the treatment groups will be used to calculate, when possible the EC50 The dose-response pattern and appropriate statistical analyses will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC)

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles The Sponsor is responsible for providing Wildlife International, Ltd verification that the test substance has been characterized according to GLPs prior to its use in the study GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning

Identification	decamethylcyclopentasiloxane, supplied as Dow Corning ® 1693 Fluid
Batch No ·	0000341832
Expiration Date:	7 Sept 2008
Source	Dow Corning Corporation, Auburn, Michigan 48611
CAS No	541-02-6

Wildlife International, Ltd.

Physical Description	Colorless liquid, refer to PDM
Chemical Stability	Stable, refer to MSDS
Purity	99 19 ± 0 005 area % purity
Solubility	Ether, refer to PDM, and/or maybe soluble in other common organic solvents
Storage Conditions	Room Temperature
Archive Requirements	A reserve sample was not retained for this study

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

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a batch of sediment to 5% The peat and test substance will be mixed overnight on a rotary mixer The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance The mixing time will be as short as possible (approximately 20 - 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data

After mixing, sediment will be placed in the bottom of the test chambers to a depth of approximately 2 cm (approximately 150 ml) The weight of the sediment added to each chamber will be measured using a top-loading balance and recorded A pre-weighed aliquot of Tetramin Flake Food (280 mg) will be added to the sediment prior to adding the overlying water and mixed into the sediment by hand using a glass stir rod Approximately 8 cm of water will be slowly added into the test chamber in such a manner as to minimize the disturbance to the sediment A 1 4 ratio of sediment to overlying water will be maintained throughout the study. A mark on each test chamber noting the level of the water will be made so that water lost due to evaporation can be replaced in the test chambers as needed with reverse osmosis water. The transfer time will be kept to a minimum

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(approximately 10 – 20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance After the test chambers are prepared, they will be placed in a randomized block arrangement in an environmental chamber at the target temperature and the sediment-water mixtures will be allowed to settle for approximately 48 hours before adding the test organisms A partial renewal of the overlying water will take place if ammonia concentrations exceed 4 mg/L.

Test Organism

The midge, *Chironomus riparius*, has been selected as the test species for this study Midges represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory Environmental Consulting and Testing (ECT), Superior, WI or some other source will supply identified egg masses Midges to be used in the test will be first instar larvae (approximately 1-4 days after hatching) at test initiation. Larvae will be collected from at least three separate egg masses to start the test and will be hatched in water from the same source and at approximately the same temperature as will be used during the test Midge larvae will be fed Tetramin Flake Food or equivalent incorporated into the sediment at the beginning of the test This ration of food is equivalent to feeding the organisms ten mg daily during the test No other supplemental food will be added during the test

Specifications for acceptable levels of contaminants in the food for midges have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later Calcium carbonate will be added as needed to adjust the pH to 7 0 ± 0.5 The organic carbon content of the final mixture of sediment should be 5 0 ± 1 0 and may be adjusted by the use of appropriate amounts of peat or sand

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A 28-day ration of food (tetramin fish food or equivalent) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms The amount of food added will be documented in the raw data The percent organic carbon is based solely on peat as the source of organic carbon The food added to the test system during the test is not included in the measurement of organic carbon

Dilution Water

Water used for the holding and testing of midges will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd site The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles Prior to use the water will be filtered to 0 45 µm in order to remove microorganisms and fine particles and passed through an ultraviolet sterilizer Water used for holding and testing is characterized as moderately hard Typical values for hardness, alkalinity, pH and specific conductance are approximately

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8 1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the analyses will be summarized in the final report

Test Apparatus

Test chambers will consist of 2-L glass beakers, or equivalent, containing approximately 2 cm of sediment and approximately 8 cm of overlying water Loose plastic covers will be placed over each test chamber during the test Aeration will be applied to each test chamber through a glass pipette that shall extend to a depth of not closer than 2 cm from the surface of the sediment Air will be bubbled into the test chambers at a rate greater than 1 bubble/sec but not so great as to disturb the sediment Test chambers will be arranged in a randomized block order in an environmental chamber.

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Test chambers will be labeled with the project number, test concentration and replicate A check will be performed at least once daily to monitor the water levels in the test chambers, the temperature in the environmental chamber where the study is being conducted and the proper function of the aeration

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e g , Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux

The target test temperature will be 20 ± 2°C Temperature will be measured using a liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group daily during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation

Dissolved oxygen will be measured in one alternating replicate of each experimental group daily during the test using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent Measurements of pH will be made at the beginning and end of the test and at least once per week on a sample of the overlying water from each experimental group. Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, then discontinued

Ammonia measurements will be made in a sample of overlying water from one or more of the "water quality" replicates at the beginning of the test, and from one alternating biological replicate from each experimental group weekly during the test Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent Hardness, alkalinity and specific conductance

will be measured in a sample of the overlying water from one or more of the "water quality" replicates at the beginning of the test and from a biological replicate at the end of the test, from the controls and the highest treatment group Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (2). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two first instar larvae will be sequentially added to test chambers until each chamber contains its complement of 20 individuals After adding the test organisms to the test chambers, the test chambers will be placed in a walk-in environmental chamber and organized in a randomized block arrangement

Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g leaving sediment, unusual swimming) During the period of expected emergence the sex and number of fully emerged midges will be recorded on a daily basis After identification the midges will be removed from chambers Egg masses deposited prior to the end of the test will be recorded and then removed to prevent re-introduction of larvae into the sediment If the total number of individuals emerged in each replicate at the end of the test are fewer than what was initially placed in each replicate, then the number of individuals not accounted for will be considered dead The test duration for *Chironomus riparius* is approximately 28 days If midges emerge earlier, the study can terminate after a minimum of five days following emergence of the last adult in the control.

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from selected concentrations at test initiation, on Day 7, and at the end of the test The overlying water will be removed from the test chamber Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e g . glass or polypropylene bottle) and stored under the appropriate conditions (frozen, refrigeration or ambient) until analyzed The sampling scheme is summarized below

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PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 7	Termination (28)
Control	1	1	1
Level 1-Low Concentration	1	1	1
Level 2	1	1	1
Level 3	1	1	1
Level 4	1	1	1
Level 5-High Concentration	1	1	1
	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report

Statistical Analyses

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software 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 method, or binomial probability with nonlinear interpolation (4,5,6). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) will be determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence rates and development rates

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, will be calculated for each replicate of each treatment and control group The emergence rate and development rate will be calculated for each

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replicate of each control and treatment group using the SAS System for Windows Version 8 2 (7) The emergence rate, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_e}{n_a}$$

where. ER = emergence rate
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The emergence rate data were arcsine transformed using the following formula to normalize the data prior to statistical analyses

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_e}{n_a}}\right)$$

where ERarc = arcsin-transformed emergence rate
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day Development rate will be calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where $\bar{x}$ = development rate
i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment ($= \sum f_i$)
x = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

where day_i = inspection day (days since application).

The data will be analyzed using Student's t-test to determine any statistical differences between solvent and negative control groups. If there are no significant differences between the solvent and negative control, the controls will be pooled and all comparisons will be made between the pooled control and treatment groups The data will be analyzed using Dunnett's test, as appropriate, to identify those treatment levels that are statistically different ($p<0.05$) from the pooled control group (5, 6). If data cannot be pooled, the most appropriate comparisons will be made to either the negative or solvent control, or both If other statistical procedures are used to analyze the data, the procedures will be described in the raw data and final report.

An ANOVA procedure looking at the interaction between sexes will be used to evaluate sensitivity between sexes for development rates The data will be analyzed for significant interactions between sex and treatments for development rate to determine if data can be pooled between sexes There is no feasible way to determine the sex of three day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence rate are not possible because calculations require knowing how many individuals of each sex are initially exposed If there are no significant interactions found between sex and treatments for the number of female and male midges that emerge, the emergence rate data for both sexes will also be pooled for analysis.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd will include, but not be limited to

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor
3. Dates of initiation and termination of the test
4. *Chironimus riparius* culture records.
5. Stock solution calculation and preparation, if applicable
6. Biological observations.
7. Water chemistry results (e g , hardness and alkalinity)
8. Statistical calculations, if applicable
9. Test conditions (light intensity, photoperiod, etc)

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10. Calculation and preparation of test concentrations
11 Copy of final report.

FINAL REPORT

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

1 Name and address of the facility performing the study
2 Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates
3 A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol
5 The test substance identification including name. chemical abstract number or code number. strength, purity, composition, and other information provided by the Sponsor
6 Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor
7 A description of the methods used to conduct the test
8 A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod
9 A description of the preparation of the test solutions
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment. and the duration of the test.
11 A description of circumstances that may have affected the quality or integrity of the data
12 The name of the Study Director and the names of other scientists professionals, and supervisory personnel involved in the study
13 A description of the transformations, calculations, and operations performed on the data a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses
14. Statistical methods used to evaluate the data.
15 The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable

16. The location where raw data and final report are to be stored
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792), OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No 6283, Agricultural Production Bureau, 1 October 1999) Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International. Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices. Standard Operating Procedures and the specified protocol A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International. Ltd The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e g. residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd and a copy of the final report will be filed by project number in archives located on the Wildlife International. Ltd site, or at an alternative location to be specified in the final report

Wildlife International, Ltd.

REFERENCES

1. **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment* Adopted 13 April 2004.

2. **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

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

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

5. **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.

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

7. **The SAS System for Windows** 1999-2001 Release 8 2, (TS2M0) SAS Institute, Inc Cary, North Carolina

Wildlife International, Ltd.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: D5: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/050307/CHI-SEDb/SUB570 **AMENDMENT NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-108

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Sampling for Analytical Measurements, Page 10:

CHANGE. The overlying water will be removed from the test chamber.

TO: A layer of overlying water will be maintained over the sediment during sampling to prevent loss of material due to volatility.

REASON: The procedure will be changed to reduce the potential loss of material due to volatility, and will be based on the methodology developed by the sponsor.

STUDY DIRECTOR — DATE 12/12/07

LABORATORY MANAGEMENT — DATE 12/12/07

Wildlife International, Ltd. Project Number 570A-108

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: D5: A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/050307/CHI-SEDb/SUB570 **DEVIATION NO.:** 1

SPONSOR: Silicones Environmemtal, Health and Safety Council **PROJECT NO.:** 570A-108

DATE OF DEVIATION: July 21, 2007

DEVIATION: The temperature measured by the fulscope had gradually increased to 24 0°C over a 7 hour period during the test which is outside of the 20 ± 2°C range established by the protocol The study was moved to another environmental chamber on July 23, 2007 so that repairs could be performed, then moved back on July 25, 2007 after completion of the repairs.

REASON: The increase in temperature was caused by a malfunction in the cooling system of the air handler for the environmental chamber. The malfunction was identified and repaired This slight increase in temperature had no adverse impact upon the results or interpretation of the study

STUDY DIRECTOR DATE 12/14/07

LABORATORY MANAGEMENT DATE 12/14/07

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: D5. A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/050307/CHI-SEDb/SUB570 **DEVIATION NO.:** 2

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-108

DATE OF DEVIATION: July 13, 2007

DEVIATION: The formulated sediment had an organic carbon content of 3 2%, which is below the the acceptable range established by the protocol (5 0 ± 1 0%)

REASON: The carbon content can vary dependent upon the organic content of the peat. The lower value did not impact the study

STUDY DIRECTOR — DATE 1/11/08

LABORATORY MANAGEMENT — DATE 1/11/08

Appendix 2

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

	Mean	Range
Specific Conductance (µmhos/cm)	309(N = 4)	305 - 310
Hardness (mg/L as $CaC0_3$)	138 (N = 4)	136 - 140
Alkalinity (mg/L as $CaC0_3$)	183 (N = 4)	182- 184
pH	8 1 (N = 4)	7.9 – 8.1

Appendix 3

Formulated Sediment Preparation [1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000

[1]The sand and clay were mixed in a PK Twinshell® mixer for 20 minutes and the dry sediment was stored under ambient conditions until used.

[2] The peat moss was added separately to each individual batch (750 gram batch) of sediment prepared. The amount of peat moss added was adjusted for the percent moisture (51%).

The pH was measured in the negative control sediment once all ingredients were mixed together and found to be 6.6.

Appendix 4

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	< 0.020	Heptachlor	< 0.010
Alpha BHC	< 0.010	Heptachlor Epoxide	< 0.024
Alpha Chlordane	< 0.010	Kepone	< 0.20
Beta BHC	< 0.024	Malathion	< 3.0
Bolstar	< 2.0	Merphos	< 6.0
Chlordane	< 0.50	Methoxychlor	< 0.10
Coumaphos	< 3.0	Methyl Parathion	< 2.0
Delta BHC	< 0.024	Mevinphos	< 4.0
Demeton-O	< 2.0	Mirex	< 0.11
Demeton-S	< 3.0	Naled	< 3.0
Diazinon	< 4.0	o,p-DDD	< 0.020
Dichlorvos	< 3.0	o,p-DDE	< 0.020
Dieldrin	< 0.020	o,p-DDT	< 0.020
Disulfoton	< 2.0	p,p-DDD	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDE	< 0.020
Endosulfan I	< 0.010	p,p-DDT	< 0.020
Endosulfan II	< 0.020	PCB-1016	< 0.50
Endosulfan Sulfate	< 0.040	PCB-1221	< 0.50
Endrin	< 0.020	PCB-1232	< 0.50
Endrin Aldehyde	< 0.10	PCB-1242	< 0.50
Endrin Ketone	< 0.040	PCB-1248	< 0.50
EPN	< 4.0	PCB-1254	< 0.50
Ethion	< 2.0	PCB-1260	< 0.50
Ethoprop	< 3.0	Phorate	< 2.0
Ethyl Parathion	< 2.0	Ronnel	< 2.0
Famphur	< 3.0	Stirophos	< 2.0
Fensulfothion	< 15	Telodrin	< 0.010
Fenthion	< 2.0	Tokuthion	< 2.0
Gamma BHC -- Lindane	< 0.010	Toxaphene	< 1.0
Gamma Chlordane	< 0.010	Trichloronate	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Trithion	< 2.0
HCB	< 0.10		

[1] Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006

Appendix 4 (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	12.8
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	7.55
Calcium	34.9	Selenium	< 0.0200
Chloride	4.8	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.0
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	1.2	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 5

Agvise Soil Characterization Report

AGVISE LABORATORIES

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Northwood, ND 58267
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Homepage www.agvise.com

AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INTERNATIONAL
Protocol or Study No = VARIOUS
Sample ID. = 570A-108 BOTTOM
Trial ID. = NEGATIVE CONTRO
Date Received = 11-20-07
Date Reported = 12-20-2007

AGVISE Lab No	07-1095
Percent Sand	73
Percent Silt	10
Percent Clay	17
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	1.07
Cation Exchange Capacity (meq/100 g)	10.3
% Moisture at 1/3 Bar	20.1
% Organic Carbon --Walkley Black	3.2
% Organic Matter--Walkley Black	5.6
pH in 1:1 soil:water ratio	7.4

Base Saturation Data

Cation	Percent	ppm
Calcium	45.7	942
Magnesium	42.3	523
Sodium	0.9	22
Potassium	0.3	11
Hydrogen	10.9	11

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

Date 12/26/07

Appendix 6

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Sediment

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.85	Heptachlor	< 0.85
Alpha BHC	< 1.0	Heptachlor Epoxide	< 0.85
Alpha Chlordane	< 0.85	Kepone	< 7.1
Beta BHC	< 2.0	Malathion	< 68
Bolstar	< 68	Merphos	< 68
Chlordane	< 17	Methoxychlor	< 8.5
Coumaphos	< 68	Methyl Parathion	< 68
Delta BHC	< 0.85	Mevinphos	< 68
Demeton-O	< 68	Mirex	< 1.7
Demeton-S	< 68	Naled	< 68
Diazinon	< 68	o,p-DDD	< 1.7
Dichlorvos	< 68	o,p-DDE	< 1.7
Dieldrin	< 1.7	o,p-DDT	< 1.7
Disulfoton	< 77	p,p-DDD	< 1.7
Dursban (Chlorpyrifos)	< 68	p,p-DDE	< 1.7
Endosulfan I	< 0.85	p,p-DDT	< 1.7
Endosulfan II	< 1.7	PCB-1016	< 17
Endosulfan Sulfate	< 1.7	PCB-1221	< 43
Endrin	< 1.7	PCB-1232	< 17
Endrin Aldehyde	< 1.7	PCB-1242	< 23
Endrin Ketone	< 1.7	PCB-1248	< 17
EPN	< 68	PCB-1254	< 17
Ethion	< 68	PCB-1260	< 17
Ethoprop	< 68	Phorate	< 68
Ethyl Parathion	< 68	Ronnel	< 68
Famphur	< 68	Stirophos	68
Fensulfothion	< 68	Telodrin	< 1.2
Fenthion	< 68	Tokuthion	< 68
Gamma BHC Lindane	< 0.85	Toxaphene	< 34
Gamma Chlordane	3.1	Trichloronate	< 68
Guthion (Azinphos-methyl)	< 68	Trithion	< 68
HCB	< 0.85		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 6 (Continued)

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

Metals			
Component	Measured Concentration (mg a.i /Kg)	Component	Measured Concentration (mg a.i./Kg)
Aluminum	9200	Magnesium	2710
Antimony	< 2.00	Manganese	4.05
Arsenic	< 2.00	Mercury	< 0.0968
Barium	61.0	Nickel	2.81
Beryllium	< 0.500	Nitrate by IC (solid)	< 1.5
Bromide by IC (solid)	< 5.1	Nitrite by IC (solid)	< 1.0
Cadmium	< 0.500	Potassium	89.7
Calcium	5000	Selenium	< 2.00
Chloride by IC (solid)	20.1	Silver	< 0.500
Chromium	8.25	Sodium	< 100
Cobalt	0.500	Sulfate by IC (solid)	91.9
Copper	1.33	Thallium	< 2.00
Fluoride by IC (solid)	4.3	Vanadium	4.97
Iron	422	Zinc	3.96
Lead	11.7		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 7

The Analysis of D5 in Sediment

Appendix 7.1

Analytical Method Flowchart for the Analysis of D5 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF D5 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample sediment through the overlying water from the test beakers. Add the requisite volume of sediment (~5 mL) to each centrifuge tube

↓

Fortify recovery samples with the appropriate stock solution.

↓

Add 10 0 mL HPLC-grade bottled water and 5 µL of the internal standard to each sample and QC.

↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately five minutes.

↓

Transfer the ethyl acetate layer (top layer) into a 50.0 mL volumetric flask containing 2.5 g sodium sulfate.

↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining the ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.

↓

Transfer 1.00 mL of extracts into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit for analysis.

Appendix 7.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Flame Ionization Detector (FID)
ANALYTICAL COLUMN:	J&W DB-5MS column (30 m x 0.25 mm, 0.25 µm film thickness)
INJECTOR TEMPERATURE:	280°C
RUN TIME:	15 25 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 8.00°C/minute Final temperature 1: 150°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 220°C Final hold time 2: 0.00 minute
DETECTOR TEMPERATURE:	310°C
TOTAL FLOW:	13.8 mL/min at split vent
HYDROGEN:	40 mL/min
AIR:	450 mL/min
INJECTION VOLUME:	1.0 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	12 psi
APPROXIMATE D5 RETENTION TIME:	10 minutes

Appendix 7.3

Analytical Stocks and Standards Preparation

Stock solutions of D5 were prepared by weighing 0.1000 g and 1.0000 g of the test substance on an analytical balance. Each aliquot of test substance was transferred to a 100-mL class A volumetric flask and brought to volume with 80% DMF : 20% toluene. These stock solutions contained 1.00 and 10.0 mg/mL of D5, respectively. The 1 00 mg/mL solution was diluted in 50% DMF : 20% toluene to prepare a 0.100 mg/mL stock solution. The 1 00 and 0.100 mg/mL stock solutions were used to prepare calibration standards in ethyl acetate. The 10.0 mg/mL stock solution was used to fortify the quality control samples.

A stock solution of Tetrakis (trimethylsilyloxy) silane (internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% DMF : 20% toluene. This stock solution (1.00 mg a.i./mL) was diluted in 80% DMF : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. Five microliters of the 0 150 mg a.i./mL stock solution were added to each test sample and quality control sample prior to analysis. Fifty microliters of the 0.150 mg a.i./mL stock solution were added to the calibration standards on Day 7 and Day 28 prior to analysis 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	100	10.0	1.00*
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10 0	30.0
1.00	500	10.0	50.0
1.00	700	1.00	70.0

*On Day 0, the lowest standard was prepared using 1000 µL of 5.00 mg/L standard in 5.00 mL of ethyl acetate

Appendix 7.4

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-108-3, with a nominal concentration of 259 mg/Kg, were calculated using the following equations:

$$\text{D5 in sample (mg /Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of D5 in sample (mg/Kg)}}{\text{nominal concentration of D5 in sample (mg/Kg)}} \text{ X } 100$$

Peak Area = 9.10142
Y-intercept = 0.57688
Slope = 1.13
Dilution factor = 9.65

$$\text{D5 in sample (mg/Kg)} = \frac{9.10142 - 0.57688}{1.13} \text{ X } 9.65$$

Concentration of D5 in sample (mg/Kg) = 72.8 mg a.i./Kg

$$\text{Percent of nominal concentration} = \frac{72.8 \text{ mg/Kg}}{259 \text{ mg/Kg}} \text{ X } 100$$

Percent of nominal concentration = 28.1%

Appendix 7.5

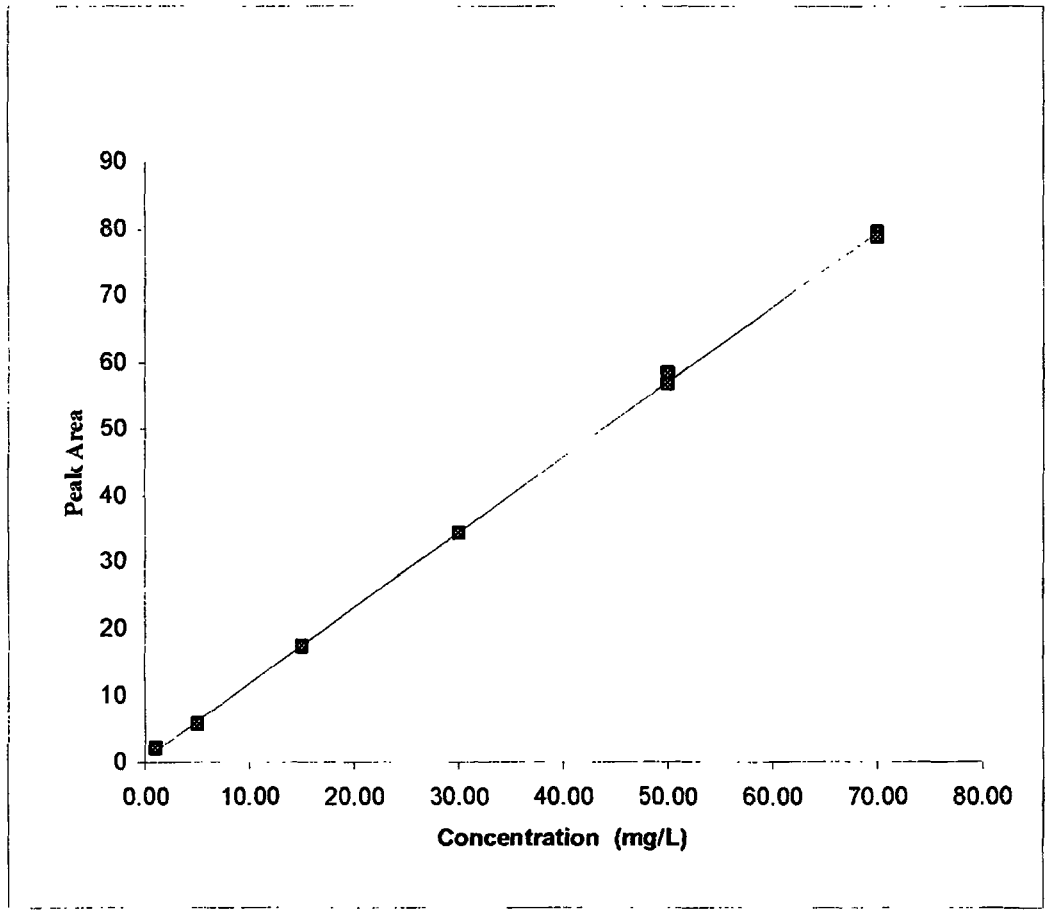
Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (570A-108-)	Sampling Interval (Day)	Concentrations of D5		Percent Recovery[1]
		Fortified (mg/Kg)	Measured (mg/Kg)	
SMAS-1	0	150	118	78.9
SMAS-2	0	500	461	92.2
SMAS-3	0	2000	1871	93 6
SMAS-4	7	150	128	85.5
SMAS-5	7	500	448	89.6
SMAS-6	7	2000	1862	93.1
SMAS-7	28	150	137	91.0
SMAS-8	28	500	467	93.5
SMAS-9	28	2000	1858	92.9
			Mean =	90.0
			Standard Deviation =	4.91
			CV =	5.45%

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

Appendix 7.6

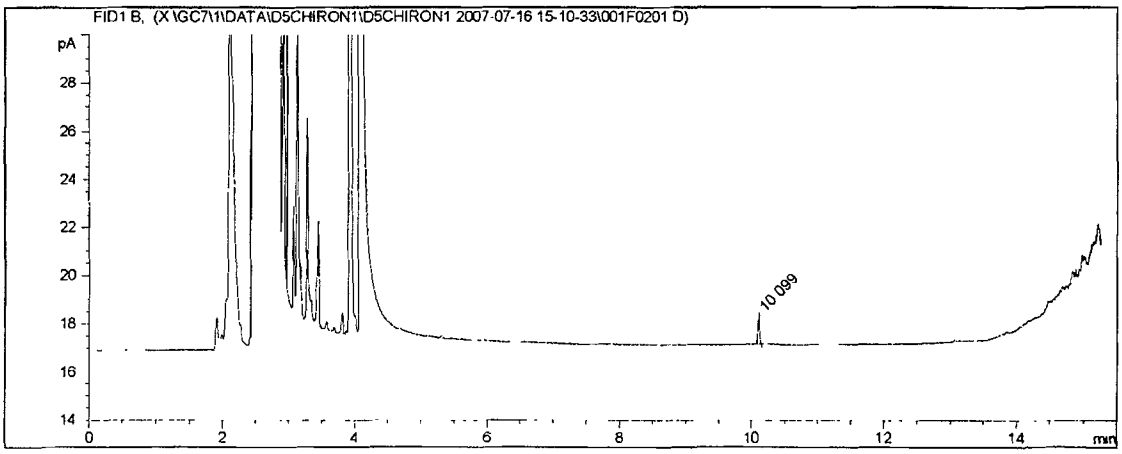
Representative Calibration Curve for D5 in Sediment



Slope = 1.13; Y-Intercept = 0.57688; $R^2 = 0.9996$

Appendix 7.7

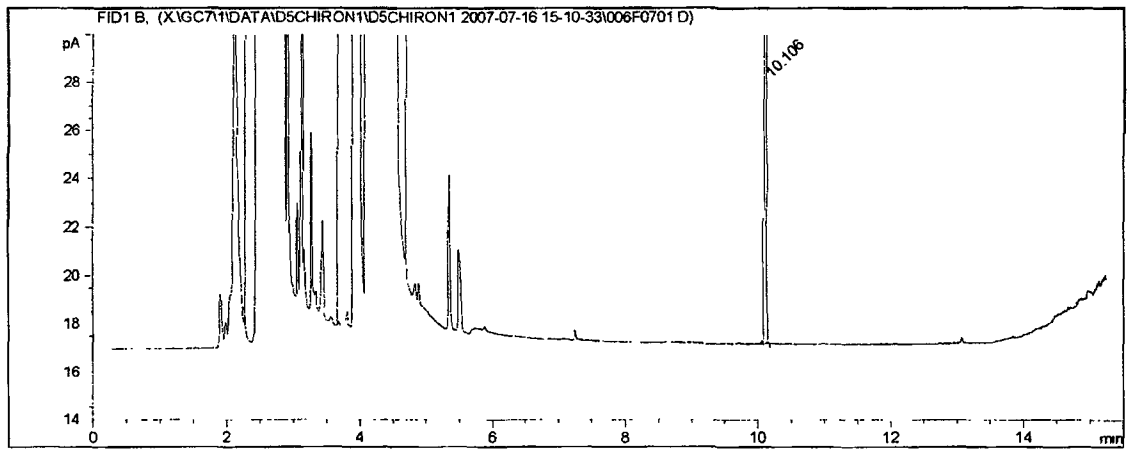
Representative Chromatogram of a Low-level D5 Calibration Standard



Nominal concentration: 1.00 mg/L

Appendix 7.8

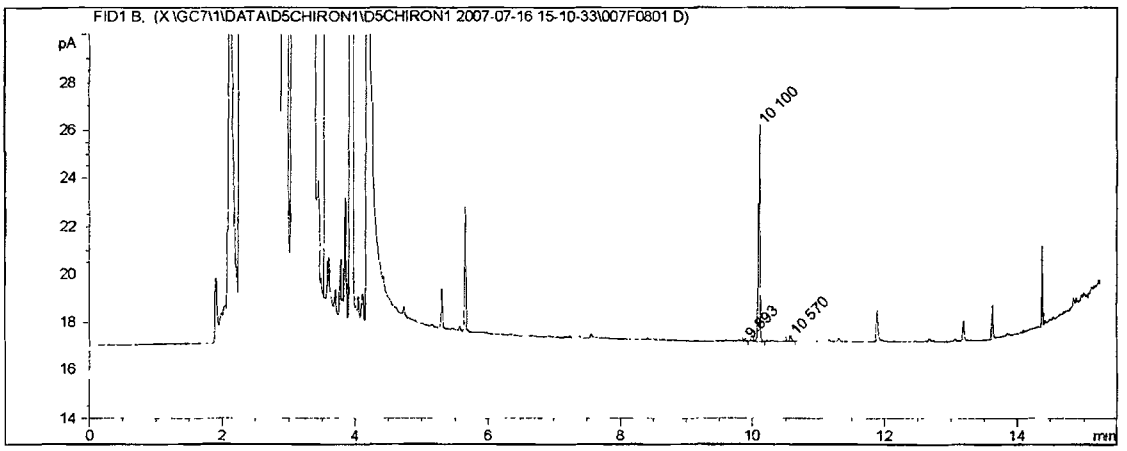
Representative Chromatogram of a High-level D5 Calibration Standard for Sediment



Nominal concentration: 70.0 mg/L

Appendix 7.9

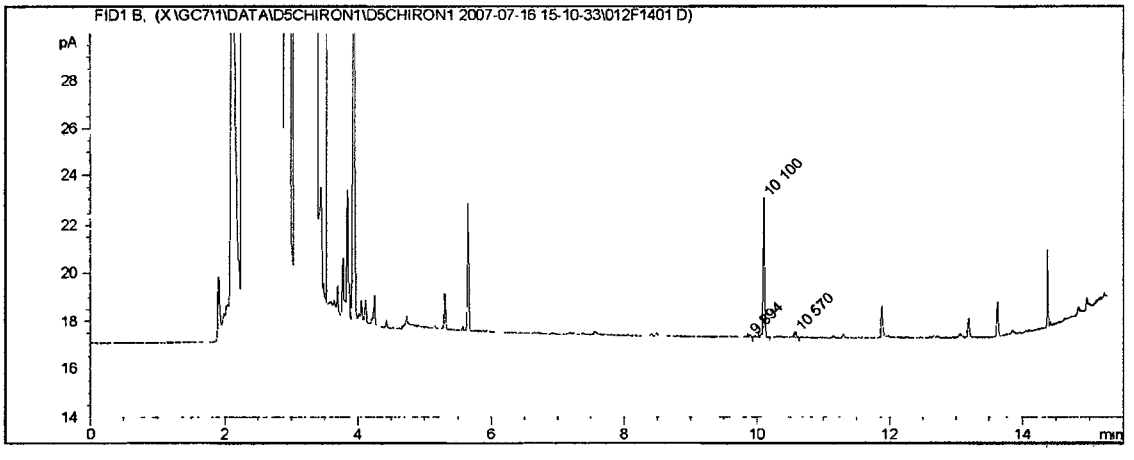
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-108-MAS-1; 150 mg/Kg, nominal concentration.

Appendix 7.10

Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-108-3; 259 mg/Kg nominal concentration

Appendix 8

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Males and Females pooled) by Day of Test														
Replicate		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	2	8	7	0	0	0	1	0	0	0	0	0	0	0	0
	B	0	2	8	5	2	0	0	0	0	0	0	0	0	0	0
	C	0	5	8	3	0	0	1	0	0	0	0	0	0	0	0
	D	0	2	7	4	4	0	1	0	0	0	0	0	0	0	0
35	A	3	11	4	1	0	0	0	0	0	0	0	0	0	0	0
	B	0	2	6	0	4	1	0	0	2	1	0	0	0	0	1
	C	0	6	4	1	1	1	2	0	1	0	0	0	0	0	0
	D	0	7	3	3	0	1	2	0	0	0	0	0	0	0	0
70	A	0	5	5	1	1	1	1	1	1	0	0	0	0	0	0
	B	0	4	3	3	4	3	1	1	0	0	0	0	0	0	0
	C	0	5	6	3	0	1	0	1	0	1	0	0	0	0	0
	D	1	2	1	3	3	1	4	1	0	0	0	1	0	0	1
160	A	0	1	2	4	3	1	3	0	1	0	1	0	0	0	0
	B	0	1	1	2	4	2	3	4	0	0	0	1	--	--	--
	C	0	0	0	4	1	3	1	1	0	0	0	0	0	0	0
	D	0	1	2	3	1	3	1	1	0	1	0	0	0	0	0
248	A	0	0	1	0	0	0	1	1	2	1	2	0	1	0	1
	B	0	0	1	0	1	2	1	3	1	1	1	1	0	0	0
	C	0	0	2	0	3	2	1	2	0	1	1	0	0	0	0
	D	0	0	0	0	0	0	1	2	2	1	1	0	0	0	1
390	A	0	0	1	0	0	1	5	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
	C	0	0	0	0	0	0	1	1	2	2	2	0	0	1	0
	D	0	0	0	0	2	0	1	2	0	1	0	0	0	0	1
759	A	0	0	0	0	0	0	0	0	0	2	1	0	0	2	0
	B	0	0	0	0	1	0	0	0	0	0	0	1	1	2	0
	C	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	D	0	0	0	0	1	0	4	2	0	0	1	0	2	0	0

Notes: -- = all emerged or dead.

Appendix 8 (Continued)

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Males) by Day of Test														
Replicate		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	2	6	1	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	2	5	0	0	0	0	0	0	0	0	0	0	0	0
	C	0	5	7	3	0	0	0	0	0	0	0	0	0	0	0
	D	0	2	6	3	1	0	1	0	0	0	0	0	0	0	0
35	A	3	10	1	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	2	4	0	0	0	0	0	2	1	0	0	0	0	0
	C	0	5	2	0	0	0	0	0	0	0	0	0	0	0	0
	D	0	5	1	1	0	0	0	0	0	0	0	0	0	0	0
70	A	0	5	3	1	0	0	0	0	0	0	0	0	0	0	0
	B	0	3	2X	2	0	0	0	0	0	0	0	0	0	0	0
	C	0	5	2	1	0	0	0	0	0	0	0	0	0	0	0
	D	1	2	1	1	1	0	1	0	0	0	0	0	0	0	0
160	A	0	1	2	3	2	0	0	0	0	0	0	0	0	0	0
	B	0	1	1	1	3	0	2	1	0	0	0	0	--	--	--
	C	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
	D	0	1	1	2	1	0	1	1	0	0	0	0	0	0	0
248	A	0	0	1	0	0	0	0	0	1	0	2	0	1	0	0
	B	0	0	1	0	1	2	0	1	1	0	0	0	0	0	0
	C	0	0	2	0	2	1	1	0	0	1	0	0	0	0	0
	D	0	0	0	0	0	0	1	2	1	0	0	0	0	0	0
390	A	0	0	1	0	0	1	3	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	C	0	0	0	0	0	0	1	1	2	1	0	0	0	0	0
	D	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0
759	A	0	0	0	0	0	0	0	0	0	2	1	0	0	2	0
	B	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0
	C	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	D	0	0	0	0	1	0	4	2	0	0	1X	0	2	0	0

Observations: X = emerged and died; -- = all emerged or dead

Appendix 8 (Continued)

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Females) by Day of Test														
Replicate		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	0	2	6	0	0	0	1	0	0	0	0	0	0	0	0
	B	0	0	3	5	2	0	0	0	0	0	0	0	0	0	0
	C	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
	D	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0
35	A	0	1	3	1	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	2	0	4	1	0	0	0	0	0	0	0	0	1
	C	0	1	2	1	1	1	2	0	1	0	0	0	0	0	0
	D	0	2	2	2	0	1	2	0	0	0	0	0	0	0	0
70	A	0	0	2	0	1	1	1	1	1	0	0	0	0	0	0
	B	0	1	1	1	3, 1X	3	1	1	0	0	0	0	0	0	0
	C	0	0	4	2	0	1	0	1	0	1	0	0	0	0	0
	D	0	0	0	2	2	1	3	1	0	0	0	1	0	0	1
160	A	0	0	0	1	1	1	3	0	1	0	1	0	0	0	0
	B	0	0	0	1	1	2	1	3	0	0	0	1	--	--	--
	C	0	0	0	1	1	3	1	1	0	0	0	0	0	0	0
	D	0	0	1	1	0	3	0	0	0	1	0	0	0	0	0
248	A	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1
	B	0	0	0	0	0	0	1	2	0	1	1	1	0	0	0
	C	0	0	0	0	1	1	0	2	0	0	1	0	0	0	0
	D	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1X
390	A	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	C	0	0	0	0	0	0	0	0	0	1	2	0	0	1	0
	D	0	0	0	0	0	0	0	2	0	1	0	0	0	0	1
759	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Observations: X = emerged and died. -- = all emerged or dead.

Appendix 9

Observations of Abnormal Behavior in Organisms

Mean Measured Concentration (mg/Kg)	Abnormal Observation[1]				
	Dead Pupae or Larvae	Adult That Emerged and Died	Swimming in the Water Column	Observed on the Surface of the Sediment	Observed Climbing the Walls of the Test Chamber
Negative Control	0	0	0	0	0
35	**1**	0	0	0	0
70	0	**3**	0	0	0
160	**5**	0	0	0	0
248	**8**	**1**	**2**	0	0
390	**11**	0	**1**	**2**	0
759	**12**	**1**	**2**	0	**1**

[1]Observations made over the course of the entire study and include observations from each replicate in the treatment group.

Appendix 10

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 & Insects
2. Willard B. Nixon, Ph.D , Director of Chemistry
3. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T Thomas, B.S., Aquatic Biologist

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DODECAMETHYLCYCLOHEXASILOXANE (D6): A BIOACCUMULATION TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-112

ASTM Standard E 1706-00

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

AUTHORS:

Henry O. Krueger, Ph.D.
Susan T. Thomas, B.S.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: July 13, 2007

STUDY COMPLETION DATE: August 15, 2008

SUBMITTED TO:

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
U.S.A
(410) 822-8600

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-112

STUDY COMPLETION: August 15, 2008

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exceptions:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods.

The stability of tissue samples during frozen storage was not determined.

Reference substance characterization and stability at the test site were not determined in compliance with Good Laboratory Practices.

STUDY DIRECTOR:

Henry O. Krueger, Ph.D — Date 8/15/08
Director of Aquatic Toxicology/Terrestrial Plants and Insects

SPONSOR APPROVAL:

Sponsor Representative — Date 8/20/08

Applicant/Submitter — Date

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR	MANAGEMENT:
Protocol	September 10, 2007	September 10, 2007	October 9, 2007
Test Substance Preparation	September 7, 2007	September 7, 2007	October 8, 2007
Dissolved Oxygen Measurements	October 18, 2007	October 18, 2007	November 8, 2007
Biological Data and Draft Report	March 18 – 19, 2008	March 19, 2008	April 28, 2008
Analytical Data and Draft Report	March 16 – 18, 2008	March 19, 2008	April 24, 2008
Final Report	August 15, 2008	August 15, 2008	August 15, 2008

All inspections were study-based unless otherwise noted.

Linda R. Mitchell

Linda R. Mitchell
Director of Regulatory and Ecotox Operations

15 AUGUST 2008
Date

REPORT APPROVAL

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-112

STUDY DIRECTOR:

Henry O. Krueger, Ph. D.
Director of Aquatic Toxicology/Terrestrial Plants and Insects

Date 8/15/08

PRINCIPAL INVESTIGATORS:

Susan T. Thomas, B.S.
Aquatic Biologist

Date 8/15/08

Willard B. Nixon for Timothy Z. Kendall

Timothy Z. Kendall, M.S.
Supervisor

Date 8/15/08

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

Willard B. Nixon, Ph. D.
Director of Chemistry

Date 8/15/08

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SUMMARY

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-112

TEST DATES:		
	Study Initiation:	July 13, 2007
	Experimental Start (OECD):	September 7, 2007
	Experimental Start (EPA):	September 10, 2007
	Biological Termination:	November 7, 2007
	Experimental Termination:	February 5, 2008

LENGTH OF EXPOSURE: 58 Days (28 days Uptake; 30 days Depuration)

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

SOURCE OF TEST ORGANISMS: Aquatic Research Organisms
Hampton, NH 03842
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
100 mg a.i./Kg	28.1 mg a.i./Kg
1000 mg a.i./Kg	484 mg a.i./Kg

RESULTS:

	28.1 mg a.i./Kg	484 mg a.i./Kg
BAF[1]	0.66	0.070
k_1[2]	0.090	0.012
k_2[3]	0.134	0.170
BAFK[4]	0.67	0.070
$t_{1/2}$ (days)[5]	5.2	4.1
t_{90} (days)[6]	17.2	13.6

[1] BAF = Bioaccumulation Factor
[2] k_1 = Kinetic Uptake Rate
[3] k_2 = Depuration Rate
[4] BAFK based on Whole Tissue Concentrations (BAFK = Kinetic Bioaccumulation Factor)
[5] $t_{1/2}$ = Calculated time for 50% clearance of D6 in tissue
[6] t_{90} = Calculated time to reach 90% of steady-state in tissues.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Silicones Environmental, Health and Safety Council at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from September 10, 2007 to November 7, 2007. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 570A-112 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the bioaccumulation potential of sediment-incorporated dodecamethylcyclohexasiloxane (D6) in the oligochaete, *Lumbriculus variegatus*. The formulated sediment used in the test had an organic content of approximately 3.6%. The sediment and a 28-day ration of food were dry mixed in the test chambers. Overlying water was added to the test chambers and the test chambers were allowed to settle for approximately 48 hours prior to the addition of the test organisms. The measured endpoints of the test were tissue concentrations after 14 and 28 days of uptake and during depuration.

EXPERIMENTAL DESIGN

The test consisted of a 28-day uptake phase followed by a 30-day depuration phase. During the uptake phase, oligochaetes were exposed to two sub-lethal test concentrations and a negative control. Eight replicate test chambers were maintained in each treatment and control group. Seven replicates were initiated with approximately one gram of tissue in each replicate. One replicate was used for analytical sampling of sediment on Day 0 and did not contain organisms. Each test chamber contained a quantity of sediment and overlying water.

Nominal test concentrations selected in consultation with the sponsor were 100 and 1000 mg a.i./Kg of sediment based on the dry weight of the sediment. The results of the study are based on the mean measured test concentrations. Sediment samples were collected and analyzed from one sacrificed replicate of each treatment group and control on days 0, 14 and 28 of the uptake phase. Results of the analyses were used to verify the exposure over time. Tissue samples were collected from the culture on Day 0 and from one sacrificed replicate from each treatment group and control on days 14 and 28 of the uptake phase and day 14 of the depuration phase. An additional replicate was sacrificed at the end of the depuration phase from each treatment group and control for the determination of lipid content in the

tissue. The tissue concentrations were used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioaccumulation factor (BAFK).

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et. Al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (1); the ASTM E 1706-00 Guideline: *Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (2); the OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (3); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (4), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (5).

Test Substance

The test substance was received from Dow Corning on June 22, 2007 and assigned Wildlife International identification number 8159. The test substance, a liquid, was identified as: Dow Corning® 246 Fluid; CAS Number 540-97-6; Lot No. LL114030. The reported purity was 99.54%, with an expiration date of June 8, 2009. The test substance was stored under ambient conditions.

Internal Standard

The internal standard was received from Sigma-Aldrich on March 30, 2006 and was assigned Wildlife International, Ltd. identification number 7566 upon receipt. The standard, described as a liquid, was identified on the label as: Tetrakis (trimethylsilyloxy) silane; 97%; Lot #: 13222MB; CAS #: 3555-47-3. The standard had a reported purity of 97%. The standard was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guidelines 218 (6) was used. The sediment was composed of approximately 10% peat moss, 70% sand and 20% silt and clay (kaolin clay). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the

peat, since the peat was added later (Appendix 2). The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry constituents of the final mixture were stored under ambient conditions until used. The results of periodic analyses performed to measure the concentrations of selected contaminants in the formulated sediment used by Wildlife International, Ltd. are presented in Appendix 3. Samples of the sediment used in the test were sent to Agvise Laboratories, Northwood, ND and summaries of the sediment characterizations are found in Appendix 8. The percent organic carbon of the sediment used during the uptake phase and depuration phase was found to be 3.6 and 3.3%, respectively.

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 measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The study was conducted in a temperature-controlled room. Each test chamber was a 9-L glass aquaria (approximately 15 x 30 x 20 cm high) holding 5 liters of water and 1 liter of sediment. Aquaria were arranged in groups of four according to treatment group.

A continuous-flow diluter was used to provide dilution water (well water) to each aquaria so that there were two volume additions per day. The flow of dilution water was controlled using rotameters. The rotameters were calibrated prior to the start of the test and approximately weekly during the test. The diluter system delivered dilution water to a mixing chamber from which one line was split into four separate supply lines that delivered water to each aquaria in the group. The proportion of water split to each replicate was checked prior to the test and approximately weekly

during the test to ensure that flow rates varied by no more that 10% of the mean flow rate of the replicates. The general operation of the diluter was checked visually at least two times per day during the test and at least one time at the beginning and end of the test.

Preparation of Test Concentrations

Concentrations of the test substance were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment). Nominal test concentrations were 100 and 1000 mg a.i./Kg. An aliquot of 159.0 g of peat (37% moisture content) was weighed into 24 Nalgene bottles. Eight bottles were used to prepare the 100 mg a.i./Kg treatment level and eight were used to prepare the 1000 mg a.i./Kg treatment level. The remaining eight bottles were used to prepare the negative control. Neat test material was then added to the peat and mixed overnight on a rotary mixer. After approximately 16 hours of mixing the treatment groups were completely processed one at a time by adding 900.0 g of the formulated sediment to each of the 24 Nalgene bottles (eight at a time) and mixed on the rotary mixer for approximately 40 minutes each.

Eight replicate test chambers were prepared for each treatment and control group for use in the uptake phase. For each replicate, 1000 mL of sediment was placed in a 9-L glass aquaria. Next, a pre-weighed amount of food (Salmon Starter), enough for 28 days (200 mg), was added to each chamber that would contain organisms during the test and was mixed into the dry sediment using a glass stir rod. Finally, approximately 5000 mL of overlying water (well water) was poured into the chamber over a baffle to minimize the disturbance to the sediment. Test chambers were indiscriminately positioned in an environmentally controlled room in groups of four according to treatment group approximately 51 hours prior to test initiation to condition the sediment prior to introduction of organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Three replicate test chambers were prepared for each treatment and control group for use in the depuration phase. For each replicate, 1000 mL of untreated sediment was placed in 9-L glass The untreated sediment used in the depuration phase of the study was prepared similarly to the treated sediment used in the uptake phase of the study, however 286.0 g of peat (with a 65% moisture content) were mixed with 900.0g of the sand/clay mixture. The reason the weight of peat added to the mixture changed between the sediment prepared for the uptake phase and the sediment prepared for the depuration phase is due to the fact that the moisture content of the peat was different. When corrected

for the percent moisture, the same amount of peat was added to each mixture. The percent organic carbon for the sediment used in the uptake phase and in the depuration phase was 3.6 and 3.3% respectively, showing that the final mixtures were comparable

A pre-weighed aliquot of salmon starter was mixed into the dry sediment prior to adding the 5000 mL of overlying water. The chambers were allowed to settle in the environmental chamber approximately two days prior to transferring the test organisms from the uptake test chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Aquatic Research Organisms, Hampton, New Hampshire. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 14-day holding period immediately preceding the test, water temperature ranged from 21.6 to 23.9°C, while the pH ranged from 8.3 to 8.5 and dissolved oxygen ranged from 6.5 to 8.0 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, approximately one gram (wet weight) of oligochaetes was impartially distributed to transfer chambers. The transfer chambers were indiscriminately assigned to test chambers and the organisms were transferred to the test chambers below the air/water interface. Observations of mortality and abnormal behavior were made daily during the test. Seven replicate test chambers in each treatment and control group contained organisms, while one test chamber per group was maintained without organisms for day 0 analytical purposes.

Oligochaetes were fed a mixture of yeast, cereal grass and trout chow (YCT) during the holding period and salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the test. A 200 mg aliquot of food was weighed for each test chamber that contained organisms during the test and was added to the test chamber after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. A 200 mg aliquot of salmon starter was added to the test

chambers prepared for the depuration phase of the test after adding the sediment, but prior to adding the overlying water and organisms.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 193 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test chamber daily during the test using a hand-held liquid-in-glass thermometer. Temperature also was measured continuously in a beaker of water adjacent to the test chambers using a Fulscope ER/C Recorder. The continuous recorder was calibrated with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a sample of overlying water collected from one alternating replicate test chamber from each treatment and control group at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (7). Specific conductance was measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test chambers at the beginning of the test, an additional 1 gram of organisms were impartially selected at the beginning of the test and measured for dry weight. The weight of the subset of organisms was used to determine the ratio of organic carbon in the sediment to the dry weight of organisms. The test chambers were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). The test was divided into two phases: uptake and depuration. The duration of the uptake phase was 28 days, and the depuration phase was 30 days. On Day 28 of the uptake phase, the organisms from three replicates of each treatment group were sieved from the sediment, enumerated and transferred to new test chambers containing untreated sediment and clean water for the duration of the test. At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group was measured.

Statistical Analyses

The equations used for BAF (bioaccumulation factor) and BAFK (kinetic bioaccumulation factor) calculations are described in draft OPPTS 850.1730 Guidance Document (8). The kinetic uptake rate (k_1) and depuration rate (k_2) were calculated for oligochaete tissue and then used to calculate a kinetic bioaccumulation factor (BAFK = k_1 / k_2). These rate constants were also used to calculate both the half-life for clearance in tissue ($t_{½}$) and time to reach 90% of steady-state (t_{90}).

Analytical Sampling

Sediment samples were collected from one test chamber of each treatment group at the beginning of the test, on Day 14 and Day 28 of the uptake phase. The replicate used for sample collection on Day 0 did not contain oligochaetes, whereas at test initiation oligochaetes were placed in the replicates used for sample collection on Days 14 and 28. The samples were analyzed in duplicate for the control and in triplicate for the treatment groups on the day of collection. Tissue samples were collected on Day 0 from the culture, on Days 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Tissue samples were also collected on Day 30 of the depuration phase from each treatment group for the determination of lipid content. The depuration phase was continued past Day 14 to Day 30 so that the analytical results of tissue concentrations could be evaluated. Tissue samples were collected by sieving the sediment to remove organisms. The organisms were rinsed free of as much sediment as possible and placed in a tared liquid scintillation

vial. The water was removed from the vial (as much as possible) and a wet weight was obtained. Tissue samples were stored frozen for 7 to 91 days until analysis.

Analytical Method

The analytical methods used for the analysis of D6 was developed by Wildlife International, Ltd. Sediment was sampled through overlying water from the test tanks and approximately 5 mL of sediment was added to each centrifuge tube. To each sample, 10.0 mL of HPLC grade bottled water and 5 or 50 µL of internal standard was added. Samples were vortexed and shaken after the addition of approximately 20 mL of ethyl acetate and the samples were centrifuged for approximately five minutes at 60% speed. The top layer was transferred into a 50 mL volumetric flask containing 2.5 g of sodium sulfate. Samples were extracted with an additional 20 mL of ethyl acetate. The ethyl acetate extracts were combined and the flask was filled to volume. The extracts (1.00 mL) were transferred into autosampler vials and 50.0 µL N-methyl, N-trimethylsillyl trifluoracetamide was added to each vial. Samples were analyzed by gas chromatography equipped with flame ionization detection. For the tissue analysis, the method consisted of extracting the samples with tetrahydrofuran (THF). Samples were analyzed by gas chromatography equipped with mass selective detection or by gas chromatography equipped with flame ionization detection.

Concentrations of D6 in the tissue samples were determined using an Agilent 6890 Gas Chromatograph (GC) equipped with an Agilent Model 5975 Mass Selective Detector operated in SIM mode or equipped with Flame Ionization Detection. Chromatographic separations were achieved using a Phenomen ZB-5 Column (0.25 mm x 30 m, 0.25 µm). Concentrations of D6 in the sediment samples were determined using an Agilent 6890 Gas Chromatograph (GC) equipped with Flame Ionization Detection (FID). Chromatographic separations were achieved using a J&W DB-5MS Column (0.25 mm x 30 m, 0.25 µm film thickness). Method flowcharts for the analyses of D6 in sediment and tissue are provided in Appendices 6.1 and 6.2, respectively. The instrumental parameters for the analyses are provided in Appendix 6.4 and 6.5, respectively.

For the sediment analyses, calibration standards of D6 test substance, ranging in concentration from 1.00 to 70.0 mg/L were prepared in ethyl acetate using a stock solution of D6 test substance in 80% dimethylformamide (DMF) : 20% toluene. Each standard was fortified with 50 µL of the internal standard prior to final volume (Appendix 6.6). For the tissue analyses, calibration standards of D6 test

substance, ranging in concentration from 0.0500 to 0.500, 0.100 to 0.800, 0.700 to 7.00 and 1.00 to 70.0 mg a.i./L were prepared using stock solutions of D6 test substance in tetrahydrofuran. Each standard was fortified with 25.0 or 100 µL of the internal standard prior to final volume (Appendix 6.6). Calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the ratio of peak area responses (test substance/internal standard) versus the respective concentrations of the calibration standards. The concentration of D6 in the samples was determined by substituting the ratio of peak area responses (test substance/internal standard) of the samples into the applicable linear regression equation. An example of the calculations for a representative sample is included in Appendix 6.7.

For sediment and tissue, the method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was calculated as the product of the low calibration standard (1.00 mg a.i./L for sediment and 0.0500 mg a.i./L for tissue) and the dilution factor of the negative control samples. Each control sample dilution factor was determined as the quotient of the final volume and the initial sample dry mass; the initial dry mass was different for each sample.

Sediment samples were fortified at 100 and 500 mg a.i./Kg using stock solutions of D6 in 80% DMF : 20% toluene (Appendix 6.6), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 89.8 to 95.8% of nominal concentrations (Appendix 6.8).

Tissue samples were fortified at 2.50, 25.0 and 250 mg a.i./Kg using stock solutions of D6 in tetrahydrofuran (Appendix 6.6), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 89.3 to 122% of nominal concentrations (Appendix 6.9).

Lipid Analyses

Vials containing tissue samples for lipid analysis were removed from the freezer and allowed to thaw. Tare weights of the vials had been pre-determined. Bottled water (10 mL) was added to each sample and samples were homogenized for approximately one minute using a hand-held tissue homogenizer. The homogenizer was rinsed with the appropriate solvent(s) in between samples. Each homogenate was transferred to a 250-mL separatory funnel containing 25 mL of chloroform and 50

mL of methanol. Each vial was rinsed with an additional 10 mL of bottled water and the rinse was poured into its respective separatory funnel. The funnels were then shaken with venting for approximately one minute, and 50 mL of chloroform followed by 50 mL of saturated sodium chloride was added to each separatory funnel. Funnels were swirled briefly with venting and the phases were allowed to separate. For each sample, the chloroform layer was drained through a powder funnel packed with Teflon® wool and anhydrous sodium sulfate into a 250-mL roundbottom flask. An additional 50-mL aliquot of chloroform was added to each separatory funnel and the extraction and draining procedures were repeated. The extracts were rotary-evaporated to near dryness in a waterbath maintained at approximately 40°C. Each sample was then transferred to a pre-weighed, labeled scintillation vial. Each 250-mL roundbottom flask was rinsed with a small volume of chloroform and the rinse was transferred to its respective scintillation vial. The remaining solvent in each vial was evaporated under a gentle stream of nitrogen or clean dry air. Each vial was reweighed and the weight recorded. The lipid content was calculated from the difference in the vial tare weight and the vial weight containing the lipid. Percent lipids were calculated from the ratio of lipid content to total tissue sample weight. Lipid content determinations are presented in Table 4, and a method flowchart is presented in Appendix 6.3.

A representative calibration curve for sediment is presented in Appendix 6.10. Representative chromatograms of low and high-level calibration standards for sediment are presented in Appendices 6.11 and 6.12, respectively. A representative chromatogram of a sediment matrix fortification sample is presented in Appendix 6.13. A representative chromatogram of a sediment test sample is presented in Appendix 6.14.

A representative calibration curve for tissue is presented in Appendix 6.15. Representative chromatograms of low and high-level calibration standards for tissue are presented in Appendices 6.16 and 6.17, respectively. A representative chromatogram of a tissue matrix fortification sample is presented in Appendix 6.18. A representative chromatogram of a tissue test sample is presented in Appendix 6.19.

RESULTS AND DISCUSSION

Measurement of Test Concentrations in Sediment

Results of analyses to measure concentrations of D6 in samples collected during the definitive test are presented in Tables 1-4 and Appendix 6. Nominal concentrations selected for use in this definitive test were 100 and 1000 mg a.i./Kg.

Samples of sediment were collected from the negative control and each of the test concentrations on Day 0, Day 14 and Day 28. The purpose of these samples was to verify the concentrations in sediment at test initiation and to monitor the test substance in the sediment over the course of the study. Results are based on mean measured concentrations. Negative control samples on Days 0, 14, and 28 were all below the limit of quantitation (LOQ).

Mean measured concentrations of D6 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 0 were 33.2 and 624 mg a.i./Kg, respectively (Table 1). Mean measured concentrations of D6 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 14 were 33.9 and 569 mg a.i./Kg, respectively. Mean measured concentrations of D6 in sediment from the 100 and 1000 mg a.i./Kg nominal test concentrations on Day 28 were 17.3 and 258 mg a.i./Kg, respectively. All concentrations showed a low but constant trend in the sediment throughout the study. The overall mean measured concentrations for the 100 and 1000 mg a.i./Kg nominal treatment groups were 28.1 and 484 mg a.i./Kg, or 28 and 48% of nominal, respectively.

Measurement of D6 in Tissue

Tissue samples were collected from the negative control and each of the test concentrations on Day 14 and 28 of the uptake phase and on Day 14 of the depuration phase. Negative control samples were below the limit of quantitation (LOQ) at each sampling interval.

The concentrations of D6 in tissues of oligochaetes exposed to 28.1 mg a.i./Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 1. Mean measured concentrations of D6 in tissues of oligochaetes from the 28.1 mg a.i./Kg nominal test concentration on Days 14 and 28 were 17.8 and 18.5 mg a.i./Kg, respectively. The BAF and BAFK values for oligochaete tissue were 0.66 and 0.67 respectively (Table 5). The estimated

time to reach 90% of steady state (t_{90}) in oligochaete tissue was 17.2 days (Table 5). The concentration of D6 depurated quickly in oligochaete tissue and the mean measured concentration of D6 in oligochaete tissue for the 28.1 mg a.i./Kg treatment group by Day 14 of depuration was 2.85 mg a.i./Kg, which is approximately 15% of the mean tissue concentration at Day 28 of uptake (Figure 1). The estimated time to reach 50% clearance ($t_{1/2}$) for oligochaete tissue was 5.2 days (Table 5).

The concentrations of D6 in tissues of oligochaetes exposed to 484 mg a.i./Kg during the uptake and depuration phases of the study are presented in Tables 2 and 3, respectively. A graph of the mean measured tissue concentrations for oligochaetes from each sampling day is presented in Figure 2. Mean measured concentrations of D6 in tissues of oligochaetes from the 484 mg a.i./Kg nominal test concentration on Days 14 and 28 were 66.2 and 33.7 mg a.i./Kg. The BAF and BAFK values for oligochaete tissue were 0.070 and 0.070, respectively (Table 5). The calculation to estimate time to reach 90% of steady state in oligochaete tissue was 13.6 days (Table 5). As in the 28.1 mg a.i./Kg treatment group, the concentration of D6 depurated quickly in oligochaete tissue and the mean measured concentration of D6 in oligochaete tissue by Day 14 of depuration was 3.13 mg a.i./kg, which is approximately 9.3% of the mean tissue concentration at Day 28 of uptake (Figure 2). Estimate of time to reach 50% clearance for oligochaete tissue was 4.1 days (Table 5).

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 6 through 11. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 84% (7.1 mg/L) of saturation throughout the test. Measurements of pH ranged from 8.0 to 8.3 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 12 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatments and control group and no treatment-related signs of toxicity. At test termination, there was an increase in the numbers of oligochaetes present in the test chambers from the number of worms originally placed in each replicate, indicating that reproduction had occurred.

At the beginning of the test, a one-gram (wet weight) aliquot of organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the dry weight of those organisms was 0.0617 g. It was determined that the formulated sediment had an organic content of 3.6%; therefore, the ratio of organic carbon to the dry weight of organisms was 583 g organic carbon : 1 g of dry tissue.

CONCLUSIONS

A kinetic BAF value for D6 in oligochaetes (*Lumbriculus variegatus*) was calculated for both the 28.1 and 484 mg a.i./Kg treatment groups. The BAF and BAFK values for oligochaete tissue in the 28.1 mg a.i./Kg treatment group were 0.66 and 0.67, respectively. The BAF and BAFK values for oligochaete tissue in the 484 mg a.i./Kg treatment group were 0.070 and 0.070, respectively. During depuration, D6 was eliminated quickly and the Day 14 depuration tissue concentrations for the two treatment groups were 9.3 - 15% of the Day 28 uptake tissue concentrations. The estimated time to reach 50% clearance for oligochaete tissue concentrations was 5.2 days for the 28.1 mg a.i./Kg treatment group, and 4.1 days for oligochaete tissue for the 484 mg a.i./Kg treatment group.

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Table 1

Measured Concentrations of D6 in Sediment Samples

Nominal Test Concentration (mg a.i./Kg)	Sample Number (570A-112-)	Sampling Time (Day)	Measured Concentration (mg a.i./Kg)	Percent of Nominal[1]	Overall Mean Measured Concentration (mg a.i./Kg)[1]	Mean Percent of Nominal[1]
Negative Control (0.0)	1A	0	< 10.5	--	--	--
	1B	0	< 9.16	--		
	4A	14	< 8.34	--		
	4B	14	< 8.36	--		
	7A	28	< 9.91	--		
	7B	28	< 10.0	--		
100	2A	0	31.7	31.7	28.1	28
	2B	0	28.2	28.2		
	2C	0	39.6	39.6		
	5A	14	27.1	27.1		
	5B	14	33.2	33.2		
	5C	14	41.3	41.3		
	8A	28	17.8	17.8		
	8B	28	19.6	19.6		
	8C	28	14.5	14.5		
1000	3A	0	668	66.8	484	48
	3B	0	558	55.8		
	3C	0	645	64.5		
	6A	14	412	41.2		
	6B	14	592	59.2		
	6C	14	702	70.2		
	9A	28	314	31.4		
	9B	28	274	27.4		
	9C	28	187	18.7		

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

Table 2

Measured Concentrations of D6 in Tissue Samples

Nominal Test Concentration (mg a.i./Kg)	Sample Number (570A-112-)	Sampling Time (Day)	Measured Concentration (mg a.i./Kg)
Negative Control (0.0)	T1	0	< 0.423
	T2	14	< 0.515
	T5	28	< 0.820
100	T3*	14	17.8
	T6*	28	18.5
1000	T4*	14	66.2
	T7*	28	33.7

* Sample reanalyzed.

Table 3

Measured Concentrations of D6 in Tissue Samples During the Depuration Phase

Nominal Test Concentration (mg a.i./Kg)	Sample Number (570A-112-)	Sampling Time (Day)	Measured Concentration (mg a.i./Kg)
Negative Control (0.0)	T8	14	< 0.457
100	T9*	14	2.85
1000	T10*	14	3.13

* Samples reanalyzed.

Table 4

Tissue Lipid Content

Sample I.D. (570A-112-)	Tissue Weight (grams)	Lipid Weight (grams)	Percent Lipids (%)
L-1	0.7085	0.0174	2.46
L-2	0.7994	0.0173	2.16
L-3	0.7205	0.0228	3.16
		Mean lipid percentage	2.59

Table 5

BAF Values

Mean Measured Test Concentration (mg a.i./Kg)	BAF[1]	BAFK[1]	k_1	k_2
28.1	0.66	0.67	0.090	0.134
484	0.070	0.070	0.012	0.170

[1] Equations for BAF, BAFK, k_1 and k_2:

BAF = (Day 28 tissue conc) ÷ (mean sediment conc)

BAFK = (k_1) ÷ (k_2)

k_1 = (k_2 x Day 28 uptake tissue concentration) ÷ (mean sediment conc × (1 − e^(−k_2t)))

k_2 = (ln(Day28uptake tissue conc) − ln(Day 14 depuration tissue conc)) ÷ (14 days of depuration)

Estimated Time to Reach 50 and 90% of Steady State

Mean Measured Test Concentration (mg a.i./Kg)	$t_{1/2}$ (days)	t_{90} (days)
28.1	5.2	17.2
484	4.1	13.6

Table 6

Temperature of Overlying Water in the Test Chambers During the Uptake Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)														
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	F	G	A
Negative Control		22.6	22.4	22.4	22.7	22.8	22.6	22.3	23.1	23.0	22.8	22.6	22.7	22.7	22.7	22.4
28.1		22.3	22.1	22.0	22.3	22.7	22.7	22.8	22.3	22.4	22.5	22.4	22.8	22.9	22.8	22.2
484		22.4	22.3	22.2	22.7	22.3	22.7	22.8	23.0	22.8	22.8	22.7	22.7	22.8	22.8	22.4

[1] Temperature measured continuously in a beaker of water adjacent to the test chambers ranged from approximately 22.0 to 23.0°C during the test.

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)													
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Negative Control		22.8	22.8	22.8	22.8	22.8	23.0	22.7	22.7	22.6	22.9	22.9	22..9	22.9	22.8
28.1		22.5	22.6	22.9	22.8	22.6	22.8	22.5	22.5	22.9	22.7	22.6	22.9	23.0	22.9
484		22.6	22.7	22.8	22.8	22.7	22.6	22.6	22.7	22.8	22.8	22.8	22.9	23.0	22.8

Table 7

Temperature of Overlying Water in the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control		22.8	22.8	22.5	21.9	22.3	22.9	22.5	22.7	22.9	22.9	22.9	22.9	22.6	22.5
28.1		23.0	22.9	22.8	22.3	22.6	23.3	22.9	23.0	23.2	23.2	23.2	23.1	22.9	22.9
484		22.8	22.8	22.8	22.2	22.4	23.1	22.9	22.9	23.1	23.1	23.1	23.0	22.8	22.7

Mean Measured Test Concentration (mg a.i./Kg)	Day	Temperature (°C)															
		15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	Replicate:	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Negative Control		22.6	22.6	22.4	22.6	22.7	22.3	22.0	22.5	22.4	22.9	22.4	22.3	22.5	22.1	22.5	22.0
28.1		23.0	23.0	22.8	22.8	23.0	22.7	22.3	23.1	22.9	23.3	22.9	22.6	22.8	22.6	23.0	22.4
484		23.0	23.0	22.9	22.8	23.0	22.6	22.3	23.0	23.0	23.3	22.9	22.6	22.7	22.4	22.7	22.3

Table 8

Dissolved Oxygen in Overlying Water in the Test Chambers During the Uptake Phase

Mean Measured Test Concentration (mg a.i./Kg)	Day	0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Replicate:	A	B	C	D	E	F	G	A	B	C	D	E	F	G	A
		Dissolved Oxygen (mg/L)														
Negative Control		8.0	7.8	7.8	7.8	7.6	7.8	7.7	7.6	7.7	7.8	7.7	7.3	8.0	8.1	8.0
28.1		8.0	7.9	7.8	7.9	7.5	7.4	7.8	7.6	7.6	7.8	7.7	7.8	7.7	8.1	8.0
484		7.8	7.9	7.6	7.5	7.6	7.5	7.7	7.5	7.6	7.7	7.8	7.5	7.8	8.1	7.9

[1] A dissolved oxygen concentration of 5.1mg/L represents 60% saturation at 23°C.

Mean Measured Test Concentration (mg a.i./Kg)	Day	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	B	C	D	E	A	B	C	D	E	A	B	C	D	E
		Dissolved Oxygen (mg/L)													
Negative Control		7.6	7.9	7.7	7.8	8.0	8.3	8.1	7.8	7.8	8.0	7.6	7.8	8.0	7.9
28.1		7.8	7.9	7.7	7.9	7.9	8.5	8.1	7.9	7.9	7.9	7.7	7.8	7.9	8.0
484		7.9	7.7	7.8	7.6	7.9	8.1	7.8	8.0	7.8	8.0	7.4	7.9	8.0	8.0

Table 9

Dissolved Oxygen in the Overlying Water of the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	Dissolved Oxygen (mg/L)													
Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Replicate:	A	B	C	A	B	C	A	B	C	A	B	C	A	B
Negative Control	7.9	7.1	7.6	7.5	7.4	7.5	7.7	7.8	7.3	7.7	7.9	8.2	7.9	7.6
28.1	7.6	7.3	7.5	7.4	7.5	7.5	7.6	7.1	7.6	8.0	7.2	7.8	8.0	7.9
484	7.6	7.3	7.6	7.4	7.3	7.7	7.2	7.2	7.5	7.5	7.7	8.1	7.8	7.5

Mean Measured Test Concentration (mg a.i./Kg)	Dissolved Oxygen (mg/L)															
Day	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Replicate:	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Negative Control	7.5	7.7	7.6	8.0	8.2	8.1	8.2	8.0	7.7	7.9	7.5	8.2	8.0	7.7	7.8	7.9
28.1	7.8	7.7	7.9	7.9	8.1	8.0	8.2	8.1	8.1	7.9	7.9	7.9	8.0	7.5	7.8	7.9
484	7.6	7.3	7.8	7.7	8.0	7.9	8.2	8.0	7.8	7.8	7.7	8.1	7.9	7.6	7.5	7.9

Table 10

pH of Overlying Water in the Test Chambers During the Uptake Phase

Mean Measured Test Concentration (mg a.i./Kg)	Replicate:	pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
		A	B	C	D	E
Negative Control		8.1	8.3	8.2	8.2	8.3
28.1		8.0	8.3	8.3	8.2	8.3
484		8.0	8.3	8.2	8.2	8.3

Table 11

pH of Overlying Water in the Test Chambers During the Depuration Phase

Mean Measured Test Concentration (mg a.i./Kg)	Replicate:	pH				
		Day 7	Day 14	Day 21	Day 28	Day 30
		A	B	A	B	A
Negative Control		8.2	8.2	8.2	8.3	8.3
28.1		8.2	8.3	8.2	8.3	8.3
484		8.2	8.2	8.2	8.3	8.3

Table 12

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Chambers During the Test

Mean Measured Test Concentration (mg a.i./Kg)	Day 0 (Uptake)				Day 30 (Depuration)			
	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (µmhos/cm)	Ammonia (mg/L)
Negative Control	128	182	340	<0.17[1]	140	182	320	<0.17
28.1	140	182	340	<0.17	140	182	335	<0.17
484	140	182	340	<0.17	140	184	335	<0.17
Replicate	A				B			

[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Figure 1.

Concentrations of D6 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 28.1 mg a.i./Kg

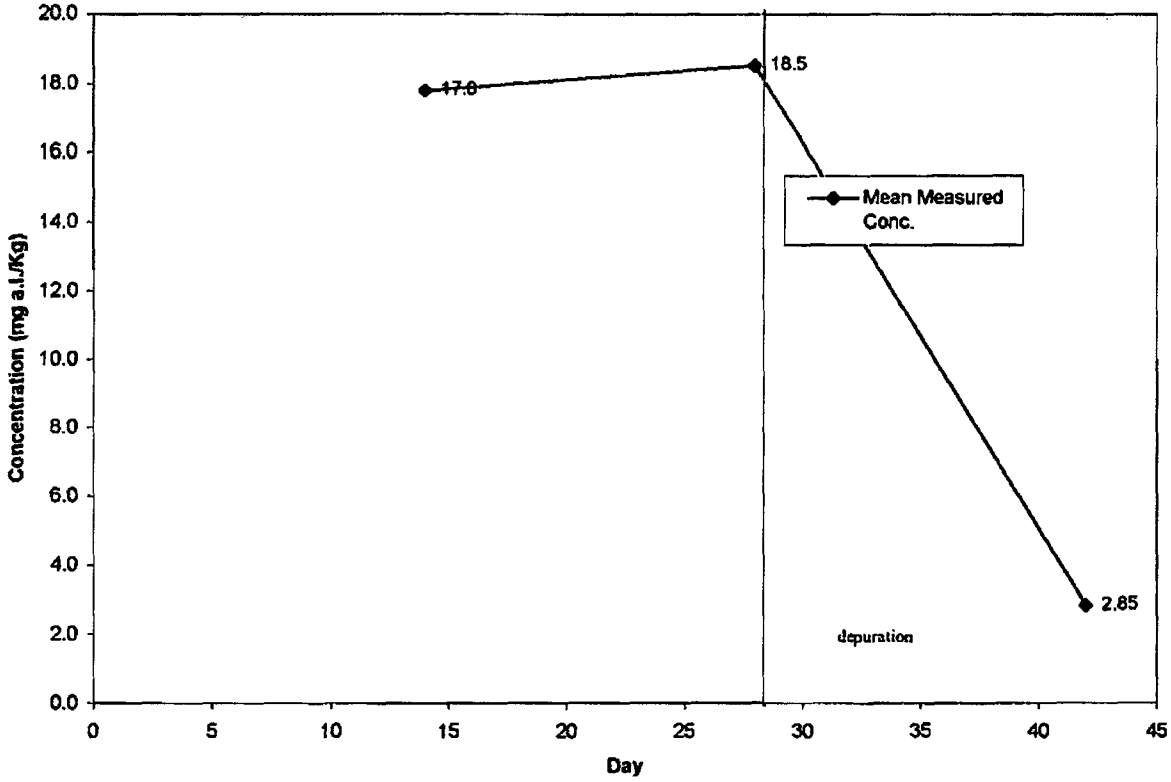
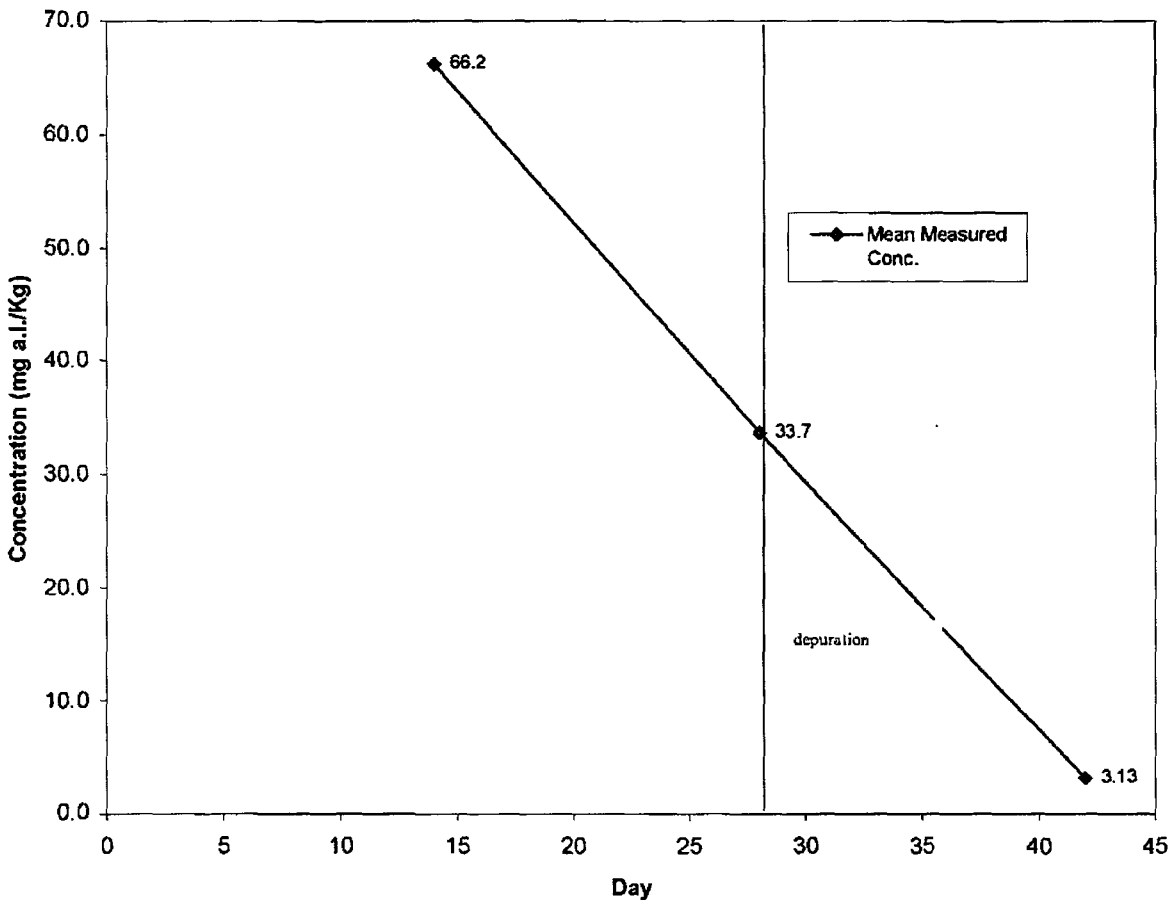


Figure 2.

Concentrations of D6 Based on Measured Concentrations in Tissues of Oligochaetes Exposed to 484 mg a.i./Kg



Appendix 1

Study Protocol, Amendments and Deviation

PROTOCOL

DODECAMETHYLCYCLOHEXASILOXANE (D_6): A BIOACCUMULATION TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

ASTM Standard E 1706-00

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

EU Technical Guidance Document on Risk Assessment

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
570A-112

SPONSOR

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

PERFORMING LABORATORY

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1 (410) 822-8600

July 6, 2007

Wildlife International, Ltd.

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DODECAMETHYLCYCLOHEXASILOXANE (D_6): A BIOACCUMULATION TEST
WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

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LABORATORY MANAGEMENT: Willard B. Nixon, Ph. D.
Director of Chemistry

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

STUDY DIRECTOR — DATE 7/13/07

LABORATORY MANAGEMENT — DATE 7/13/07

SPONSOR'S REPRESENTATIVE — DATE 7/6/07

PROTOCOL NO.: 570/070607/LUM-BIOb/SUB570

Wildlife International, Ltd.

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FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: _____ Experimental Termination Date: _____

Project No.: 570A-112

Test Concentrations: _____

Test Substance No.: _____ Reference Substance No. (if applicable): _____

INTRODUCTION

Wildlife International, Ltd. will conduct a bioaccumulation test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1); the U.S. EPA 600/R-99/064 *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.) (2), the ASTM Standard E 1706-00: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (5), and the Federal Environment Agency: Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (6). Raw data for all work performed at Wildlife International, Ltd. and 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. A copy of the final report will be archived at Dow Corning.

OBJECTIVE

The objective of this study is to determine the bioaccumulation potential of a sediment-incorporated test substance in the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions followed by a depuration period of at least 14 days in untreated sediment. The sediment used in the test will have an organic content of approximately 5%. A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after the sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding organisms. The measured endpoints of the test are tissue concentrations after 14 and 28 days of uptake and during depuration

EXPERIMENTAL DESIGN

Oligochaetes will be exposed to two test concentrations (100 and 1000 mg a.i./kg) and a negative control. Each group will consist of eight replicate test chambers. Seven replicates will contain up to approximately one gram of tissue (approximately 300 organisms) in each chamber. One

replicate will be used for analytical sampling of sediment on Day 0. The test will be divided into two phases: uptake and depuration. During the uptake phase, oligochaetes in the treatment groups will be exposed to sublethal concentrations of sediment incorporated D-5, while oligochaetes in the control group will be exposed only to untreated sediment. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Days 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Each test chamber will contain a quantity of sediment and overlying water. Only sediment will be analyzed in test systems, since the water solubility of D6 is 5.13 µg/L (7). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of D6 in water. Therefore, only sediment samples from the analytical sampling test chambers will be collected from each control and treatment group at specified intervals for analysis of the test substance. No oligochaetes will be placed in "analytical" replicate(s) sampled at the beginning of the test. However, the "analytical" replicate(s) sampled after the beginning of the test (e.g., on Day 14 and 28) will contain oligochaetes. Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be 100 and 1000 mg a.i./kg. Results of the analyses will be used to verify the exposure over time.

To control bias, oligochaetes will be impartially assigned to exposure chambers at test initiation. No other potential sources of bias are expected to affect the results of the study.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to

GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	Dodecamethylcyclohexasiloxane (D6) (Supplied as Dow Corning® 246 Fluid)
Lot No.:	LL114030
Expiration Date:	June 8, 2009
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	540-97-6
Physical Description:	Colorless liquid
Chemical Stability:	Stable
Purity:	99.54 ± 0.007 area percent purity
Solubility:	Soluble in common organic solvents such as acetone and dimethylsulfoxide
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained for this study

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1.5 kg batch of sediment to 5%. The peat and test substance will be mixed overnight on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data. Approximately 1- 2 L of the dry mix will then be transferred to each of eight test chambers. The same volume of sediment will be added to each chamber. The transfer time will be kept to a

minimum (approximately 10-20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance.

After all 8 chambers have been filled with sediment, a 28 day ration of food (approximately 200 mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 5 L of well water will be added to the chambers. Test chambers will be maintained in an environmentally controlled room. Dilution water will be delivered to each test chamber at a rate that will result in two turnovers of dilution water per day. After approximately 48 hours of acclimation and settling, organisms will be added to the chambers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study. Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd. Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Organisms will be fed YCT during holding. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test. Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (8) will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon.

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report.

Test Apparatus

The study will be conducted in a temperature-controlled room. Each test chamber will be a 9-L aquarium (approximately 15 X 30 X 20 cm high) holding approximately 5 liters of water. Each aquarium will be filled with the same amount of sediment (1 to 2 L). Aquaria will be set out in groups of four.

A continuous-flow diluter will be used to provide dilution water (well water) to each aquaria so that there are two volume additions per day. The flow of dilution water will be controlled using rotameters. The rotameters will be calibrated prior to test initiation and at approximately weekly intervals thereafter. The diluter system delivers dilution water to a mixing chamber from which one line is split into four separate supply lines that deliver water to each aquaria in a group. The proportion of water split to each replicate will be checked prior to the test and at approximately weekly intervals thereafter to ensure that these flow rates vary by no more than ±10% of the mean flow rate of the replicates. The general operation of the diluter will be checked visually at least two times per day during the test and at least once at the beginning and end of the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured daily using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured daily in the overlying water from one alternating replicate of each experimental group. Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week.

Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a sample of the overlying water from one replicate of each experimental group at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (9). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, approximately 1 g (approximately 300) adult oligochaetes will be impartially distributed to transfer containers (e.g., glass beakers) until each container holds its complement of approximately 1 g. The organisms in each transfer container then will be transferred below the air/water interface to the test chambers (glass aquaria). An additional batch of individuals (~1 g) will be impartially selected at the beginning of the test and will be measured for dry weight. The weight of the subset of organisms will be used to determine the ration of organic carbon in the sediment to the dry weight of organisms. After completing the transfer of organisms, the test chambers will be maintained in a temperature-controlled room. Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming). At test termination, the total wet weight of all individuals remaining in one replicate of each treatment group will be measured.

The test will be divided into two phases: uptake and depuration. The duration of the uptake phase will be 28 days and the depuration phase will last at least 14 days. The test may be extended pending the results of the 14-day analysis. Sediment samples will be collected on Day 0, 14 and 28. Tissue samples will be collected from the culture on Day 0 and from test systems on Day 14 and 28 of the uptake phase, and on Day 14 of the depuration phase. Additional samples will be collected during depuration phase if necessary. Tissue concentrations will be used to calculate the uptake rate constant (k_1), the depuration rate constant (k_2), and the kinetic bioconcentration factor (BCF).

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from one test chamber of each concentration at the beginning of the test (no more than one hour after completing the addition of organisms to the test system), at 14 days and at the end of the uptake phase (Day 28). The overlying water will be removed from the test chamber. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below:

Phase	Sample Interval	Sediment[1]	Oligochaetes[2]	Action
Uptake	0 hours	X	X[3]	Organisms added
	14 days	X	X	
	28 days	X	X	Organisms removed And purged
Depuration[4]	14 days		X	

[1] There will be two concentration tested (100 and 1000 mg a.i./kg dry weight). Sufficient sediment will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[2] Approximately 1g of oligochaetes will be collected in each replicate to allow the preparation of three replicate samples from each treatment group and duplicate samples from the control group for the analysis of the test substance.

[3] One sample of approximately 1g of oligochaetes from the culture will be used to determine background levels in oligochaetes.

[4] Additional depuration samples will be added if necessary pending the outcome of the Day 14 samples.

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one or more appropriate test chambers and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

The kinetic uptake rate (k_1) and depuration rate (k_2) will be calculated for oligochaete tissue using the calculations described in draft OPPTS 850.1730 Guidance Document (10). These rate constants will be used to calculate a kinetic bioconcentration factor (BCFK = k_1/k_2) and also to calculate the half-life for clearance in tissue ($t_{½}$), time to reach 90% of steady-state (t_{90}), and BCFK values using the equations and graphical methods outlined in the draft OPPTS 850.1730 Guidance Document.

The BCFK of D6 can be defined as the ratio of the uptake rate constant, k_1 to the elimination (depuration) rate constant, k_2. That is,

$$BCFK = \frac{k_1}{k_2}. \quad \text{(Eq. 1).}$$

This kinetic definition of the BCFK is valid regardless of whether a steady-state concentration of a chemical is achieved during uptake as long as first-order kinetics can be assumed for uptake and elimination processes. Both the uptake rate, k_1, and elimination rate, k_2, of the chemical can be estimated by measuring concentrations of the chemical in water and fish tissues at several points in time.

If elimination follows first order kinetics, the decline in log concentration of the chemical in tissues over time is a straight line. The slope of this line is the elimination rate constant, k_2. Thus, if measurements of chemical concentration in tissues are made at only two time points, the estimate can be obtained using the simple algebraic relationship

$$k_2 = \frac{(\ln C_m - \ln C_t)}{t_e} \quad \text{(Eq. 2).}$$

where C_m is the concentration in fish tissue at the end of the uptake period and C_t is the concentration in the fish tissue after an elimination period of length t_e. Alternatively, k_2 can be estimated as the

slope calculated by linear regression if measurements are made at more than two time points.

Finally, the uptake rate is calculated as

$$k_1 = \frac{k_2 C_{ss}}{C_w(1 - e^{-k_2 t})} \quad \text{(Eq. 3).}$$

where t is the length of the uptake (exposure) period and C_w is the concentration of D6 in the water during the exposure period.

The half-life for clearance in tissue during the depuration phase was determined by the following equation:

$$t_{½} = (1/k_2)\ln 2$$

It is assumed that the two chamber, two-parameter model applies. This means that the time to reach 90% steady-state in the uptake phase is about equal to the time it takes to reach 90% of depuration in the depuration phase. Therefore, k_2, the depuration rate constant, was used in the following equation to determine the time to reach 90% steady-state for uptake:

$$0.9 = 1 - e^{-k_2 t}$$

or

$$t_{90} = 2.3/k_2$$

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Lumbriculus variegatus* culture records.
5. Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations.
6. Biological observations.
7. Test conditions (light intensity, photoperiod, etc.).

8. Water chemistry results (e.g., hardness and alkalinity).
9. Statistical calculations, if applicable.
10. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.

14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates of any findings reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

REFERENCES

1 **Phipps, G.L., G.T. Ankley, D.A. Benoit, and V.R. Mattson.** 1993. Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants. Environ. Toxicol. Chem. 12:269-279.

2 **U.S. Environmental Protection Agency.** 2000. *Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Fresh Water Invertebrates* (2nd ed.). EPA 600/R-99/064

3 **ASTM Standard E 1706-00.** 2000. *Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates.* American Society for Testing and Materials.

4 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater.*

5 **European Chemicals Bureau.** 2003. *Technical Guidance Document on Risk Assessment, Part II.* Institute for Health and Consumer Protection.

6 **Federal Environment Agency.** 2001. *Validation of the Preliminary EU- Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances.* Institute of Hydrobiology Dresden University of Technology.

7 **Varaprath, S., C.L. Frye and J. Hamelink.** 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263 – 1265.

8 **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Organization for Economic Cooperation and Development. Adopted 13 April 2004.

9 **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.

10 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.1730: *Fish BCF.*

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Dodecamethylcyclohexasiloxane (D_6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOb/SUB570 **AMENDMENT NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-112

EFFECTIVE DATE: September 7, 2007

AMENDMENT: Page 3:

ADD:	Proposed Dates:	Experimental Start Date: September 10, 2007 Experimental Termination Date: October 22, 2007
	Test Concentrations:	Negative Control, 100 and 1000 mg a.i./Kg
	Test Substance No.:	8159
	Reference Substance No.:	NA

REASON: This information was not available at the time the protocol was signed by the Study Director.

AMENDMENT: Preparation of Test Concentrations, Page 6:

Sediment will be prepared in 1 kg batches instead of 1.5 kg batches.

REASON: Smaller batches will be prepared for ease of handling and to reduce the amount of sediment left over after preparation of test chambers.

Wildlife International, Ltd.

AMENDMENT: Test Organism, Page 7:

CHANGE: Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test.

TO: Prior to the test, the organisms will be held in a container with ground up paper towels and overlying water from the same source and at approximately the same temperature as will be used in the test.

REASON: Ground up paper towels have been found to be an easier substrate to work with when culturing oligochaetes.

STUDY DIRECTOR — DATE 9/12/07

LABORATORY MANAGEMENT — DATE 9/13/07

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Dodecamethylcyclohexasiloxane (D_6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOb/SUB570 **AMENDMENT NO.:** 2

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-112

EFFECTIVE DATE: September 26, 2007

AMENDMENT: Experimental Design, Page 5:

CHANGE: During the uptake phase, oligochaetes in the treatment groups will be exposed to sublethal concentrations of sediment incorporated D-5, while oligochaetes in the control group will be exposed only to untreated sediment.

TO: During the uptake phase, oligochaetes in the treatment groups will be exposed to sublethal concentrations of sediment incorporated D6, while oligochaetes in the control group will be exposed only to untreated sediment.

REASON: The test material being used in this study is D6, not D-5.

AMENDMENT: Experimental Design, Page 5 and Test Procedures and Biological Measurements, Page 10:

The correct abbreviation for kinetic bioconcentration factor is BCFK.

REASON: To clarify the correct abbreviation for kinetic bioconcentration factor.

AMENDMENT: Test Apparatus, Page 9:

ADD: Test chambers will be labeled with the project number, test concentration and replicate.

REASON: To add that the test chambers would be labeled with the project number, test concentration and replicate.

Wildlife International, Ltd. Project Number 570A-112

AMENDMENT: Data Analysis, Pages 12 and 13:

CHANGE: Fish tissue.

TO: Oligochaete tissue.

REASON: The tissue being analyzed was incorrectly being identified as fish tissue but is actually oligochaete tissue.

AMENDMENT: Final Report, Page 14:

CHANGE: 9. A description of the preparation of the test solutions.

TO: 9. A description of the preparation of the test sediment.

REASON: The test material is being administered through the sediment.

STUDY DIRECTOR — DATE 10/18/02

LABORATORY MANAGEMENT — DATE 10/18/02

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: Dodecamethylcyclohexasiloxane (D6): A Bioaccumulation Test with *Lumbriculus variegates* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOb/SUB570 **AMENDMENT NO.:** 3

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-112

EFFECTIVE DATE: July 13, 2007

AMENDMENT: Page 3:

ADD: Reference Substance No.: 7566

REASON: The above information was not know at the time the study director signed the protocol.

STUDY DIRECTOR — DATE 4/29/08

LABORATORY MANAGEMENT — DATE 4/29/08

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Dodecamethylcyclohexasiloxane (D_6): A Bioaccumulation Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/070607/LUM-BIOb/SUB570 **DEVIATION NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-112

DATE OF DEVIATION: September 8, 2007 and October 6, 2007

DEVIATION: The organic carbon content of the sediment used in the uptake and depuration phases of the study was 3.6 and 3.3%, respectively and not between 4.0 and 6.0% as described in the protocol.

REASON: The sediment was prepared using the recipe and the procedure described in the protocol. The BSAF calculations took into account the measured organic carbon content of the sediment during the uptake phase, therefore, this deviation from the protocol had no adverse impact upon the results or interpretation of the study.

DATE OF DEVIATION: September 24, 2007, October 8, 2007, October 22, 2007

DEVIATION: The approximately 1 gram tissue samples were not analyzed in duplicate for the control group or in triplicate for the treatment groups, but were analyzed as single samples.

REASON: The procedure was changed, in consultation with the sponsor, to analyze the tissue as single samples for each treatment group and control.

Wildlife International, Ltd.

DATE OF DEVIATION: November 5, 2007

DEVIATION: A second check on the operation of the diluter system was not performed on depuration Day 28 of the test (November 5, 2007).

REASON: Biologist oversight. The operation of the diluter system was inadvertently not checked a second time on depuration Day 28 of the test. Diluter checks on depuration Day 29 show the diluter system was working properly. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

STUDY DIRECTOR — DATE 4/27/08

LABORATORY MANAGEMENT — DATE 4/28/08

Appendix 2

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000[2]

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used.

[2] The amount of peat stated above is a theoretical value, based on 10% of the target final weight of the recipe. The peat was added to individual batches, based on the same percentage, and all ingredients were mixed on a rotary mixer.

Appendix 3

Analyses of Pesticides, Organics and Metals in
Wildlife International, Ltd. Formulated Sediment

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.85	Heptachlor	< 0.85
Alpha BHC	< 1.0	Heptachlor Epoxide	< 0.85
Alpha Chlordane	< 0.85	Kepone	< 7.1
Beta BHC	< 2.0	Malathion	< 68
Bolstar	< 68	Merphos	< 68
Chlordane	< 17	Methoxychlor	< 8.5
Coumaphos	< 68	Methyl Parathion	< 68
Delta BHC	< 0.85	Mevinphos	< 68
Demeton-O	< 68	Mirex	< 1.7
Demeton-S	< 68	Naled	< 68
Diazinon	< 68	o,p-DDD	< 1.7
Dichlorvos	< 68	o,p-DDE	< 1.7
Dieldrin	< 1.7	o,p-DDT	< 1.7
Disulfoton	< 77	p,p-DDD	< 1.7
Dursban (Chlorpyrifos)	< 68	p,p-DDE	< 1.7
Endosulfan I	< 0.85	p,p-DDT	< 1.7
Endosulfan II	< 1.7	PCB-1016	< 17
Endosulfan Sulfate	< 1.7	PCB-1221	< 43
Endrin	< 1.7	PCB-1232	< 17
Endrin Aldehyde	< 1.7	PCB-1242	< 23
Endrin Ketone	< 1.7	PCB-1248	< 17
EPN	< 68	PCB-1254	< 17
Ethion	< 68	PCB-1260	< 17
Ethoprop	< 68	Phorate	< 68
Ethyl Parathion	< 68	Ronnel	< 68
Famphur	< 68	Stirophos	< 68
Fensulfothion	< 68	Telodrin	< 1.2
Fenthion	< 68	Tokuthion	< 68
Gamma BHC – Lindane	< 0.85	Toxaphene	< 34
Gamma Chlordane	< 3.1	Trichloronate	< 68
Guthion (Azinphos-methyl)	< 68	Trithion	< 68
HCB	< 0.85		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 3 (Continued)

Analyses of Pesticides, Organics and Metals in
Wildlife International, Ltd. Formulated Sediment

Metals			
Component	Measured Concentration (mg/kg)	Component	Measured Concentration (mg/kg)
Aluminum	9200	Magnesium	2710
Antimony	< 2.00	Manganese	4.05
Arsenic	< 2.00	Mercury	< 0.0968
Barium	61.0	Nickel	2.81
Beryllium	< 0.500	Nitrate by IC (solid)	< 1.5
Bromide by IC (solid)	< 5.1	Nitrite by IC (solid)	< 1.0
Cadmium	< 0.500	Potassium	89.7
Calcium	5000	Selenium	< 2.00
Chloride by IC (solid)	20.1	Silver	< 0.500
Chromium	8.25	Sodium	< 100
Cobalt	< 0.500	Sulfate by IC (solid)	91.9
Copper	1.33	Thallium	< 2.00
Fluoride by IC (solid)	4.3	Vanadium	4.97
Iron	422	Zinc	3.96
Lead	11.7		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 4

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

	Mean	Range
Specific Conductance (µmhos/cm)	315 (N = 4)	310 - 320
Hardness (mg/L as $CaCO_3$)	133 (N = 4)	128 - 140
Alkalinity (mg/L as $CaCO_3$)	180 (N = 4)	180 - 180
pH	8.2 (N = 4)	8.1 - 8.2

Appendix 5

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	< 0.020	Heptachlor	< 0.010
Alpha BHC	< 0.010	Heptachlor Epoxide	< 0.024
Alpha Chlordane	< 0.010	Kepone	< 0.20
Beta BHC	< 0.024	Malathion	< 3.0
Bolstar	< 2.0	Merphos	< 6.0
Chlordane	< 0.50	Methoxychlor	< 0.10
Coumaphos	< 3.0	Methyl Parathion	< 2.0
Delta BHC	< 0.024	Mevinphos	< 4.0
Demeton-O	< 2.0	Mirex	< 0.11
Demeton-S	< 3.0	Naled	< 3.0
Diazinon	< 4.0	o,p-DDD	< 0.020
Dichlorvos	< 3.0	o,p-DDE	< 0.020
Dieldrin	< 0.020	o,p-DDT	< 0.020
Disulfoton	< 2.0	p,p-DDD	< 0.020
Dursban (Chlorpyrifos)	< 2.0	p,p-DDE	< 0.020
Endosulfan I	< 0.010	p,p-DDT	< 0.020
Endosulfan II	< 0.020	PCB-1016	< 0.50
Endosulfan Sulfate	< 0.040	PCB-1221	< 0.50
Endrin	< 0.020	PCB-1232	< 0.50
Endrin Aldehyde	< 0.10	PCB-1242	< 0.50
Endrin Ketone	< 0.040	PCB-1248	< 0.50
EPN	< 4.0	PCB-1254	< 0.50
Ethion	< 2.0	PCB-1260	< 0.50
Ethoprop	< 3.0	Phorate	< 2.0
Ethyl Parathion	< 2.0	Ronnel	< 2.0
Famphur	< 3.0	Stirophos	< 2.0
Fensulfothion	< 15	Telodrin	< 0.010
Fenthion	< 2.0	Tokuthion	< 2.0
Gamma BHC – Lindane	< 0.010	Toxaphene	< 1.0
Gamma Chlordane	< 0.010	Trichloronate	< 2.0
Guthion (Azinphos-methyl)	< 4.0	Trithion	< 2.0
HCB	< 0.10		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 5

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	12.8
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	7.55
Calcium	34.9	Selenium	< 0.0200
Chloride	4.8	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.0
Cobalt	< 0.0050	Sulfate	< 5.0
Copper	< 0.0100	Thallium	< 0.0200
Fluoride	1.2	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 13, 2006.

Appendix 6

The Analysis of D6 in Sediment and Tissue

Appendix 6.1

Analytical Method Flowchart for the Analysis of D6 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF
D6 IN SEDIMENT

Rinse all glassware with ethyl acetate.
↓

Sample sediment through the overlying water from the test tank. Add the requisite volume of sediment (~5 mL) to each centrifuge tube.
↓

Fortify recovery samples with the appropriate stock solution.
↓

Add 10.0 mL HPLC-grade bottled water and 5 or 50 µL of internal standard to each sample and QC.
↓

Add approximately 20 mL of ethyl acetate to each sample. Vortex and shake each sample for one minute. Centrifuge at 60% speed for approximately five minutes.
↓

Transfer the ethyl acetate layer (top layer) into a 50 mL volumetric flask containing 2.5 g sodium sulfate.
↓

Repeat the extraction procedure with an additional 20 mL of ethyl acetate combining the ethyl acetate portions in the proper volumetric flask. Bring to volume with ethyl acetate.
↓

Transfer 1.00 mL of extracts into autosampler vials and add 50.0 µL MSTFA (N-methyl, N-trimethylsilyl trifluoroacetamide) to each vial. Submit samples for analysis.

Appendix 6.2

Analytical Method Flowchart for the Analysis of D6 in Tissue

METHOD OUTLINE FOR THE ANALYSIS OF
D6 IN TISSUE

Rinse all glassware with tetrahydrofuran (THF).
↓

Extract whole samples in scintillation vials as received. Add 10 mL of THF to each sample and matrix blank. Fortify quality control samples with the appropriate stock solutions. Fortify all samples and QC samples with internal standard (250 or 10,000 µg a.i./L at instrument).
↓

Homogenize tissues with a spatula. Minimize the time the contents of the vial is open to air. Vortex for approximately two minutes. Sonicate for 10 minutes and vortex again briefly to wash the sides of the vial.
↓

Transfer approximately 2 mL of the extract to a second vial and add approximately 500 mg of Na_2SO_4. Vortex for 15 seconds and allow to dry for approximately 15 minutes.
↓

Transfer an aliquot of the dried extract to a microcentrifuge tube and ultra-centrifuge each sample for approximately five minutes at 14,000 rpm.
↓

Transfer an aliquot of each extract to an autosampler vial. Submit samples for either GC/MS or GC/FID analysis.

Appendix 6.3

Analytical Method Flowchart for the Analysis of Lipids in Tissue

METHOD OUTLINE FOR THE PROCESSING OF LIPIDS IN TISSUE FOR D6 CONCENTRATIONS

Remove vials to be analyzed from freezer and allow samples to thaw.

↓

To each sample, add 10 mL of HPLC-grade bottled water and homogenize for approximately one minute using a hand-held tissue homogenizer. Rinse the homogenizer with the appropriate solvent(s) in between samples.

↓

Transfer each homogenate to a 250-mL separatory funnel that contains 25 mL of chloroform and 50 mL of methanol.

↓

Rinse each vial with an additional 10 mL of bottled water and pour rinse into respective separatory funnel.

↓

Shake each separatory funnel for approximately one minute with venting.

↓

Add 50 mL of chloroform followed by 50 mL of saturated sodium chloride to each separatory funnel.

↓

Briefly swirl each separatory funnel with venting.

↓

Allow the phases to separate. For each sample, drain the chloroform layer through a powder funnel packed with Teflon® wool and anhydrous sodium sulfate into a 250-mL round-bottom flask.

↓

Add an additional 50-mL aliquot of chloroform to each separatory funnel and repeat the extraction and draining procedures.

↓

Rotary evaporate the extracts in a water bath maintained at approximately 40°C to near dryness.

↓

Transfer each extract to a pre-weighed, labeled scintillation vial.

↓

Rinse each 250-mL round-bottom flask with a small volume of chloroform and transfer rinse to respective scintillation vial.

↓

Evaporate the remaining solvent in each vial under a gentle stream of nitrogen or clean dry air.

↓

Reweigh each vial and record weight.

Appendix 6.4

Typical Gas Chromatograph (GC) Operational Parameters for Sediment/Tissue Analysis

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Flame Ionization Detector (FID)
ANALYTICAL COLUMN:	J&W DB-5MS column (30 m x 0.25 mm, 0.25 µm)
INJECTOR TEMPERATURE:	280°C
RUN TIME:	15.25 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 8.00°C/minute Final temperature 1: 150°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 220°C Final hold time 2: 0.00 minute
DETECTOR TEMPERATURE:	310°C
INJECTION VOLUME:	1.0 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	Approximately 12 psi
APPROXIMATE D6 RETENTION TIME:	13.1 minutes

Appendix 6.5

Typical Gas Chromatograph (GC/MS) Operational Parameters for Tissue Analysis

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)	
DETECTOR:	Agilent Model 5975 Mass Selective Detector operated in SIM mode	
ANALYTICAL COLUMN:	Phenomen ZB-5 column (30 m x 0.25 mm, 0.25 µm)	
INJECTOR TEMPERATURE:	280°C	
RUN TIME:	15.25 minutes	
OVEN:	Initial temperature:	50°C
	Initial hold time:	1.00 minute
	Ramp 1:	8.00°C/minute
	Final temperature 1:	150°C
	Final hold time 1:	0.00 minute
	Ramp 2:	40.00°C/minute
	Final temperature 2:	220°C
	Final hold time 2:	0.00 minute
DETECTOR TEMPERATURE:	310°C	
INJECTION VOLUME:	1.0 µL	
MAKE-UP GAS:	Helium	
HEAD PRESSURE:	Approximately 12 psi	
APPROXIMATE D6 RETENTION TIME:	12.6 minutes	

Appendix 6.6

Analytical Stocks and Standards Preparation

For the sediment analysis, stock solutions of D6 were prepared by weighing 1.0006 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 80% DMF : 20% toluene. This primary stock solution (10.0 mg a.i./mL) was diluted in 80% DMF : 20% toluene to produce a 1.00 mg a.i./mL stock solution. The 10.0 and 1.00 mg a.i./mL stock solutions were used to fortify the sediment quality control samples. Calibration standards were prepared in ethyl acetate using the 1.00 mg/mL stock solution. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
1.00	10.0	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D6 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using 80% DMF : 20% toluene. This primary stock solution (1.00 mg a.i./mL) was diluted in 80% DMF : 20% toluene to prepare a 0.150 mg a.i./mL stock solution. The 0.150 mg a.i./mL stock solution was added to all sediment samples and calibration standards (50 µL).

For the tissue analysis, stock solutions of D6 was prepared by weighing 0.1000 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. This primary stock solution (1.00 mg a.i./mL) was diluted in tetrahydrofuran to produce a 0.100 and 0.0100 mg a.i./mL stock solution. The 1.00, 0.100 and 0.0100 mg a.i./mL stock solutions were used to fortify the tissue quality control samples. Calibration standards were prepared in tetrahydrofuran using the 1.00, 0.100 and 0.0100 mg a.i./mL stock solutions. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.100	100	10.0	1.00
1.00	50.0	10.0	5.00
1.00	150	10.0	15.0
1.00	300	10.0	30.0
1.00	500	10.0	50.0
1.00	700	10.0	70.0

A stock solution of Tetrakis (trimethylsilyloxy) silane (D6 internal standard) was prepared by weighing 0.1031 g (weight corrected for purity) on an analytical balance. The internal standard was transferred to a 100-mL Class A volumetric flask and brought to volume using tetrahydrofuran. This primary stock solution (1.00 mg a.i./mL) was diluted in tetrahydrofuran to prepare a 0.100 mg a.i./mL stock solution. The 1.00 and 0.100 mg a.i./mL stock solutions were added to all tissue samples and calibration standards (25 µL or 100 µL).

Appendix 6.7

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-112-2B, with a nominal concentration of 100 mg a.i./Kg, were calculated using the following equations:

$$\text{D6 in sample (mg a.i./Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of D6 in sample (mg a.i./Kg)}}{\text{nominal concentration of D6 in sample (mg a.i./Kg)}} \text{ X } 100$$

Peak Area = 3.87433
Y-intercept = 0.74939
Slope = 1.09
Dilution factor = 9.86

$$\text{D6 in sample (mg a.i./Kg)} = \frac{3.87433 - 0.74939}{1.09} \text{ X } 9.86$$

Concentration of D6 in sample (mg a.i./Kg) = 28.2 mg a.i./Kg

$$\text{Percent of nominal concentration} = \frac{28.2 \text{ mg a.i./Kg}}{100 \text{ mg a.i./Kg}} \text{ X } 100$$

Percent of nominal concentration = 28.2%

Appendix 6.8

Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

Sample Number (570A-112-)	Sampling Interval (Day)	Concentrations of D6		Percent Recovery[1]
		Fortified (mg a.i./Kg)	Measured (mg a.i./Kg)	
MAS-1	0	100	90.4	90.4
MAS-2	0	500	472	94.5
MAS-3	14	100	89.8	89.8
MAS-4	14	500	457	91.4
MAS-5	28	100	95.8	95.8
MAS-6	28	500	475	95.1
			Mean =	92.8
			Standard Deviation =	2.60
			CV =	2.81

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

Appendix 6.9

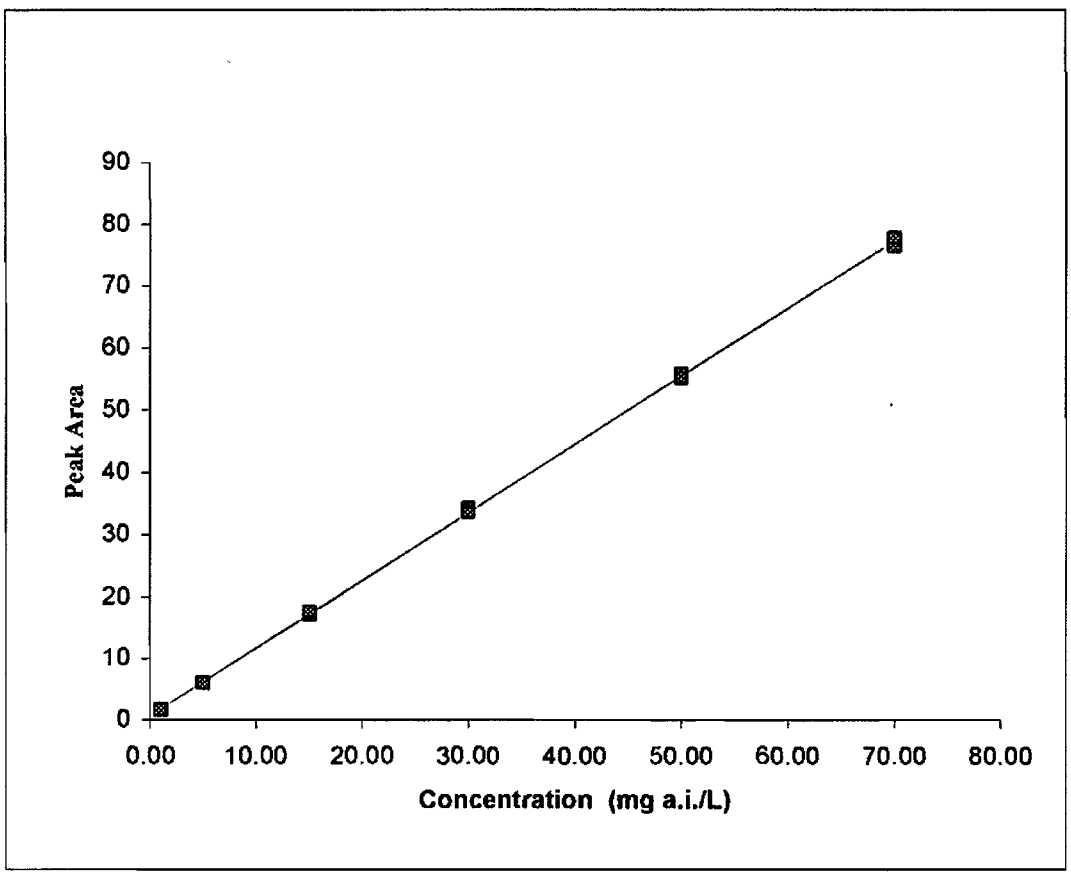
Matrix Fortifications Analyzed During Sample Analyses in Tissue

Sample Number (570A-112-)	Concentrations of D6		Percent Recovery[1]
	Fortified (mg a.i./Kg)	Measured (mg a.i./Kg)	
TMAS-1	2.50	3.05	122
TMAS-2	25.0	27.7	111
TMAS-3	250	223	89.3
		Mean =	107
		Standard Deviation =	16.6
		CV =	15.6

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

Appendix 6.10

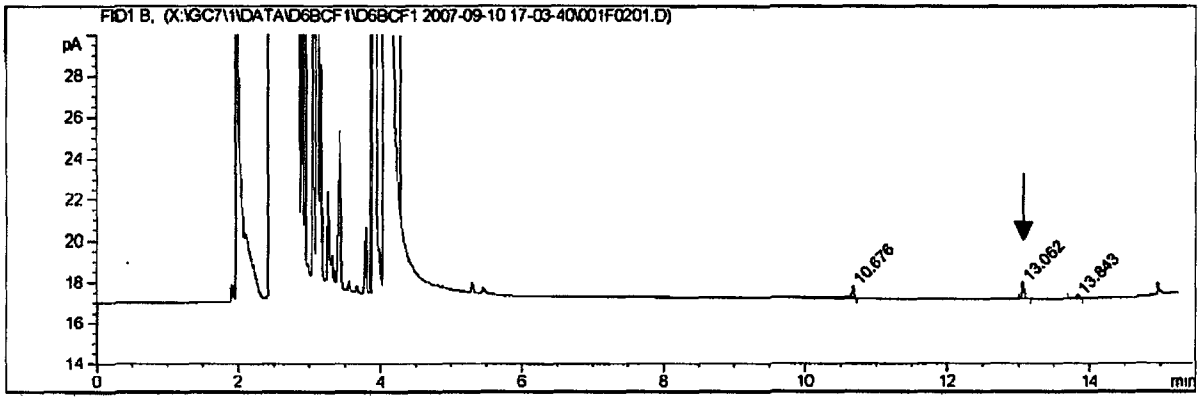
Representative Calibration Curve for D6 in Sediment



Slope = 1.09; Y-Intercept = 0.74939; $R^2 = 0.9998$

Appendix 6.11

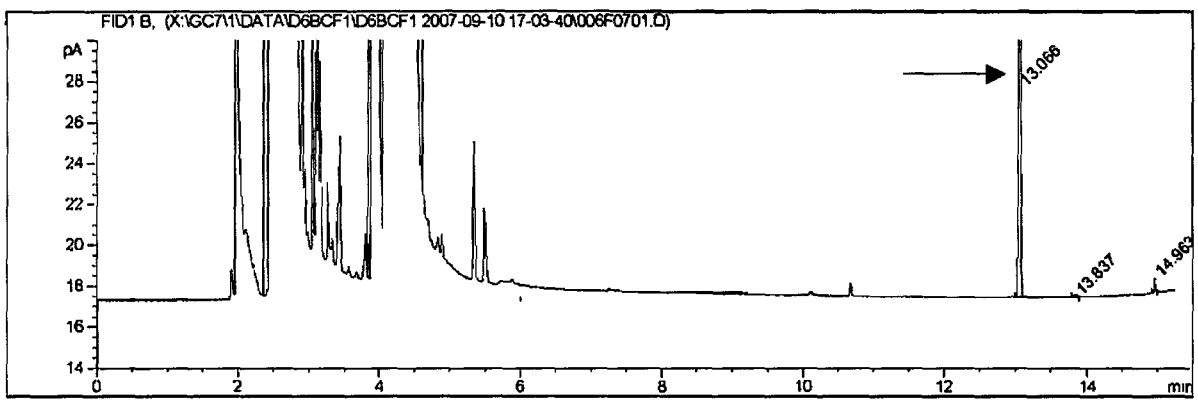
Representative Chromatogram of a Low-level D6 Calibration Standard for Sediment



Nominal concentration: 1.00 mg a.i./L. The arrow indicates the approximate retention time of D6.

Appendix 6.12

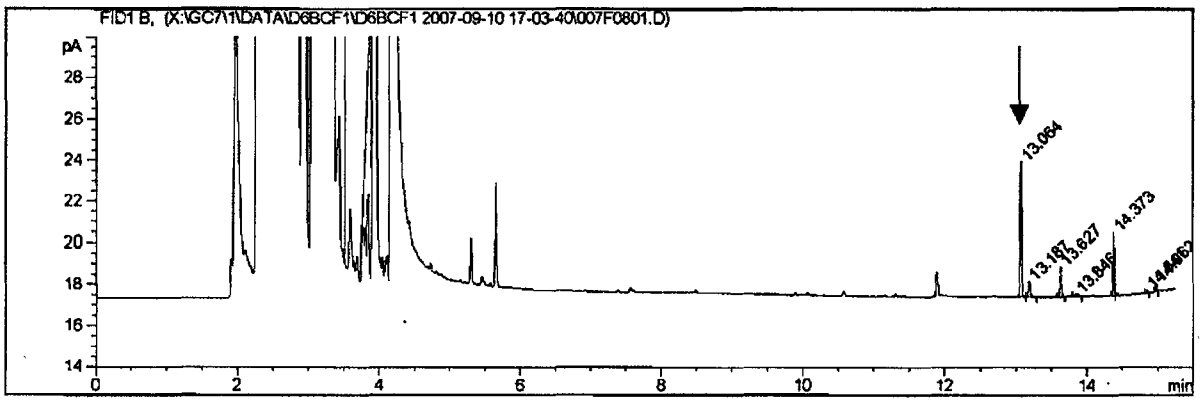
Representative Chromatogram of a High-level D6 Calibration Standard for Sediment



Nominal concentration: 70.0 mg a.i./L. The arrow indicates the approximate retention time of D6.

Appendix 6.13

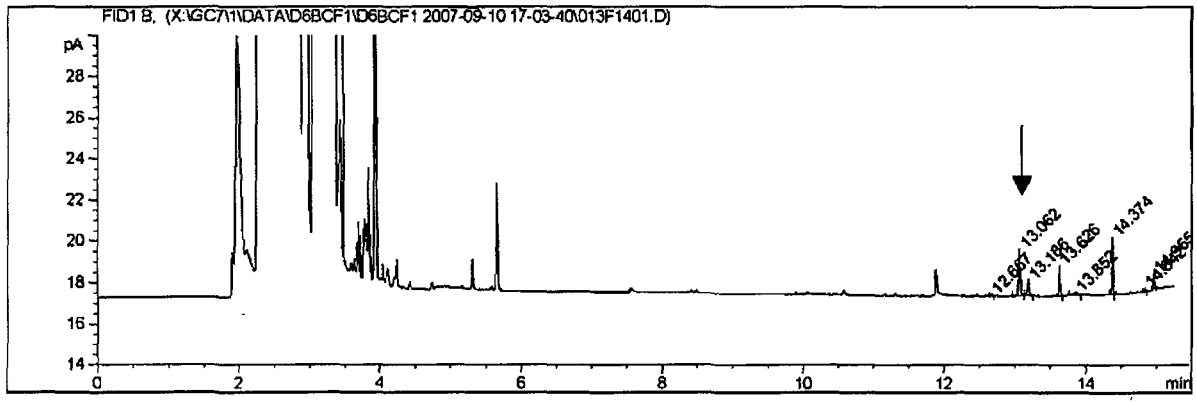
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-112-MAS-1; 100 mg a.i./Kg, nominal concentration. The arrow indicates the approximate retention time of D6.

Appendix 6.14

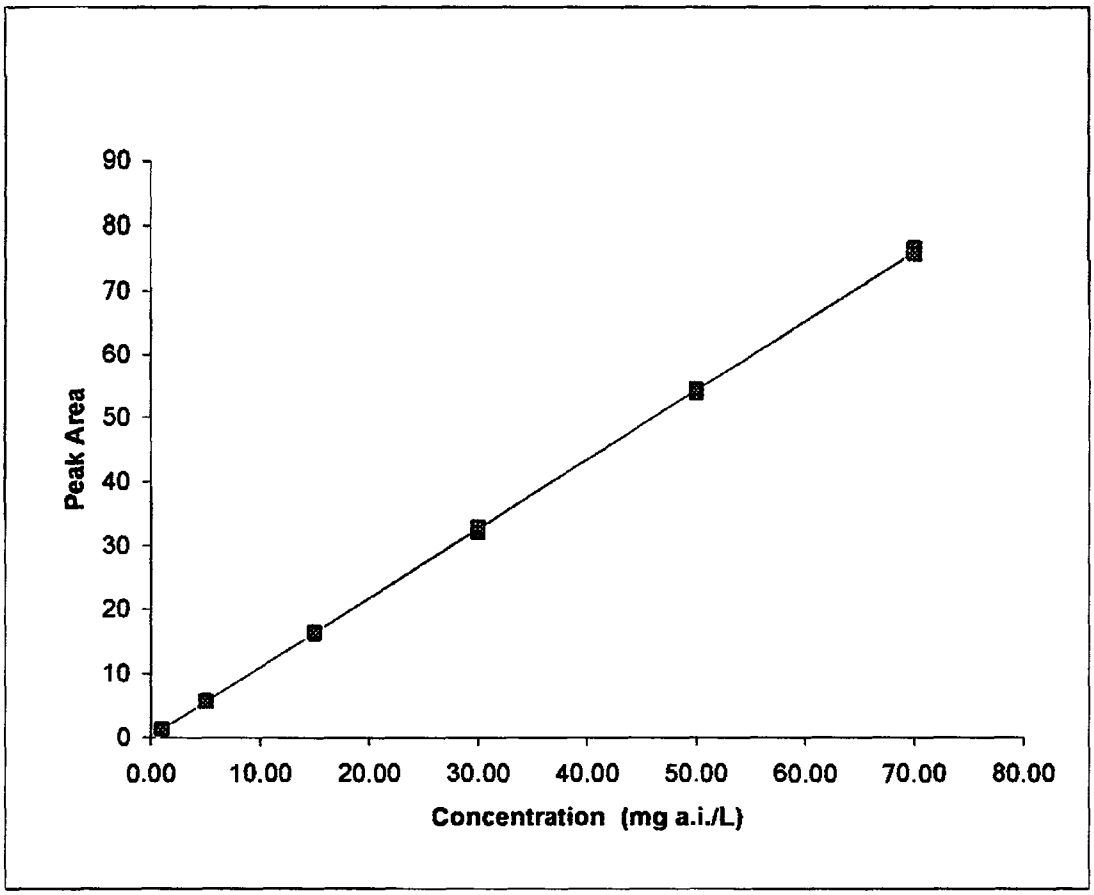
Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-112-2B; 100 mg a.i./Kg nominal concentration. The arrow indicates the approximate retention time of D6.

Appendix 6.15

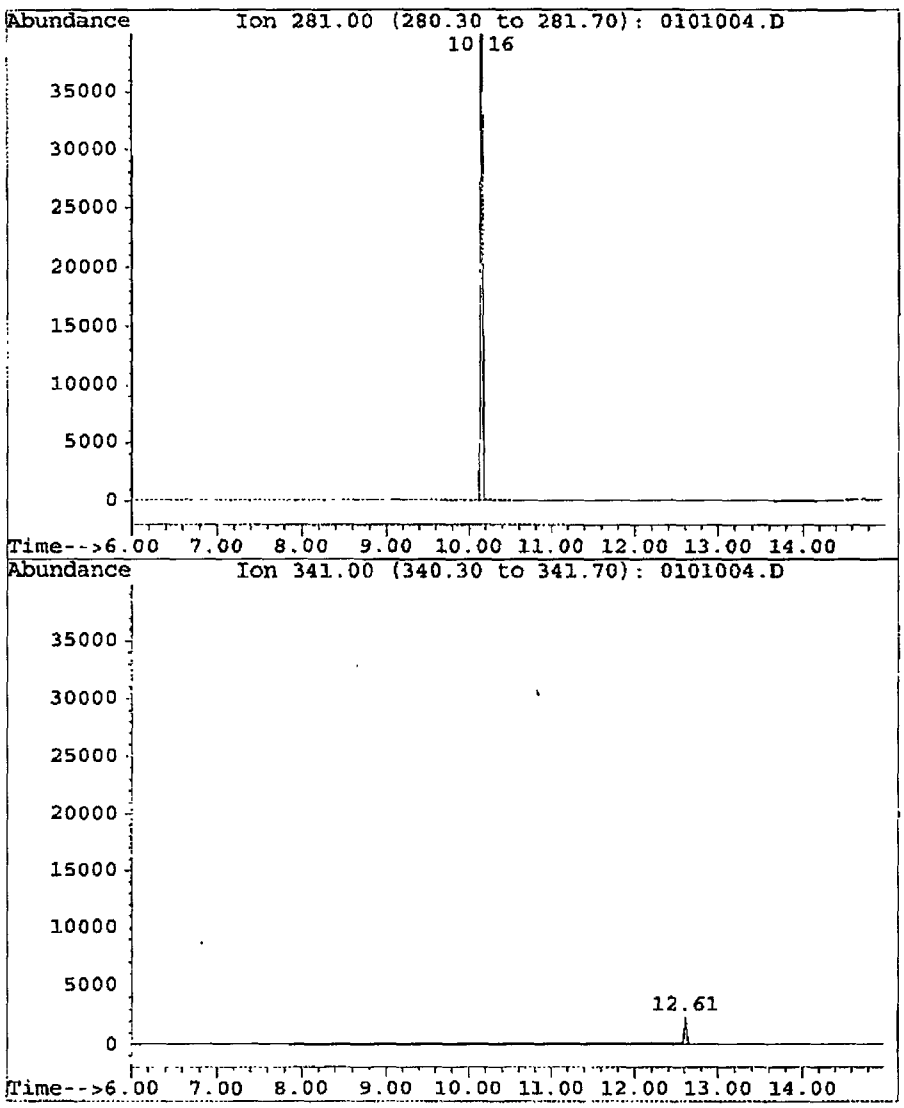
Representative Calibration Curve for D6 in Tissue



Slope = 1.09; Y-Intercept = 0.13467; R^2 = 0.9999

Appendix 6.16

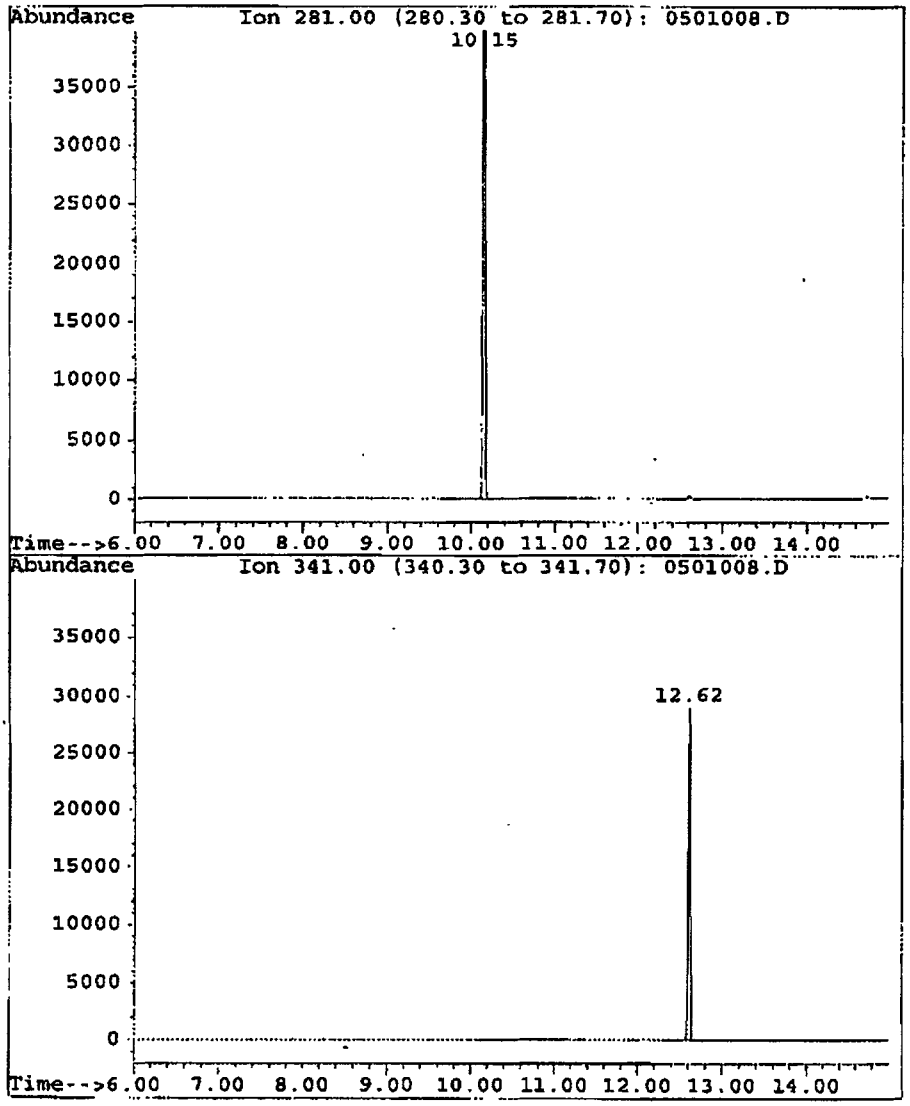
Representative Chromatogram of a Low-level D6 Calibration Standard for Tissue



Nominal concentration: 0.700 mg a.i./L.

Appendix 6.17

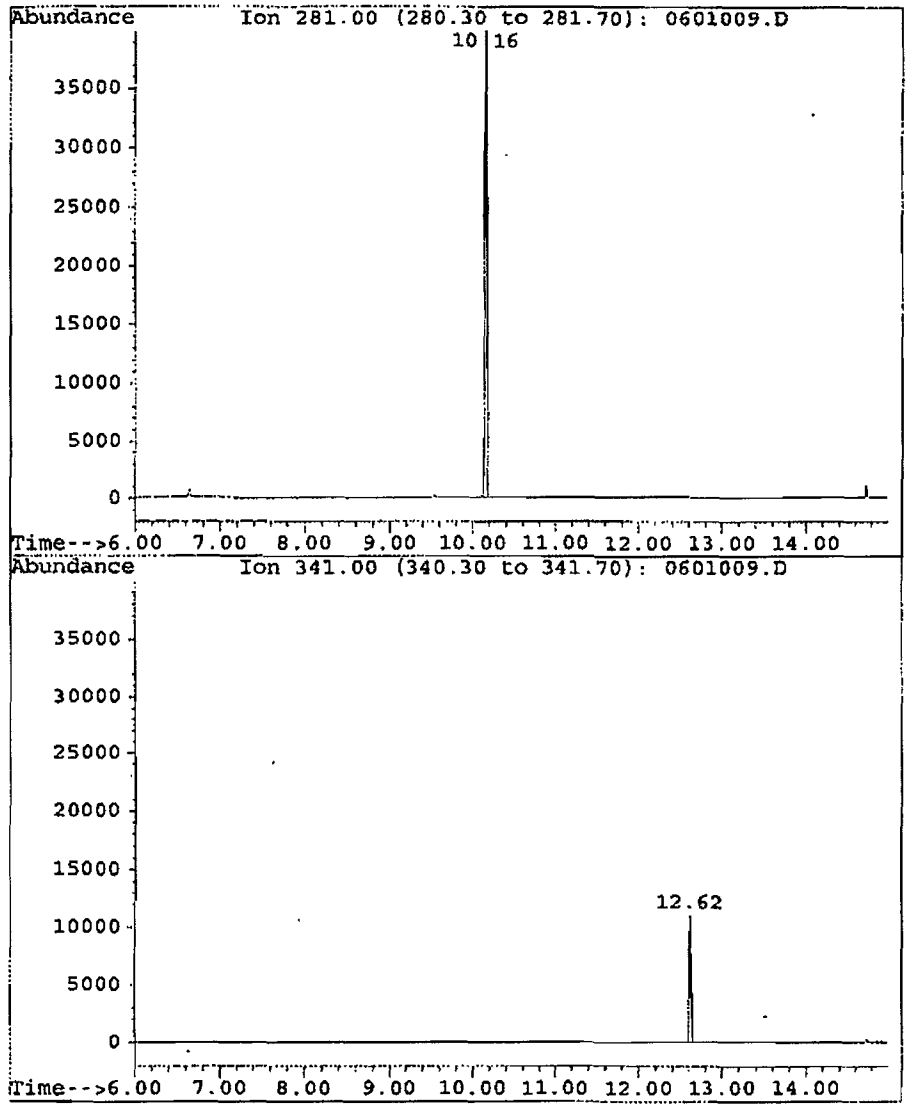
Representative Chromatogram of a High-level D6 Calibration Standard for Tissue



Nominal concentration: 7.00 mg a.i./L.

Appendix 6.18

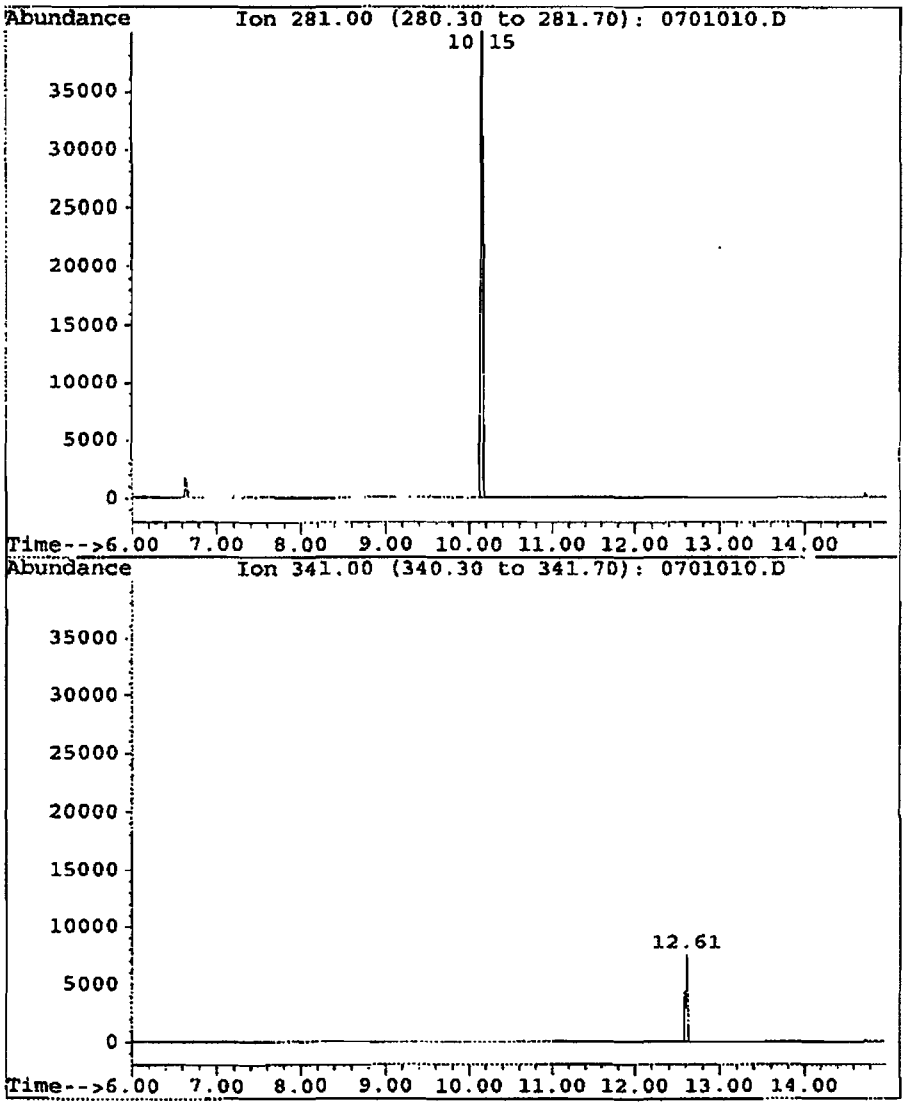
Representative Chromatogram of a Tissue Matrix Fortification



Sample number 570A-112-TMAS-2; 25.0 mg a.i./Kg, nominal concentration..

Appendix 6.19

Representative Chromatogram of a Day 0 Tissue Sample



Sample number 570A-112-T3; 100 mg a.i./Kg nominal concentration.

Appendix 7

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	3G; rest AN	0	0	1G; rest AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	1G; rest AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 166 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved. 145 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	3G; rest AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	1G; rest AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	0

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 175 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved. 0.6082 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	3G; rest AN	0	0	1G; rest AN	0	0
9	AN	0	0	AN	0	0
10	1G; rest AN	0	0	1G; rest AN	0	0
11	1G; rest AN	0	0	AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	1G; rest AN	0	0	3G; rest AN	0	0
14	AN	0	0	AN	0	[3]
15	AN	0	0	--	--	--
16	AN	0	0	--	--	--
17	AN	0	0	--	--	--
18	AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved. 0.9702 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	AN	0	0
8	4G; rest AN	0	0
9	AN	0	0
10	AN	0	0
11	AN	0	0
12	AN	0	0
13	2G; rest AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

28.1 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	3G; rest AN	0	0	2G; rest AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	1G; rest AN	0	0	AN	0	0
14	1G; rest AN	0	0	1G; rest AN	0	0
15	2G; rest AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	1G; rest AN	0	0	1G; rest AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment

[2] The sediment was sieved. 162 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

[3] The sediment was sieved. 176 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

28.1 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	2G; rest AN	0	0
11	1G; rest AN	0	0	AN	0	0
12	1G; rest AN	0	0	AN	0	0
13	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	1G; rest AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 130 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved. 0.7304 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

28.1 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	1G; rest AN	0	0	AN	0	0
14	AN	0	0	AN	0	[3]
15	1G; rest AN	0	0	--	--	--
16	1G; rest AN	0	0	--	--	--
17	AN	0	0	--	--	--
18	1G; rest AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved. 1.0566g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

28.1 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	AN	0	0
8	AN	0	0
9	AN	0	0
10	1G; rest AN	0	0
11	AN	0	0
12	AN	0	0
13	1G; rest AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

484 mg a.i./Kg – Uptake Phase

Day	Replicate A			Replicate B		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	4G; rest AN	0	0	6G; rest AN	0	0
9	AN	0	0	AN	0	0
10	1G; rest AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	1G; rest AN	0	0	AN	0	0
13	1G; rest AN	0	0	2G; rest AN	0	0
14	1G; rest AN	0	0	1G; rest AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	1G; rest AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 128 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved. 127 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

484 mg a.i./Kg – Uptake Phase

Day	Replicate C			Replicate D		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	2G; rest AN	0	0	5G; rest AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	1G; rest AN	0	0
11	AN	0	0	1G; rest AN	0	0
12	AN	0	0	1G; rest AN	0	0
13	AN	0	0	1G; rest AN	0	0
14	1G; rest AN	0	0	AN	0	0
15	AN	0	0	AN	0	0
16	AN	0	0	AN	0	0
17	AN	0	0	AN	0	0
18	AN	0	0	AN	0	0
19	AN	0	0	1G; rest AN	0	0
20	AN	0	0	AN	0	0
21	AN	0	0	AN	0	0
22	AN	0	0	AN	0	0
23	AN	0	0	AN	0	0
24	AN	0	0	AN	0	0
25	AN	0	0	AN	0	0
26	AN	0	0	AN	0	0
27	AN	0	0	AN	0	0
28	AN	0	[2]	AN	0	[3]

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 126 organisms were found and transferred to a new tank containing clean sediment for the depuration phase of the study.
[3] The sediment was sieved. 0.7294 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

484 mg a.i./Kg – Uptake Phase

Day	Replicate E			Replicate F		
	Observations[1]	Cumulative Number Dead	Number Sampled	Observations	Cumulative Number Dead	Number Sampled
0	AN	0	0	AN	0	0
1	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0
13	2G; rest AN	0	0	AN	0	0
14	AN	0	0	AN	0	[3]
15	AN	0	0	--	--	--
16	1G; rest AN	0	0	--	--	--
17	1G; rest AN	0	0	--	--	--
18	1G; rest AN	0	0	--	--	--
19	AN	0	0	--	--	--
20	AN	0	0	--	--	--
21	AN	0	0	--	--	--
22	AN	0	0	--	--	--
23	1G; rest AN	0	0	--	--	--
24	AN	0	0	--	--	--
25	1G; rest AN	0	0	--	--	--
26	AN	0	0	--	--	--
27	AN	0	0	--	--	--
28	AN	0	[2]	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 28.
[3] The sediment was sieved. 0.9210 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

484 mg a.i./Kg – Uptake Phase

Day	Replicate G		
	Observations[1]	Cumulative Number Dead	Number Sampled
0	AN	0	0
1	AN	0	0
2	AN	0	0
3	AN	0	0
4	AN	0	0
5	AN	0	0
6	AN	0	0
7	AN	0	0
8	8G; rest AN	0	0
9	AN	0	0
10	1G; rest AN	0	0
11	AN	0	0
12	AN	0	0
13	AN	0	0
14	AN	0	[2]
15	--	--	--
16	--	--	--
17	--	--	--
18	--	--	--
19	--	--	--
20	--	--	--
21	--	--	--
22	--	--	--
23	--	--	--
24	--	--	--
25	--	--	--
26	--	--	--
27	--	--	--
28	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] Replicate was used for analytical confirmation in the sediment on Day 14.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Negative Control – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Obs.[1]	Cumulative Number Dead	Number Sampled	Obs.	Cumulative Number Dead	Number Sampled	Obs.	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	0	AN	0	0	--	--	--
24	AN	0	0	AN	0	0	--	--	--
25	AN	0	0	AN	0	0	--	--	--
26	AN	0	0	AN	0	0	--	--	--
27	AN	0	0	AN	0	0	--	--	--
28	AN	0	0	AN	0	0	--	--	--
29	AN	0	0	AN	0	0	--	--	--
30	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal.
[2] The sediment was sieved. 196 organisms were found and used for lipid analysis on Depuration Day 30 (0.7085 g wet weight of organism tissue).
[3] The sediment was sieved. 1.0938 g (wet weight of organism tissue) were collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment –Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

28.1 mg a.i./Kg – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Obs.[1]	Cumulative Number Dead	Number Sampled	Obs.[1]	Cumulative Number Dead	Number Sampled	Obs.	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	1G; rest AN	0	0	2G; rest AN	0	0
6	AN	0	0	AN	0	0	2G; rest AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	1G; rest AN	0	0	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	AN	0	0	AN	0	0	AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	0	AN	0	0	--	--	--
24	AN	0	0	AN	0	0	--	--	--
25	AN	0	0	AN	0	0	--	--	--
26	AN	0	0	AN	0	0	--	--	--
27	AN	0	0	AN	0	0	--	--	--
28	1G; rest AN	0	0	AN	0	0	--	--	--
29	AN	0	0	AN	0	0	--	--	--
30	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 188 organisms were found and used for lipid analysis on Depuration Day 30 (0.7994 g wet weight of organism tissue).
[3] The sediment was sieved. 0.9004 g (wet weight of organism tissue) was collected for analytical measurement.

Appendix 7 (Continued)

Cumulative Mortality and Treatment -Related Effects in a
Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

484 mg a.i./Kg – Depuration Phase

Day	Replicate A			Replicate B			Replicate C		
	Obs.[1]	Cumulative Number Dead	Number Sampled	Obs.[1]	Cumulative Number Dead	Number Sampled	Obs.	Cumulative Number Dead	Number Sampled
1	AN	0	0	AN	0	0	AN	0	0
2	AN	0	0	AN	0	0	AN	0	0
3	AN	0	0	AN	0	0	AN	0	0
4	AN	0	0	AN	0	0	AN	0	0
5	AN	0	0	AN	0	0	AN	0	0
6	AN	0	0	AN	0	0	AN	0	0
7	AN	0	0	AN	0	0	AN	0	0
8	AN	0	0	AN	0	0	AN	0	0
9	AN	0	0	AN	0	0	AN	0	0
10	1G; rest AN	0	0	1G; rest AN	0	0	1G; rest AN	0	0
11	AN	0	0	AN	0	0	AN	0	0
12	AN	0	0	AN	0	0	AN	0	0
13	AN	0	0	AN	0	0	AN	0	0
14	AN	0	0	AN	0	0	AN	0	[3]
15	AN	0	0	AN	0	0	--	--	--
16	AN	0	0	AN	0	0	--	--	--
17	AN	0	0	AN	0	0	--	--	--
18	AN	0	0	AN	0	0	--	--	--
19	AN	0	0	AN	0	0	--	--	--
20	AN	0	0	AN	0	0	--	--	--
21	AN	0	0	AN	0	0	--	--	--
22	AN	0	0	AN	0	0	--	--	--
23	AN	0	0	AN	0	0	--	--	--
24	AN	0	0	AN	0	0	--	--	--
25	AN	0	0	AN	0	0	--	--	--
26	AN	0	0	AN	0	0	--	--	--
27	AN	0	0	AN	0	0	--	--	--
28	AN	0	0	AN	0	0	--	--	--
29	AN	0	0	AN	0	0	--	--	--
30	AN	0	[2]	AN	0	0	--	--	--

[1] Observations: AN = organisms appear normal; G = organisms observed on the surface of the sediment or leaving the sediment.
[2] The sediment was sieved. 177 organisms were found and used for lipid analysis on Depuration Day 30 (0.7205 g wet weight of organism tissue).
[3] The sediment was sieved. 0.8646 g (wet weight of organism tissue) was collected for analytical measurement.

Appendix 8

Agvise Soil Characterization Report

AGVISE LABORATORIES

604 Highway 15 West
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013
email: agvise@polarcomm.com
Homepage: www.agvise.com

AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INT.
Protocol or Study No = VARIOUS
Sample ID. = 570A-112
Trial ID. = NEGATIVE CONTRO
Date Received = 12-21-07
Date Reported = 01-02-2008

AGVISE Lab No	07-1152
Percent Sand	69
Percent Silt	12
Percent Clay	19
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	1.02
Cation Exchange Capacity (meq/100 g)	11.5
% Moisture at 1/3 Bar	22.3
% Organic Carbon --Walkley Black	3.6
% Organic Matter--Walkley Black	6.2
pH in 1:1 soil:water ratio	7.3

Base Saturation Data

Cation	Percent	ppm
Calcium	45.3	1040
Magnesium	43.0	592
Sodium	1.0	26
Potassium	0.3	12
Hydrogen	10.5	12

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

1/2/08
Date

AGVISE LABORATORIES

604 Highway 15 West
P.O. Box 510
Northwood, ND 58267
(701) 587-6010
FAX (701) 587-6013
email: agvise@polarcomm.com
Homepage: www.agvise.com

AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INT.
Protocol or Study No = VARIOUS
Sample ID. = 570A-112-DEP
Trial ID. = NEGATIVE CONTRO
Date Received = 12-21-07
Date Reported = 01-02-2008

AGVISE Lab No	07-1153
Percent Sand	71
Percent Silt	14
Percent Clay	15
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	0.88
Cation Exchange Capacity (meq/100 g)	9.8
% Moisture at 1/3 Bar	25.5
% Organic Carbon --Walkley Black	3.3
% Organic Matter--Walkley Black	5.6
pH in 1:1 soil:water ratio	7.3

Base Saturation Data

Cation	Percent	ppm
Calcium	44.8	880
Magnesium	42.8	504
Sodium	1.4	33
Potassium	0.3	13
Hydrogen	10.6	10

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

1/2/08
Date

Appendix 9

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. Timothy Z. Kendall, M.S., Supervisor
4. Amy S. Blankinship, M.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

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OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *LUMBRICULUS VARIEGATUS* USING SPIKED ARTIFICIAL SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-115

U. S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.1735

OECD Guideline 225

AUTHORS:

Susan T. Thomas, B.S.
Henry O. Krueger, Ph.D.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: March 25, 2009

STUDY COMPLETION DATE: October 2, 2009

SUBMITTED TO:

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard
Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601
U.S.A
(410) 822-8600

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-115

STUDY COMPLETION: October 2, 2009

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Parts 160 and 792, 17 August 1989; OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999, with the following exception:

Periodic analyses of well water and sediment for potential contaminants were not conducted in accordance with Good Laboratory Practices; however, these analyses were performed using a certified laboratory and standard U.S. EPA analytical methods.

STUDY DIRECTOR:

Susan Thomas — 10/2/09

Susan T. Thomas, B.S.
Biologist — Date

SPONSOR APPROVAL:

10/8/09

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 in 40 CFR Parts 160 and 792, 17 August 1989, OECD Principles of Good Laboratory Practice, (ENV/MC/CHEM(98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. The dates of all inspections and audits and the dates that any findings were reported to the Study Director and Study Director's Management were as follows:

		DATE REPORTED TO:	
ACTIVITY:	DATE CONDUCTED:	STUDY DIRECTOR	MANAGEMENT:
Protocol	September 18, 2008	September 18, 2008	September 30, 2008
Test Substance Preparation	March 30, 2009	March 30, 2009	April 1, 2009
Dissolved Oxygen Measurements	April 28, 2009	April 28, 2009	May 4, 2009
Dry Weights	May 6, 2009	May 6, 2009	May 11, 2009
Analytical Data and Draft Report	August 20 and 21, 2009	August 21, 2009	August 24, 2009
Biological Data and Draft Report	August 18, 19 and 20, 2009	August 20, 2009	September 10, 2009
Final Report	October 2, 2009	October 2, 2009	October 2, 2009

All inspections were study-based unless otherwise noted.

Marshall T. Hynson
Quality Assurance Program Supervisor

10/2/2009
Date

REPORT APPROVAL

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxieity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-115

STUDY DIRECTOR:

Susan Thomas — 10/2/09

Susan T. Thomas, B.S.
Aquatic Biologist — Date

PRINCIPAL INVESTIGATOR:

Tim Kendall — 10/2/09

Timothy Z. Kendall, M.S.
Supervisor — Date

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

Hen Krueger — 10/2/09

Henry O. Krueger, Ph. D.
Director of Aquatic, Plant and Insect Toxicology — Date

Willard B. Nixon — 10/2/09

Willard B. Nixon, Ph. D.
Director of Chemistry — Date

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SUMMARY

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Artificial Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-115

TEST SUBSTANCE: Octamethyltrisiloxane (L3; CAS No. 107-51-7); Dow Corning® 200 Fluid, 1 cst.; Lot 0002680862; CAS Number: 107-51-7; Purity: 99.9%; Expiration Date: May 27, 2010

TEST DATES:

Study Initiation:	March 25, 2009
Experimental Start (OECD):	March 30, 2009
Experimental Start (EPA):	April 2, 2009
Biological Termination:	April 30, 2009
Experimental Termination:	May 6, 2009

LENGTH OF EXPOSURE: 28 Days

TEST ORGANISM: Oligochaete (*Lumbriculus variegatus*)

AGE OF TEST ORGANISMS: Adult

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
Superior, Wisconsin 54880
U.S.A.

TEST CONCENTRATIONS:

Nominal	Mean Measured (dry weight)
Negative Control	<LOQ
3.1 mg/Kg	0.65 mg/Kg
6.3 mg/Kg	1.1 mg/Kg
13 mg/Kg	1.6 mg/Kg
25 mg/Kg	4.8 mg/Kg
50 mg/Kg	8.9 mg/Kg
100 mg/Kg	17 mg/Kg

RESULTS: Based on mean measured test concentrations
28 Day EC50: >17.0 mg/Kg, the highest concentration tested
NOEC: 1.1 mg/Kg
LOEC: 1.6 mg/Kg

Executive Summary

This study was conducted by Wildlife International, Ltd. for the Silicones Environmental, Health and Safety Council at the Wildlife International, Ltd. aquatic toxicology laboratory in Easton, Maryland. The in-life phase of the test was conducted from April 2, 2009 to April 30, 2009. The objective of the study was to determine the effects of sediment incorporated octamethyltrisiloxane (L3) on the oligochaete, *Lumbriculus variegatus*, during a 28 day exposure period under flow-through test conditions. The endpoints of the study were survivorship and growth (dry weight). Oligochaetes were exposed to nominal concentrations of 3.1, 6.3, 13, 25, 50 and 100 mg L3/kg. A negative control was also tested concurrently. The test sediment was formulated sediment prepared based on OECD Guideline 218. The organic carbon content of the formulated sediment was measured to be 1.9 %. Sediment samples were collected on Days 0, 7 and 28 to measure concentrations of L3. Overlying water and pore water samples were not analyzed. Eight replicate test compartments were maintained in each treatment and the control with 10 oligochaetes per test compartment for a total of 80 oligochaetes per test concentration. Five additional replicates were added in each treatment group for analytical sampling of sediment. A diluter system was used to provide approximately two volume additions of overlying water per day. The test was performed at 23 ± 1°C with a photoperiod of 16 hours of light and 8 hours of darkness. Overlying water was well water with a hardness of approximately 144 mg/L as $CaCO_3$. Mean measured sediment concentrations were 0.65, 1.1, 1.6, 4.8, 8.9 and 17 mg/kg which represented 21, 17, 12, 19, 18 and 17% of nominal, respectively. All water quality measurements remained within acceptable limits during the test. Oligochaetes generally appeared normal during the test with a few observations of abnormal behavior in the L3 and control treatment groups. At test termination there was an increase in the numbers of oligochaetes in some test compartments indicating that reproduction had occurred. After 28 days of exposure the mean number of oligochaetes in the control was 17.3. The mean number of worms in the 0.65, 1.1, 1.6, 4.8, 8.9 and 17 mg/kg treatment groups was 15.8, 15.1, 11.8, 11.5, 9.1 and 9.8, respectively. Dunnett's test showed that the number of worms was significantly reduced in the 1.6, 4.8, 8.9 and 17 mg/kg treatment groups in comparison to the negative control ($p \leq 0.05$). Mean dry weight was not significantly reduced in any L3 treatment group in comparison to the negative control ($p > 0.05$). The LOEC, based on the final number of worms was 1.6 mg/kg. The NOEC for *Lumbriculus variegatus* exposed to L3 was 1.1 mg/kg.

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Silicones Environmental, Health and Safety Council at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from April 2, 2009 to April 30, 2009 and dry-weight measurements were completed on May 6, 2009. Raw data generated by Wildlife International, Ltd. and a copy of the final report are filed under Project Number 570A-115 in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated L3, on the oligochaete, *Lumbriculus variegatus* during a 28-day exposure period under flow-through test conditions. The measured endpoints of the test were survivorship (original organisms and/or offspring) and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of oligochaetes were exposed to a geometric series of six test concentrations and a negative control for 28 days under flow-through test conditions. Eight replicate test compartments were maintained in each treatment and control group, with 10 oligochaetes in each test compartment, for a total of 80 oligochaetes per test concentration. Each test compartment contained a quantity of sediment and overlying water. An additional five replicates were added in each treatment and control group for analytical sampling of sediment. Organisms were added at test initiation to one analytical replicate for the Day 7 and Day 28 analyses. The remaining three analytical replicates for Days 0, 7 and 28 were "abiotic" and did not contain organisms.

Nominal test concentrations selected in consultation with the sponsor were 3.1, 6.3, 13, 25, 50 and 100 mg/Kg of sediment based on the dry weight of the sediment. The results of the study are based on mean measured test concentrations. Due to the low solubility of L3 in water (34.49 µg/L) only sediment samples were collected and analyzed from the "analytical replicates" of each treatment group and control (1). Samples were colleeted immediately after the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test. Results of the analyses were used to verify the exposure over time.

Test compartments were indiscriminately positioned in a diluter unit according to treatment group approximately 48 hours prior to test initiation to condition the sediment. Oligochaetes were impartially assigned to exposure compartments at test initiation. Observations of mortality and abnormal behavior were made daily during the test. Survivorship/reproduction and growth (dry weights) were determined at the end of the 28-day test period. The percent reduction in the numbers of organisms present in the treatment groups at test termination in comparison to the control group was used to determine the 28-day EC50 value. The lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) were determined by the concentration-response pattern and statistical analysis of the survival/reproduction and dry weight data.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment" (Appendix 1). The protocol was based on a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-Associated Contaminants (2); ASTM E 1706-00: *Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); OPPTS 850.1735 Guideline: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4); European Chemicals Bureau *Technical Guidance Document on Risk Assessment*, Part II (5), and the Federal Environment Agency: *Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances* (6). This study also generally meets OECD Guideline 225, Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment (7).

Test Substance

The test substance was received from Dow Corning Corporation on August 19, 2008 and assigned Wildlife International identification number 8690. The test substance, a liquid, was identified as: Dow Corning® 200 Fluid, 1 cst; CAS Number 107-51-7; Lot No. 0002680862. The reported purity was 99.9%, with an expiration date of May 27, 2010 (Appendix 2). The test substance was stored under ambient conditions.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 (8) was used as the test sediment. The sediment was composed of approximately 10% peat, 20% kaolin clay, and 70% industrial quartz sand (Appendix 3). The dry constituents of the sediment were mixed in a mixer for approximately 20 minutes without the peat. The peat was mixed with the test material and added to the rest of the ingredients on a later date. The pH of the final mixture of sediment was found to be 7.1. The percent organic carbon of the sediment was 1.9. The sediment was stored under ambient conditions until used.

A 28-day ration of food (200 mg salmon starter) was dry mixed into the test compartments after the sediment was added, but immediately before adding the water to the test compartments, and 48 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. Just as in the OECD 218 Guideline, the food added to the test system during the test was not included in the measurement of organic carbon.

Preparation of Test Concentrations

Nominal test concentrations (3.1, 6.3, 13, 25, 50 and 100 mg/Kg) were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer. Second, a predetermined amount of sand and clay were added to the peat and test material in the Nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 30 minutes).

After mixing, approximately 100 mL of dosed formulated sediment was transferred to 13 replicate test beakers (300 mL glass beakers with stainless steel mesh covered holes on opposite sides). The transfer time was kept to a minimum (approximately 10-20 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 13 beakers had approximately 100 mL of sediment added, a 28-day ration of food (approximately 200 mg of Salmon Starter) was added and gently stirred into the dry sediment. Finally, approximately 150 mL of well water was added to the beakers. After the water was added to the beakers, the beakers were placed in the water bath that was used for the study. The overlying water in the test beakers was exchanged two times daily.

Of the thirteen replicate test compartments prepared for each treatment and control group, eight test compartments were used for the evaluation of survival and growth and five compartments for analytical purposes. The sediment/water mixtures were allowed to acclimate for approximately 48 hours prior to the introduction of the test organisms. At test initiation and termination, the overlying water in all test chambers appeared clear and colorless.

Test Organism

The oligochaete, *Lumbriculus variegatus*, was selected as the test species for this study. Oligochaetes are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. The organisms were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The identity of the species was verified by the supplier.

The organisms were held in water from the same source as the water used during the test. During the 14-day holding period immediately preceding the test, water temperature ranged from 22.3 to 23.4°C, while the pH ranged from 8.1 to 8.6 and dissolved oxygen ranged from 8.0 to 8.7 mg/L.

During holding, the oligochaetes appeared normal. At test initiation, adult oligochaetes were collected from the culture and impartially added one and two at a time into transfer chambers until each chamber contained 10 organisms. Each group of ten organisms was impartially assigned and transferred to a test compartment. All transfers were made below the water surface using wide-bore pipettes. Ten replicate test compartments in each treatment and control group contained organisms (eight test compartments for the evaluation of effects plus two compartments for analytical purposes), while three test compartment per group were maintained without organisms for analytical purposes.

Oligochaetes were fed YCT, TertraMin® flake food (Drs Foster and Smith and Tetra, Rhinelander, Wisconsin) and salmon starter (Ziegler Brothers, Inc., Gardners, Pennsylvania) during the holding period and salmon starter during the test. A 200 mg aliquot of food (enough food for the entire study) was weighed for each test compartment that contained organisms during the test and was added to the test compartments after adding the sediment, and mixed with a glass stir rod into the sediment before the overlying water was added. No additional food supplementation was made during the study.

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 measurements of the well water during the four-week period immediately preceding the test are presented in Appendix 4.

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 again was filtered (0.45 µm) to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5.

Test Apparatus

The test apparatus consisted of test compartments placed in stainless steel diluter tanks. The tanks were placed in a temperature-controlled water bath in a Wildlife International, Ltd. diluter unit. One tank was assigned to each treatment or control group. Standpipes were placed in each tank to maintain the water height. Each diluter tank had above it a splitting chamber. Dilution water was delivered on a timer to the splitting chamber two times per day. Each splitting chamber contained 14 syringes. Each syringe delivered dilution water to individual test compartments which were indiscriminately and carefully positioned in diluter tanks according to treatment group.

Test compartments were 300-mL glass beakers with two stainless steel mesh-covered holes on opposite sides to allow for the flow of water through the test compartment. Each beaker contained approximately 100 mL of sediment (2.4 cm in a representative test chamber) and approximately 150 mL of overlying water. The depth of overlying water in each test compartment was maintained by the water levels in the diluter tanks (e.g. 7.5 cm in a representative test compartment). The diluter was adjusted so that approximately 769 ml of water was delivered per minute for four minutes to each splitting chamber two times per day resulting in approximately two and a half volume additions in each test compartment per day. Test compartments were labeled with the project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 334 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 23 ± 1°C. Temperature was measured in the overlying water of one alternating replicate test compartment at the beginning and end of the test, and daily during the test, using a liquid-in-glass thermometer. Temperature also was measured continuously in a beaker beside the test chambers in the negative control diluter tank using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one replicate (alternating) test compartment of each treatment and control group at the beginning and end of the test and daily during the test. Measurements of pH were made on samples of overlying water colleeted from one replicate (alternating) test compartment of each treatment and control group at test initiation, once each week during the test, and at test termination. Hardness, alkalinity, specific conductance and ammonia were measured in a composite of the overlying water from the control group replieates and the highest concentration treatment group (100 mg/Kg) replicates at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (9). Specific conductance was measured using a Acorn Series Model CON6 Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion 720Aplus pH/ISE meter.

Observations

In addition to the organisms placed in the test compartments at the beginning of the test, an additional 20 organisms were impartially selected at the beginning of the test and measured for dry weight. The test compartments were observed daily to make visual assessments of abnormal behavior (e.g. leaving the sediment, climbing the walls of the test compartment). On Day 28 of the test, oligochaetes were removed from the sediment, and the numbers of live or dead worms were enumerated. Since *Lumbriculus variegatus* generally reproduces over a 28-day period, an increase in the number or size of worms in each test compartment is not unexpected. However, it is not possible to differentiate between adults and young. Therefore, survival and reproduction were considered one endpoint, i.e. the total number of organisms present at test termination.

Dry Weight Measurements

At test termination, each surviving worm was rinsed of excess sediment, placed in a pre-weighed labeled aluminum pan and dried for approximately six days at approximately 60°C. The worms were weighed after allowing the pans to cool at room temperature for approximately one hour.

Statistical Analyses

The results of the test were based on the mean measured sediment concentrations (calculated using the measured concentrations in the biotic replicates only). The 28-day EC50 value, based on survival and reproduction was determined using the statistical program TOXSTAT version 3.5 (10).

The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival/reproduction and dry weight data. Using the statistical program TOXSTAT Version 3.5 the survival/reproduction and dry weight (growth) data were evaluated for normality (Chi-Square Test) and homogeneity of variances (Bartlett's Test or Levene's Test). After the data was deemed normal with homogeneous variance, the survival/reproduction and dry weight data were analyzed using Dunnett's t-test to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group (10,11).

Analytical Sampling

Analyses of sediment were conducted using separate test compartments collected from the control group and from each concentration treatment groups just after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28. The replicate used for sample collection on Day 0 did not contain oligochaetes. The Day 7 and Day 28 analytical replicates each had a biotic and an abiotic replicate. The samples were analyzed on the day of collection.

Analytical Method

The analytical method used for the analysis of L3 was developed by Wildlife International, Ltd. Sediment was sampled from the test beakers and approximately 5 g of sediment was added to each French square bottle. To each sample, approximately 50 mL of ethyl acetate was added. Samples were sonic disrupted for five minutes and the sediment was allowed to settle for approximately two minutes. An aliquot of the extract was removed and centrifuged at 14,500 for approximately 10 minutes. Secondary dilutions were made in ethyl acetate, as necessary and the extracts were transferred into autosampler vials and submitted for analysis.

Concentrations of L3 in the samples were determined using an Agilent Model 6890 Gas Chromatograph (GC) with an Agilent Model 5975 Mass Selective Detector operated in SIM mode. Chromatographic separations were achieved using a DB-5 column (30 m x 0.25 mm, 0.25 µm film thickness). A method flowchart for the analysis of L3 is presented in Appendix 3.1 and typical instrumental parameters are summarized in Appendix 6.2.

Calibration standards of L3, ranging in concentration from 0.0700 to 2.50 mg a.i./L were prepared in ethyl acetate using a stock solution of L3 in acetone. Calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using peak area responses versus the respective concentrations of the calibration standards. The concentration of L3 in the samples was determined by substituting the ratio 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 6.4.

The method limit of quantitation (LOQ) varied slightly from sample to sample. The LOQ was 0.07 mg a.i./L, calculated as the product of the low calibration standard (0.007 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).

Sediment samples were fortified at 3.00, 20.0 and 100 mg a.i./Kg using stock solutions of L3 in acetone (Appendix 6.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 51.8 to 85.5% of nominal concentrations (Appendix 6.5).

A representative calibration curve is presented in Appendix 6.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 6.7 and 6.8, respectively. Representative chromatograms of a matrix blank and matrix fortification sample are presented in Appendices 6.9 and 6.10, respectively. A representative chromatogram of a test sample is presented in Appendix 6.11.

RESULTS AND DISCUSSION

Measurement of Test Concentrations

Results of analyses to measure concentrations of L3 in samples collected during the definitive test are presented in Table 1 and Appendix 6. Nominal concentrations selected for use in this definitive test were 3.1, 6.3, 13, 25, 50 and 100 mg/Kg.

Samples of sediment were collected from the negative control and each of the test concentrations on Day 0, Day 7 and Day 28. The purpose of these samples was to verify the concentrations in sediment at test initiation and to monitor the test substance over the course of the study. Results are based on mean measured concentrations. Negative control samples on Days 0, 7, and 28 were all below the limit of quantitation (LOQ).

Measured concentrations of L3 in sediment are presented in Table 1. On Day 0, there were 0.759, 1.47, 1.68, 5.45, 9.55 and 19.9 mg/Kg measured in the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg treatment groups, respectively. Measured concentrations of L3 in sediment from the "biotic" replicates from the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg nominal test concentrations on Day 7 were 0.565, 1.08, 1.29, 4.34, 9.98 and 18.4 mg/Kg, respectively. Measured concentrations of L3 in

sediment from the "abiotic" replicates from the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg nominal test concentrations on Day 7 were 0.581, 0.818, 1.67, 4.96, 7.20 and 16.5 mg/Kg, respectively. Measured concentrations of L3 in sediment from the "biotic" replicates from the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg nominal test concentrations on Day 28 were 0.620, 0.895, 1.72, 4.62, 7.26 and 12.8 mg/Kg, respectively. Measured concentrations of L3 in sediment from the "abiotic" replicates from the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg nominal test concentrations on Day 28 were 0.598, 0.866, 1.64, 3.33, 6.05 and 18.5 mg/Kg, respectively. All concentrations showed a stable trend in the sediment throughout the study. The mean measured test concentrations (using the results of the biotic replicates only) for the 3.1, 6.3, 13, 25, 50 and 100 mg/Kg treatment groups were 0.65, 1.1, 1.6, 4.8, 8.9 and 17 mg/Kg respectively, representing 21, 17, 12, 19, 18 and 17 percent of nominal, respectively.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4, respectively. Temperatures were within the 23 ± 1°C range established for the test. Dissolved oxygen concentrations were ≥ 85% (7.2 mg/L) of saturation throughout the test. Measurements of pH ranged from 8.2 to 8.4 during the test. Measurements of hardness, alkalinity, conductivity and ammonia are presented in Table 5 and are typical for Wildlife International, Ltd. well water.

Observations of oligochaetes in individual replicates at each observation interval are presented in Appendix 7. All replicates appeared normal during the test, with few observations of abnormal behavior in the treatment and control groups. At test termination, there was an increase in the numbers of oligochaetes present in some test compartments from the 10 worms originally placed in each replicate, indicating that reproduction had occurred. The mean number of worms in the negative control group at test termination was 17.3 worms (Table 6). The mean number of worms in the 0.65, 1.1, 1.6, 4.8, 8.9 and 17 mg/Kg treatment groups was 15.8, 15.1, 11.8, 11.5, 9.1 and 9.8, respectively, at test termination. There were statistically significant differences ($p < 0.05$) in the 1.6, 4.8, 8.9 and 17 mg/Kg treatment groups when compared to the negative control group, therefore the no observed effect concentration (NOEC) was 1.1 mg/Kg, and the lowest observed test concentration (LOEC) was determined to be 1.6 mg/Kg.

At the beginning of the test, a group of 20 organisms from the culture were impartially selected and used for the determination of dry weight. It was determined that the average individual dry weight of those twenty organisms was 0.59 mg (Appendix 8). The average individual dry weights for the surviving worms in each replicate at test termination are presented in Appendix 8. The average dry weight per worm in the negative control group was 1.34 mg (Table 6). The average dry weight per worm in the 0.65, 1.1, 1.6, 4.8, 8.9 and 17 mg/Kg treatment groups was 1.48, 0.91, 1.15, 0.98, 1.75 and 1.47 mg, respectively. The dry weights of the treatment groups were not significantly different ($p > 0.05$) from the negative control weights, therefore the NOEC was determined to be 17 mg/Kg and the LOEC was determined to be >17 mg/Kg.

CONCLUSIONS

The 28-day EC50 value for oligochaetes (*Lumbriculus variegatus*) exposed to L3 in sediment was >17 mg/Kg, the highest concentration tested. Determination of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) was based on an evaluation of the survival/reproduction and dry weight data. Based on the survival/reproduction results of this study, the LOEC was 1.6 mg/Kg dry weight of sediment and the NOEC was 1.1 mg/Kg dry weight of sediment.

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Table 1

Measured Concentrations of L3 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-115-)	Sampling Time (Days)	Measured Concentration (mg/Kg)	Pereent of Nominal[1]	Mean Measured Concentration (mg/Kg)	Mean Percent of Nominal[1]
Negative Control	1*	0	<LOQ	--		
	8	7	<LOQ	--		
	9[3]	7	<LOQ	--		
	22	28	<LOQ	--		
	23[3]	28	<LOQ	--		
3.1	2*	0	0.759	24.5	0.65	21
	10	7	0.565	18.2		
	11[3]	7	0.581	18.7		
	24	28	0.620	20.0		
	25[3]	28	0.598	19.3		
6.3	3*	0	1.47	23.4	1.1	17
	12	7	1.08	17.2		
	13[3]	7	0.818	13.0		
	26	28	0.895	14.2		
	27[3]	28	0.866	13.8		
13	4	0	1.68	12.9	1.6	12
	14	7	1.29	9.95		
	15[3]	7	1.67	12.9		
	28	28	1.72	13.3		
	29[3]	28	1.64	12.7		
25	5	0	5.45	21.8	4 8	19
	16	7	4.34	17.4		
	17[3]	7	4.96	19.8		
	30	28	4.62	18.5		
	31[3]	28	3.35	13.4		

[1] The limit of quantitation (LOQ) was 0.07 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.007 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).
[2] Results were generated using Exeel 2000 in the full precision mode. Manual calculations may differ slightly.
[3] Abiotic samples; not included in the mean calculation.
* Sample reanalyzed.

Table 1 (Continued)

Measured Concentrations of L3 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Lumbriculus variegatus*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-115-)	Sampling Time (Days)	Measured Concentration (mg/Kg)	Percent of Nominal[1,2]	Mean Measured Concentration (mg/Kg)	Mean Percent of Nominal[1,2]
50	6	0	9.55	19.1	8.9	18
	18	7	9.98	20.0		
	19[3]	7	7.20	14.4		
	32	28	7.26	14.5		
	33[3]	28	6.05	12.1		
100	7	0	19.9	19.9	17	17
	20	7	18.4	18.4		
	21[3]	7	16.5	16.5		
	34	28	12.8	12.8		
	35[3]	28	18.5	18.5		

[1] The limit of quantitation (LOQ) was 0.07 mg a.i./Kg, calculated as the product of the concentration of the lowest calibration standard (0.007 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).
[2] Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.
[3] Abiotic samples; not included in the mean calculation..
* Sample reanalyzed.

Table 2

Temperature of Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day	Temperature (°C)													
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13
	Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control		22.7	22.8	21.8	22.0	22.8	22.2	22.7	22.7	22.5	22.4	22.3	22.5	22.6	22.6
0.65		22.9	22.9	21.5	21.8	22.8	22.2	22.5	22.9	22.7	22.6	22.4	22.6	22.7	22.8
1.1		22.8	22.6	21.6	21.7	22.7	22.2	22.7	22.9	22.6	22.6	22.5	22.6	22.6	22.7
1.6		22.7	22.8	21.5	21.6	22.8	22.1	22.6	23.0	22.5	22.6	22.4	22.7	22.7	22.8
4.8		22.8	22.9	21.8	21.6	22.7	22.1	22.5	22.8	22.6	22.6	22.4	22.6	22.7	22.8
8.9		22.8	22.8	21.5	21.5	22.8	22.1	22.6	22.7	22.5	22.7	22.6	22.6	22.7	22.8
17		22.8	22.9	21.5	21.5	22.8	22.2	22.5	22.7	22.5	22.5	22.6	22.6	22.7	22.8

[1] Temperature measured continuously in the negative control diluter tank ranged from approximately 21.5 to 23.0°C during the test.

Table 2 (Continued)

Temperature of Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day	Temperature (°C)														
		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E
Negative Control		22.7	22.6	22.5	22.5	22.2	22.8	22.7	22.5	22.6	22.8	22.5	22.5	22.8	22.5	22.7
0.65		22.7	22.9	22.7	22.7	22.2	22.7	22.8	22.5	22.7	22.8	22.6	22.5	22.8	22.7	22.7
1.1		22.7	23.0	22.5	22.7	22.3	22.8	22.8	22.5	22.6	22.8	22.7	22.6	22.8	22.7	22.8
1.6		22.5	22.9	22.6	22.7	22.1	22.8	22.8	22.4	22.5	22.7	22.6	22.6	22.8	22.4	22.7
4.8		22.6	22.7	22.6	22.5	22.1	22.7	22.8	22.5	22.5	22.9	22.7	22.5	22.9	22.5	22.8
8.9		22.7	22.8	22.6	22.6	22.1	22.7	22.7	22.5	22.7	22.7	22.5	22.5	22.9	22.6	22.6
17		22.6	22.8	22.6	22.7	22.2	22.7	22.8	22.6	22.5	22.8	22.7	22.6	22.8	22.7	22.8

Table 3

Dissolved Oxygen in Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day	Dissolved Oxygen (mg/L)													
		0[1]	1	2	3	4	5	6	7	8	9	10	11	12	13
	Replicate:	A	B	C	D	E	F	G	H	A	B	C	D	E	F
Negative Control		8.6	8.2	8.0	7.7	8.2	8.3	8.4	7.7	8.4	8.3	8.2	8.0	8.0	8.3
0.65		8.5	8.1	8.0	7.7	8.0	8.2	8.3	7.6	8.4	8.0	8.1	7.9	7.7	8.3
1.1		8.5	8.1	8.1	7.6	8.1	8.1	8.3	7.6	8.4	7.9	8.0	7.7	7.7	8.4
1.6		8.5	8.1	8.1	7.6	8.2	8.1	8.3	7.4	8.4	7.9	8.1	7.8	7.8	8.1
4.8		8.5	8.0	8.0	7.7	8.3	8.0	8.3	7.5	8.5	8.0	8.0	7.7	7.7	8.3
8.9		8.5	8.2	8.0	7.8	8.1	8.0	8.3	7.4	8.5	8.0	8.1	7.8	7.7	8.3
17		8.6	8.0	8.1	7.8	8.1	8.0	8.3	7.3	8.4	8.0	7.9	7.7	7.7	8.2

[1] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

Table 3 (Continued)

Dissolved Oxygen in Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day	Dissolved Oxygen (mg/L)														
		14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Replicate:	G	H	A	B	C	D	E	F	G	H	A	B	C	D	E
Negative Control		7.9	8.3	8.3	8.4	8.5	8.6	7.9	8.1	8.1	8.3	7.9	7.6	7.6	7.7	7.6
0.65		7.7	8.1	8.2	8.2	8.4	8.6	7.7	7.9	8.0	8.2	7.7	7.2	7.5	7.7	7.6
1.1		7.6	7.9	8.2	8.2	8.4	8.6	7.7	7.9	8.2	8.2	7.7	7.7	7.5	7.7	7.3
1.6		7.5	7.8	8.1	8.1	8.5	8.6	7.7	7.9	7.8	8.1	7.7	7.8	7.5	7.7	7.6
4.8		7.4	7.8	8.1	8.1	8.5	8.6	7.6	8.2	7.9	8.0	7.5	7.4	7.5	7.2	7.4
8.9		7.4	7.9	8.2	8.1	8.5	8.6	7.6	7.8	7.8	8.0	7.2	7.6	7.5	7.8	7.5
17		7.4	7.8	8.1	8.1	8.5	8.6	7.6	7.8	7.9	8.0	7.3	7.6	7.6	7.7	7.6

[1] A dissolved oxygen concentration of 5.1 mg/L represents 60% saturation at 23.0°C.

Table 4

pH of Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Replicate:	pH				
		Day 0	Day 7	Day 14	Day 21	Day 28
		A	H	G	F	E
Negative Control		8.3	8.2	8.3	8.3	8.4
0.65		8.4	8.2	8.3	8.3	8.3
1.1		8.4	8.2	8.3	8.3	8.3
1.6		8.4	8.2	8.3	8.3	8.4
4.8		8.4	8.2	8.3	8.3	8.4
8.9		8.4	8.2	8.3	8.3	8.4
17		8.4	8.2	8.3	8.3	8.4

Table 5

Hardness, Alkalinity, Conductivity and Ammonia of the Overlying Water in the Test Compartments

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/cm)	Day 0 Ammonia (mg/L)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)	Day 28 Ammonia (mg/L)
Negative Control	144	182	370	<0.17[1]	132	178	380	0.375
17	144	184	375	<0.17[1]	136	178	382	0.371

Measurements were taken in a composite made from each of the replicates of the respective treatment groups.
[1]0.17 mg/L = the lowest standard used to calibrate the meter.

Table 6

Mean Survival/Reproduction and Growth of Oligochaetes (*Lumbriculus variegatus*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg dry weight)	Mean Number of Surviving Worms (± Stdev)[1]	Percent Reduction[2] (%)	Mean Individual Dry Weight (mg)	Percent Reduction[2,3] (%)
Negative Control	17.3 (± 4.4)	-	1.34	--
0.65	15.8 (± 6.8)	8.7	1.48	-10
1.1	15.1 (± 2.5)	13	0.91	32
1.6	11.8 (± 3.4)	32*	1.15	14
4.8	11.5 (± 2.6)	34*	0.98	27
8.9	9.1 (± 3.3)	47*	1.75	-31
17	9.8 (± 1.8)	43*	1.47	-9.7

[1] Each replicate contained 10 oligochaetes at test initiation. The mean number of surviving worms is based on the total number of living worms at test termination since offspring cannot be differentiated from adults.

[2] Percent reduction was calculated in relation to the mean of the negative control.

* There were statistically significant differences ($p<0.05$) in the mean number of surviving worms in comparison to the negative control group using Dunnett's test.

[3] There were no statistically significant differences ($p>0.05$) in the mean individual dry weight of surviving worms in comparison to the negative control group using Dunnett's test.

Appendix 1

Study Protocol and Deviation

PROTOCOL

OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

Guideline:

OECD Guideline 225

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
570A-115

SPONSOR

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

PERFORMING LABORATORY

Wildlife International, Ltd.

8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

September 11, 2008

Wildlife International, Ltd.

OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *Lumbriculus variegatus* USING SPIKED SEDIMENT

SPONSOR: Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

SPONSOR'S REPRESENTATIVE: Kurt R. Drottar
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Health and Environmental Sciences
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STUDY DIRECTOR: Susan T. Thomas, Biologist
Wildlife International, Ltd.

LABORATORY MANAGEMENT: Henry O. Krueger Ph.D.
Director of Aquatic, Plant and Insect Toxicology

LABORATORY QUALITY ASSURANCE MANAGER: Linda R. Mitchell
Director of Regulatory
and Ecotox Operations
Wildlife International, Ltd.

PROTOCOL APPROVAL

Susan Thomas — 3/25/09
STUDY DIRECTOR — DATE

[illegible] — 3/25/09
LABORATORY MANAGEMENT — DATE

[illegible] — 9/12/08
SPONSOR'S REPRESENTATIVE — DATE

Wildlife International, Ltd.

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: April 2, 2009 Experimental Termination Date: April 30, 2009

Project No.: 570A-115

Test Concentrations: Negative Control, 3.1, 6.3, 13, 25, 50 and 100 mg/Kg

Test Substance No.: 8690 Reference Substance No. (if applicable): NA

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INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the oligochaete, *Lumbriculus variegatus*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon a study design proposed by Phipps et al. (1993): Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants (1); OECD Guideline 225, *Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment* (2), the ASTM Standard E 1706-00: *Standard Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Fresh Water Invertebrates* (3); the U.S. EPA Series 850 - Ecological Effects Test Guidelines, OPPTS Number 850.1735: *Whole Sediment Acute Toxicity Invertebrates, Freshwater* (4), the European Chemicals Bureau Technical Guidance Document on Risk Assessment, Part II (5), and the Federal Environment Agency: Validation of the Preliminary EU-Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances (6). Raw data for all work performed at Wildlife International, Ltd. and 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.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the oligochaete, *Lumbriculus variegatus*, during a 28-day exposure period under flow-through conditions. Flow-through test conditions will be used to maintain the overlying water quality. The sediment used in the test will have an organic content of approximately 5%. A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after the sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding organisms. The measured endpoints of the test are survivorship and growth as determined by dry weight measurements.

EXPERIMENTAL DESIGN

Groups of oligochaetes will be exposed to a geometric series of at least five test concentrations and a negative (untreated sediment) control for approximately 28 days. Eight replicate test compartments will be maintained in each treatment and control group, with 10 oligochaetes in each compartment for a total of 80 individuals per test concentration. Each test compartment will

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contain a quantity of sediment and overlying water. Additional replicate test compartments will be included for analytical sampling of sediment. No oligochaetes will be placed in "analytical" replicate(s) sampled at the beginning of the test However, the "analytical" replicate(s) sampled after the beginning of the test (e.g., on Day 7 and at the end of the test) will contain oligochaetes. An additional two " abiotic replicates" (analytical replicates without oligochaetes) will be added to each treatment group for analysis on Day 7 and Day 28 of the test.

Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate.

The water solubility of Octamethyltrisiloxane (L3; CAS No. 107-51-7) is 34.49 µg/L (7). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of Octamethyltrisiloxane in water. Therefore, only sediment samples from the analytical sampling test compartments will be collected from each control and treatment group at specified intervals for analysis of the test substance. Results of the analyses will be used to verify the exposure over time. The results of the study will be based on the nominal test concentrations in sediment, when appropriate.

To control bias, oligochaetes will be impartially assigned to exposure compartments at test initiation. No other potential sources of bias are expected to affect the results of the study. Survivorship and growth (dry weight) will be determined at the end of the test period (maximum of 28 days). Mortality in the treatment groups will be used to calculate, when possible, the EC50 value. The dose-response pattern and appropriate statistical analyses of survivorship and growth will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC).

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MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	Octamethyltrisiloxane (L3) (Supplied as Dow Corning® 200 Fluid, 1 est.)
Lot No.:	0002680862
Expiration Date:	May 27, 2010
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	107-51-7
Physical Description:	Colorless Liquid
Chemical Stability:	Stable according to the MSDS
Purity:	99.9 ± 0.03 area % purity
Solubility:	34.49 µg/L
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained.

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a 1.5 kg batch of sediment to 5%. The peat and test substance will be mixed overnight (approximately 12 hours) on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. Approximately 100 mL of the dry mix will then be transferred to 13 replicate test beakers. The test replicates will be

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300-mL glass beakers with stainless steel mesh-covered holes on opposite sides of the beaker. The transfer time will be kept to a minimum (approximately 10-20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance.

After all 13 beakers have been filled with sediment, a 28 day ration of food (approximately 200mg of Salmon Starter) will be added and gently stirred into the dry sediment. Finally, approximately 150mL of well water will be added to the beakers. After the beakers have been filled with water, they will be transferred to a water bath that will be used for the test. In the water bath the overlying water will be exchanged twice per day by adding one volume addition every 12 hours to each beaker and allowing water to passively flow out the holes in the sides of the beakers. After approximately 48 hours of acclimation and settling, organisms will be added to the beakers.

Test Organism

The oligochaete, *Lumbriculus variegatus*, has been selected as the test species for this study. Oligochaetes represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Adult oligochaetes will be obtained from a commercial supplier or other reliable source, or from cultures maintained by Wildlife International, Ltd. Prior to the test, the organisms will be held in a container with sand and overlying water from the same source and at approximately the same temperature as will be used in the test. Enough food for 28 days (salmon starter) will be incorporated into the sediment during the test. Specifications for acceptable levels of contaminants in feed for oligochaetes have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 225 (2) will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20%silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon

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content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (salmon starter) will be dry mixed into the test compartments after sediment has been added, but immediately before adding water to the test compartment, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. Just as in the OECD 218 testing guideline, the food added to the test system during the test is not included in the measurement of organic carbon.

Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of a similar representative batch of formulated sediment and results of the analyses will be summarized in the final report. In addition, a sample of the sediment used for the study will be sent to Agvise Laboratories, Northwood, ND (or similar facility) to determine the sediment composition.

Dilution Water

Water used for the holding and testing of oligochaetes will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove fine particles. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test will be provided in the final report. Analyses will be performed at least once

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annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the most recent analyses will be summarized in the final report.

Test Apparatus

The test apparatus will consist of a Wildlife International, Ltd. diluter unit that is designed to hold up to 14 tanks in a temperature controlled water bath. Each tank can receive renewal rates that can range from static to greater than 10 tank volume additions per day.

Test compartments will consist of 300 mL glass beakers with stainless-steel mesh-covered holes on opposite sides of the beaker. The compartments will be labeled with the project number, test concentration and replicate. All replicate beakers in each treatment group will be indiscriminately positioned in one or more diluter tanks. Each beaker will contain approximately 100 ml of sediment and approximately 150 ml of overlying water. Dilution water will be delivered directly into each beaker, passively forcing water out through the holes in the sides of the beakers and exchanging the water overlying the sediment. Each replicate beaker will receive approximately two-volume additions of water per day. Dilution water delivery will be verified by calibration prior to initiation of the test. Proper system operation will be verified at least one time daily during the test.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 23 ± 1°C. Temperature will be measured using a hand-held liquid-in-glass thermometer in the overlying water in one alternating replicate test compartment of each experimental group at the beginning and end of the test and at least three times per week during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test compartments or in the negative control diluter tank. Recorder measurements will be verified with a hand-held liquid-in-glass thermometer prior to test initiation.

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Dissolved oxygen will be measured in the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least three times per week during the test. Dissolved oxygen will be measured using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent. In the event that dissolved oxygen levels approach or fall below 60% saturation, the Sponsor will be notified, and appropriate corrective actions will be taken, if necessary, to maintain dissolved oxygen levels above 60% saturation.

Measurements of pH will be made on a sample of the overlying water from one alternating replicate of each experimental group at the beginning and end of the test and at least once per week. Water pH measurements will be made using a Thermo Orion Model 525Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, and then discontinued.

Hardness, alkalinity, specific conductance and ammonia will be measured in a composite sample of overlying water from the control group replicates and the highest concentration treatment group replicates at the beginning and end of the test. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (8). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two adult oligochaetes will be impartially distributed to transfer containers (e.g., glass beakers) until each container holds its complement of 10 individuals. The organisms in each transfer container then will be transferred below the air/water interface to the test compartments. An additional batch of 20 individuals will be impartially selected at the beginning of the test and will be measured for dry weight. After completing the transfer of organisms, the test compartments will be maintained in temperature-controlled diluter tanks. Test compartments will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming).

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Observations will be made at test termination to determine the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior. On Day 28, oligochaetes will be segregated from the sediment and live and dead organisms will be enumerated. When the total numbers of individuals found in each replicate at test termination are fewer than the number initially placed in each replicate, then those missing will be considered dead. Dry weights of surviving oligochates in each replicate will be measured at the end of the test to evaluate effects on growth.

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test compartments will be collected from each concentration at the beginning of the test (no more than one hour after completing the addition of organisms to the test system), on Day 7, and at the end of the test. There will be two additional abiotic replicates in each test group so that additional samples can be collected on Day 7 and Day 28. The overlying water will be removed from the test compartment. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen) until analyzed. The sampling scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

	Day 0	Day 7		Termination (28)	
Experimental Group	Sed	Sed	Abiotic	Sed	Abiotic
Control	1	1	1	1	1
Solvent Control (if needed)	1	1	1	1	1
Level 1-Low Concentration	1	1	1	1	1
Level 2	1	1	1	1	1
Level 3	1	1	1	1	1
Level 4	1	1	1	1	1
Level 5-High Concentration	1	1	1	1	1
	7	7	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study. At the discretion of the Study Director, samples from one

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or more appropriate test compartments and/or stock solutions will be collected and analyzed if an analytical error in sampling or analysis is suspected. The reason for the additional samples will be described by the Study Director and documented in the raw data and final report.

Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Data Analysis

When the dose-response pattern allows calculation of an EC50 value, the mortality data will be analyzed using the computer software of C.E. Stephan (9). The program was designed to calculate the EC50 value and the 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (10, 11, 12). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test.

Growth (dry weight) data will be evaluated for normality and homogeneity of variances using TOXSTAT Version 3.5 (13). If the data are deemed normal with homogeneous variances, hypothesis testing using analysis of variance (ANOVA) and multiple means tests (e.g., Dunnett's, Bonferroni, Scheffe) will be used (12,13). If the data fail the tests for normality or homogeneity, then data transformations will be tried in an attempt to correct the condition. When the data transformations fail to correct for non-normality or heterogeneity of variances, nonparametric procedures will be used to identify statistically significant differences among the experimental groups. Additional analysis of data may be conducted if deemed appropriate by the Study Director. The results of the analysis will be documented in the raw data and summarized in the final report.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.

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2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Lumbriculus variegatus* culture records.
5. Stock solution calculation and preparation, if applicable, and calculation and preparation of test concentrations.
6. Biological observations.
7. Test conditions (light intensity, photoperiod, etc.).
8. Water chemistry results (e.g., hardness and alkalinity).
9. Statistical calculations, if applicable.
10. The methods used to analyze test substance concentrations and the results of analytical measurements, if applicable.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.
6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.

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10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and compartments per chamber per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the quality Assurance Unit listing the dates that study inspections and audits were made and the dates that any findings were reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

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

GOOD LABORATORY PRACTICES

This study will be conducted in accordance with Good Laboratory Practice Standards for EPA (40 CFR Part 160 and/or Part 792); OECD Principles of Good Laboratory Practices (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with

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Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

1 **Phipps, G.L., G.T. Ankley, D.A. Benoit, and V.R. Mattson.** 1993. Use of the Aquatic Oligochaete *Lumbriculus variegatus* for Assessing the Toxicity and Bioaccumulation of Sediment-associated Contaminants. Environ. Toxicol. Chem. 12:269-279.

2 **OECD Guideline 225.** 2007. Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment. Organization for Economic Cooperation and Development. Adopted 16 October 2007.

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6 **Federal Environment Agency.** 2001. *Validation of the Preliminary EU- Concept of Assessing the Impact of Chemicals to Organisms in Sediment by Using Selected Substances.* Institute of Hydrobiology Dresden University of Technology.

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DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Octamethyltrisiloxane (L3; CAS No 107-51-7): A Prolonged Sediment Toxicity Test with *Lumbriculus variegatus* Using Spiked Sediment

PROTOCOL NO.: 570/091108/LUM-SED 28/SUB570 **DEVIATION NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-115

DATE OF DEVIATION: March 31, 2009

DEVIATION: The percent organic carbon content of the formulated sediment was 1.9 instead of 5.0 ± 1.0 as stated in the protocol.

REASON: The sediment was prepared using the composition described in the protocol. The carbon content can vary dependent upon the organic content of the peat. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

Susan Thomas
STUDY DIRECTOR

9/10/09
DATE

LABORATORY MANAGEMENT

9/10/09
DATE

Appendix 2

Certificate of Analysis

Dow Corning INTERNAL

The L3 was characterized according to GLPs [1]:

Identification:	Octamethyltrisiloxane (L3) (Supplied as Dow Corning® 200 Fluid, 1 cst.)
Batch Number:	0002680862
Expiration Date:	May 27, 2010
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS Number:	107-51-7
Physical Description:	Colorless Liquid
Stability:	Stable according to the MSDS
Purity:	99.9 ± 0.03 area % purity
Water Solubility:	34.49 µg/L [2]
Storage Conditions:	Room Temperature
Archive:	A reserve sample was not retained.

References:

1. Stutts, D.H. 2006. Characterization of Octamethyltrisiloxane (L3) (Batch Number 0002680862). Dow Corning Report Number 2006-10000-56652.
2. Varaprath, S., C.L. Frye and J. Hamelink. 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263-1265.

Appendix 3

Formulated Sediment Preparation[1]

Constituents	Weight (g)
Quartz Sand	28,000
Kaolin Clay	8,000
Peat Moss	4,000

[1] The sand and clay were mixed in PK Twinshell mixer for 20 minutes and the dry sediment was stored under ambient conditions until used. The peat was mixed with neat test material and added at a later date. The pH of the final sediment mixture was 7.1.

Appendix 4

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

	Mean	Range
Specific Conductance (µS/cm)	359 (N = 4)	358 – 360
Hardness (mg/L as $CaC0_3$)	135 (N = 4)	132 – 140
Alkalinity (mg/L as $CaC0_3$)	178 (N = 4)	176 – 180
pH	8.1 (N = 4)	8.1 – 8.1

Appendix 5

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

Pesticides and Organics			
Component	Measured Concentration (µg/L)	Component	Measured Concentration (µg/L)
Aldrin	<0.010	Gamma BHC – Lindane	<0.010
Alpha BHC	<0.010	Gamma Chlordane	<0.010
Alpha Chlordane	<0.010	Guthion (Azinphos-methyl)	<3.0
Beta BHC	<0.010	HCB	<0.010
Bolstar	<2.0	Heptachlor	<0.010
Chlordane	<0.51	Heptachlor Epoxide	<0.010
Coumaphos	<3.0	Kepone	<0.20
Delta BHC	<0.010	Malathion	<2.0
Demeton-O	<2.0	Merphos	<6.1
Demeton-S	<2.0	Methoxychlor	<0.10
Diazinon	<3.0	Methyl Parathion	<2.0
Dichlorvos	<3.0	Mevinphos	<3.0
Dieldrin	<0.020	Mirex	<0.033
Disulfoton	<2.0	Naled	<2.0
Dursban (Chlorpyrifos)	<2.0	o,p-DDD	<0.020
Endosulfan I	<0.010	o,p-DDE	<0.020
Endosulfan II	<0.020	o,p-DDT	<0.020
Endosulfan Sulfate	<0.020	p,p-DDD	<0.020
Endrin	<0.020	p,p-DDE	<0.020
Endrin Aldehyde	<0.10	p,p-DDT	<0.020
Endrin Ketone	<0.020	Phorate	<2.0
EPN	<2.0	Ronnel	<2.0
Ethion	<2.0	Stirophos	<2.0
Ethoprop	<3.0	Telodrin	<0.010
Ethyl Parathion	<2.0	Tokuthion	<2.0
Famphur	<2.0	Toxaphene	<3.0
Fensulfothion	<9.1	Trichloronate	<2.0
Fenthion	<2.0	Trithion	<2.0

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 5

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Well Water[1]
Page 2

Metals			
Component	Measured Concentration (mg/L)	Component	Measured Concentration (mg/L)
Aluminum	< 0.200	Magnesium	12.3
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	7.23
Calcium	32.5	Selenium	< 0.0200
Chloride	3.1	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.4
Cobalt	< 0.0050	Sulfate	5.8
Copper	< 0.0100	Thallium	< 0.0300
Fluoride	0.52	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 6

The Analysis of L3 in Sediment

Appendix 6.1

Analytical Method Flowchart for the Analysis of L3 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF L3 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample sediment from the test beakers. Add the requisite volume of sediment (~5 g) 4 oz. French square bottle.

↓

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

↓

Add approximately 50 mL of ethyl acetate to each sample. Sonic disrupt the mixture for five minutes and allow the sediment to settle for approximately two minutes.

↓

Remove an aliquot of extract and centrifuge at 14,500 rpm for 10 minutes.

↓

Make secondary dilutions in ethyl acetate, as necessary.

↓

Transfer the extracts into autosampler vials and submit for analysis.

Appendix 6.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Agilent Model 5975 Mass Selective Detector operated in SIM mode
ANALYTICAL COLUMN:	DB-5 column (30 m x 0.25 mm, 0.25 µm)
INJECTOR TEMPERATURE:	325°C
RUN TIME:	14.52 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 7.00°C/minute Final temperature 1: 100°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 315°C Final hold time 2: 1.00 minute
DETECTOR TEMPERATURE:	250°C
INJECTION VOLUME:	1.00 µL
MAKE-UP GAS:	Helium
APPROXIMATE L3 RETENTION TIME:	5.5 minutes

Appendix 6.3

Analytical Stocks and Standards Preparation

Stock solutions of L3 were prepared by weighing 0.1001 g (weight corrected for purity) 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 acetone. This primary stock solution (1.00 mg a.i./mL) was diluted in acetone to produce a 0.100 mg a.i./mL stock solution. The 1.00 and 0.100 mg/mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in ethyl acetate using the 0.100 mg a.i./mL stock solution. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.100	70.0	100	0.0700
0.100	400	100	0.400
0.100	800	100	0.800
0.100	1300	100	1.30
0.100	1800	100	1.80
0.100	2500	100	2.50

Appendix 6.4

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-115-4, with a nominal concentration of 13 mg/Kg, were calculated using the following equations:

$$\text{L3 in sample (mg/Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor} \div \text{Dry/Wet Soil Mass}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of L3 in sample (mg/Kg)}}{\text{nominal concentration of L3 in sample (mg/Kg)}} \text{ X } 100$$

Peak Area = 112536
Y-intercept = 12227.7443
Slope = 1175427.3725
Dilution factor =10.0
Dry Soil Mass (g)/Wet Soil Mass (g) = 2.56 g/5.04 g = 0.508

$$\text{L3 in sample (mg/Kg)} = \frac{112536 - 12227.7443}{1175427.3725} \text{ X } 10.0 \div 0.508$$

Concentration of L3 in sample (mg/Kg) = 1.68 mg/Kg

$$\text{Percent of nominal concentration} = \frac{1.68 \text{ mg/Kg}}{13 \text{ mg/Kg}} \text{ X } 100$$

Percent of nominal concentration = 12.9%

Appendix 6.5

Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

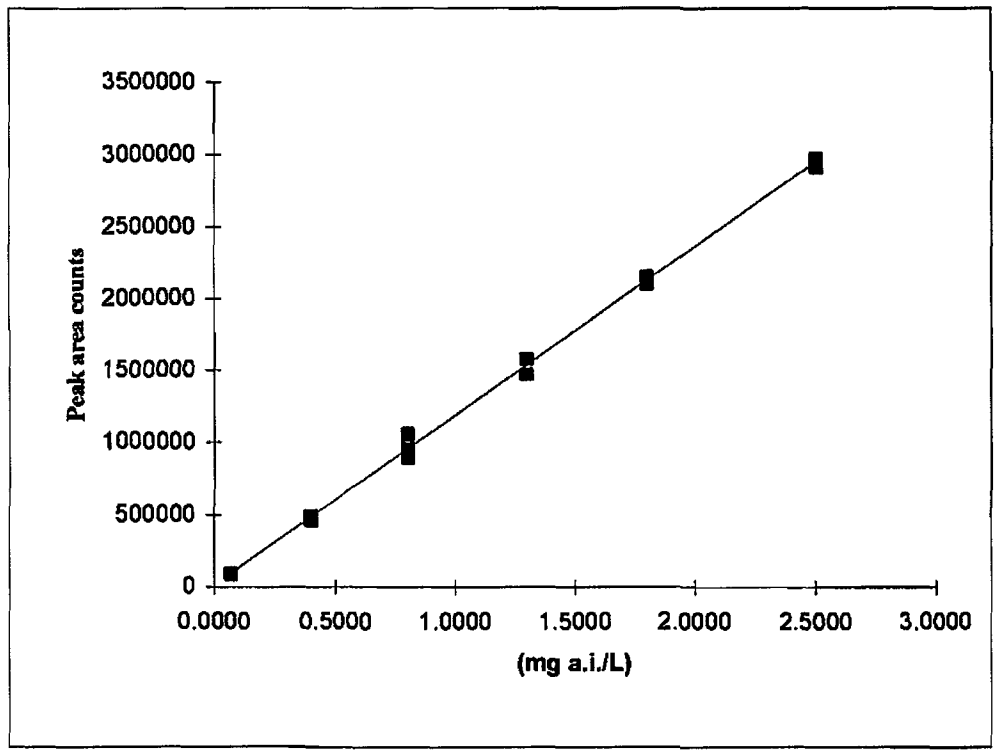
Sample Number (570A-115-)	Sample Type	Fortified Concentration (mg a.i./Kg)	Measured Concentration (mg a.i./Kg)[1]	Percent Recovery[2]
MAB-1	Matrix Blank	0.0	< LOQ	--
MAB-2	Matrix Blank	0.0	< LOQ	--
MAB-3	Matrix Blank	0.0	< LOQ	--
MAS-1	Matrix Fortification	3.00	2.09	69.6
MAS-2	Matrix Fortification	20.0	15.4	77.2
MAS-3	Matrix Fortification	100	85.5	85.5
MAS-4	Matrix Fortification	3.00	1.55	51.8
MAS-5	Matrix Fortification	20.0	10.9	54.3
MAS-6	Matrix Fortification	100	71.9	71.9
MAS-7	Matrix Fortification	3.00	2.03	67.6
MAS-8	Matrix Fortification	20.0	14.5	72.3
MAS-9	Matrix Fortification	100	83.1	83.1
			Mean =	70.4
			Standard Deviation =	11.5
			CV =	16.3%

[1] The limit of quantitation (LOQ) was 0.07 mg a.i./Kg, calculated as the product of the concentration of the lowest calibration standard (0.007 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).

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

Appendix 6.6

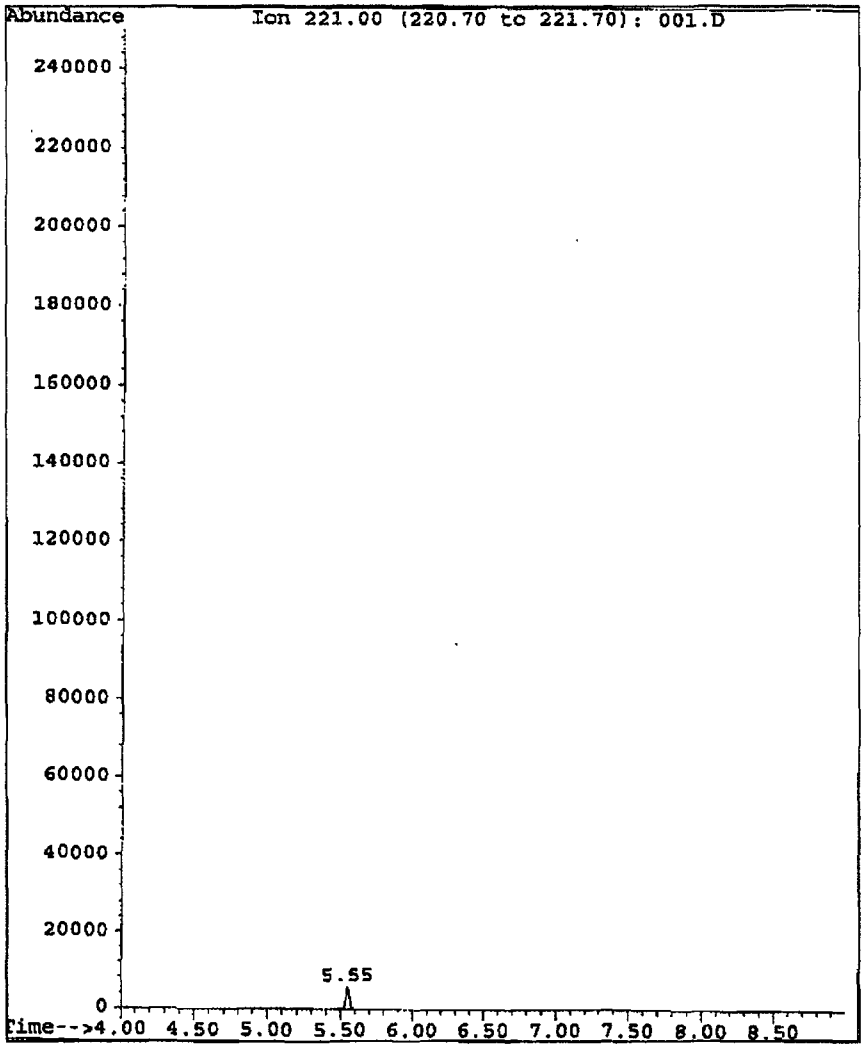
Representative Calibration Curve for L3 in Sediment



Slope = 1175427.3725; Y-Intercept = 12227.7443; $R^2 = 0.99777$

Appendix 6.7

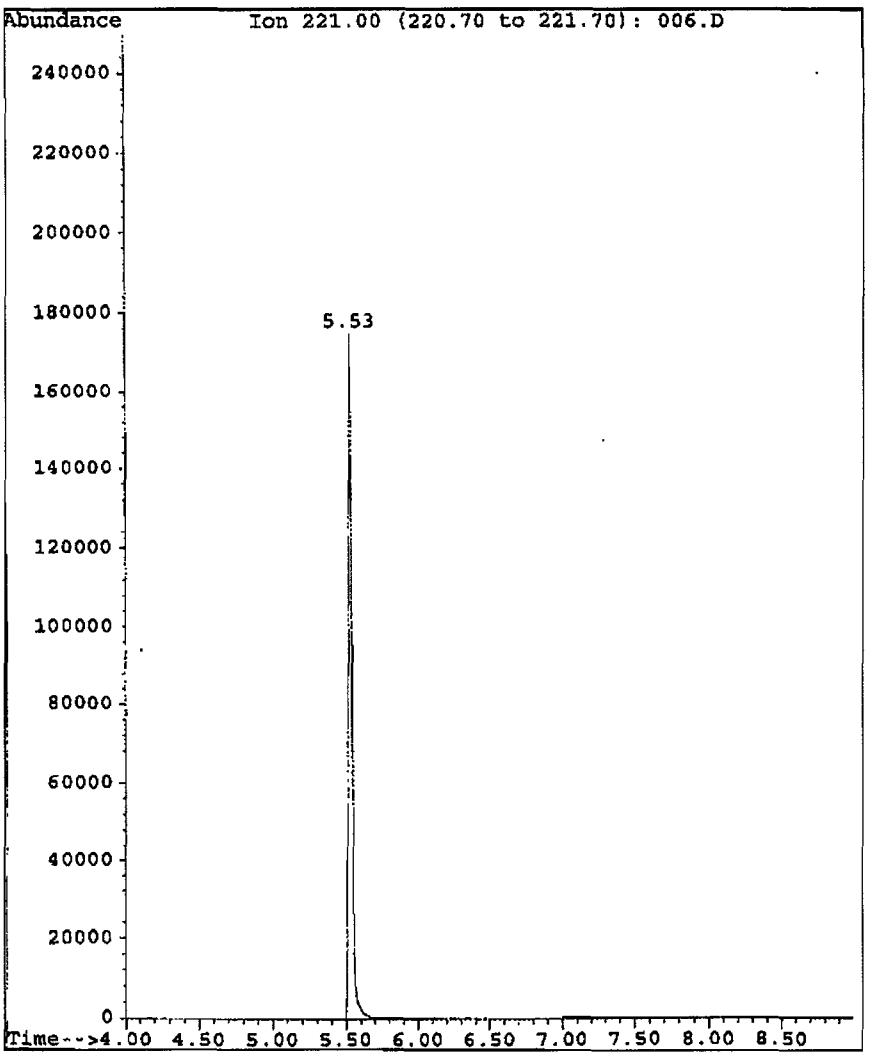
Representative Chromatogram of a Low-level L3 Calibration Standard



Nominal concentration: 0.0700 mg a.i./L

Appendix 6.8

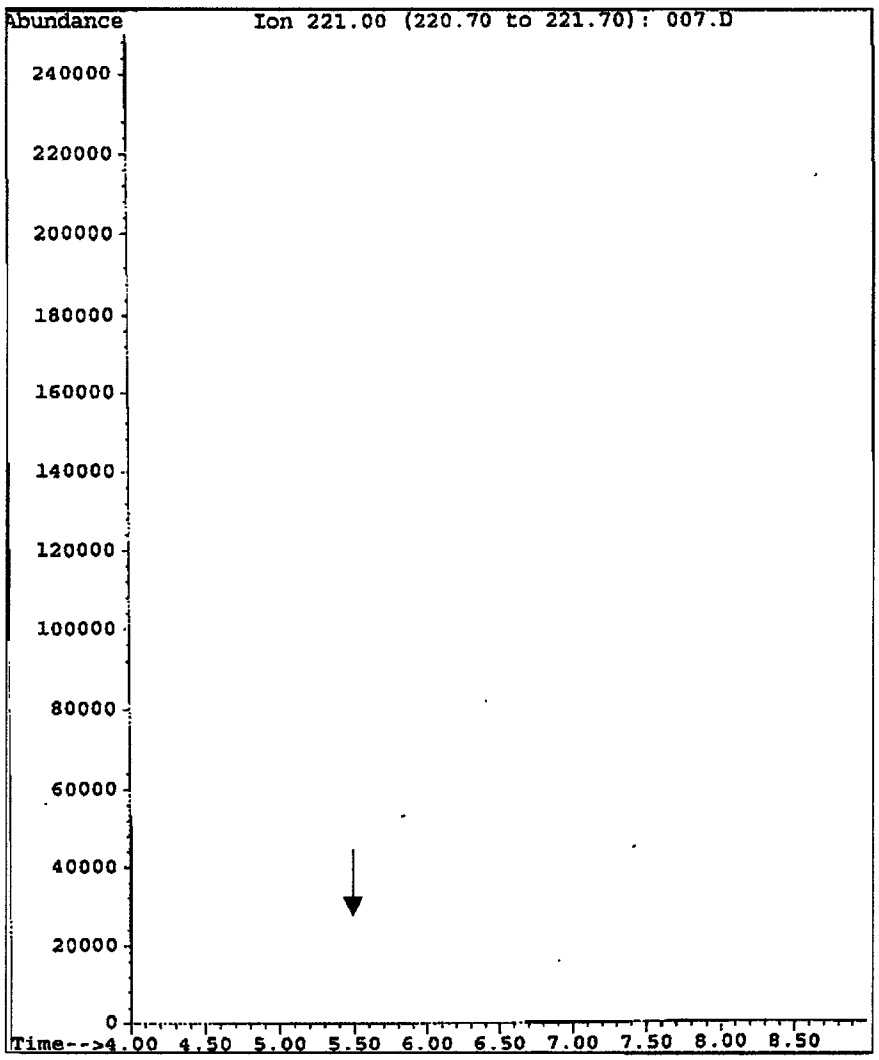
Representative Chromatogram of a High-level L3 Calibration Standard for Sediment



Nominal concentration: 2.50 mg a.i./L

Appendix 6.9

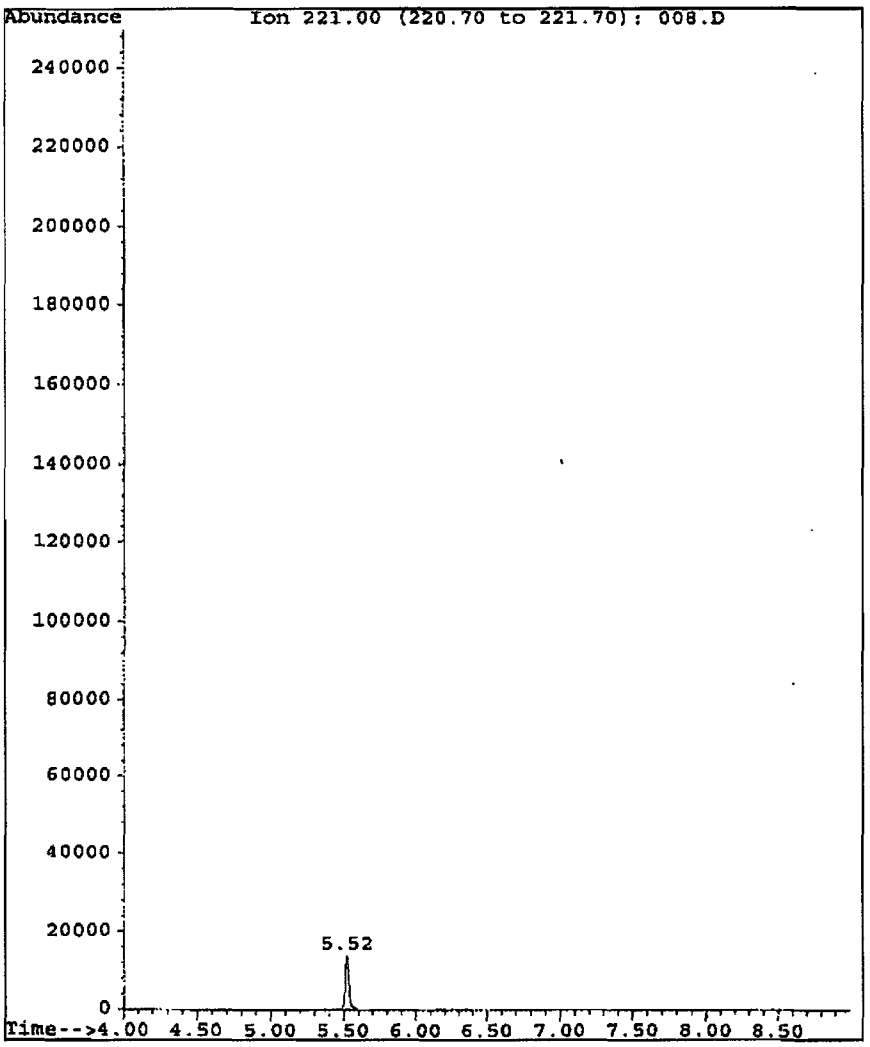
Representative Chromatogram of a Sediment Matrix Blank



Sample number 570A-115-MAB-1. The arrow indicates the approximate retention time of L3.

Appendix 6.10

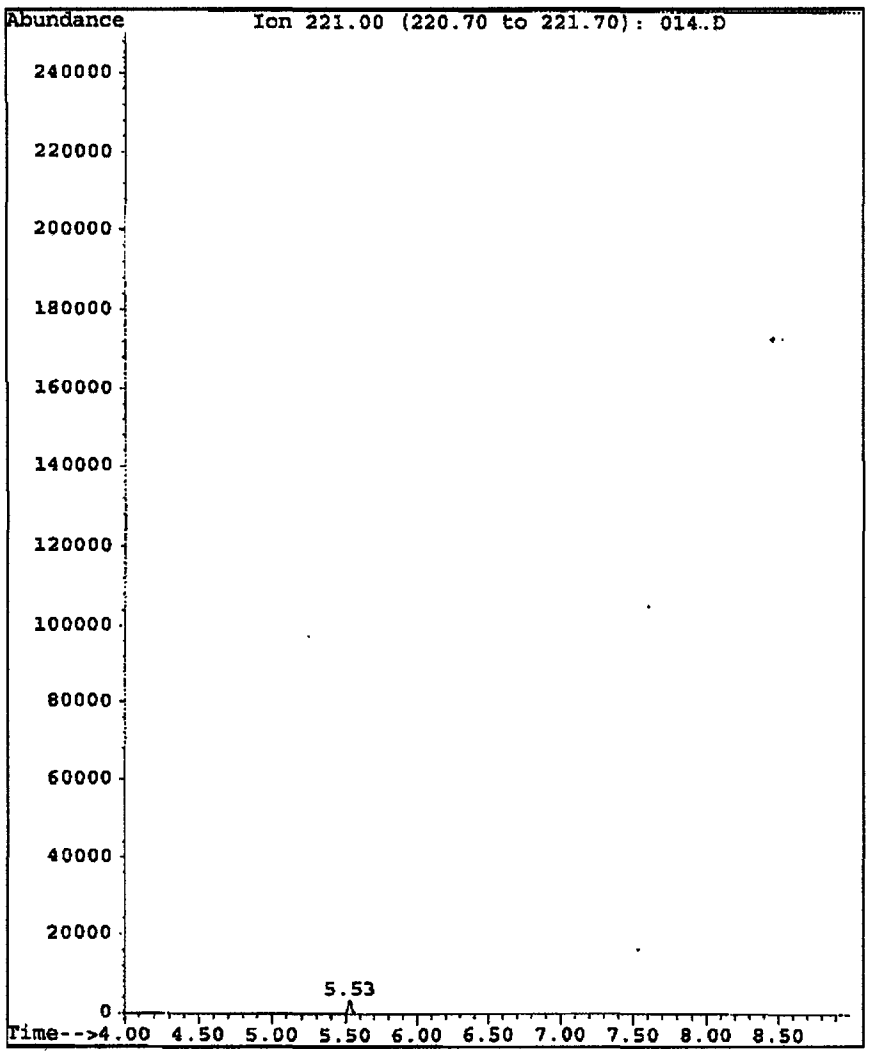
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-115-MAS-1; 3.00 mg/Kg, nominal concentration.

Appendix 6.11

Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-115-3; 6.3 mg/Kg nominal concentration

Appendix 7

Observations[1] of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
Negative Control	A	10	AN	AN	AN	AN	1G; AN	1H; AN	AN	1J; AN	1J; AN	AN	AN	1J; AN	2J; AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	1H; AN	1J; 1H; AN	1J; 1H; AN	AN	1J; AN	1J; AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	1J; AN	AN	3J; AN	AN	AN
	D	10	AN	AN	AN	AN	1H; AN	AN	AN	AN	AN	2J; 2H; AN	AN	1J; AN	1J; AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	1J; AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	1H; AN	AN	AN	AN	1J; 1H; AN	1H; AN	2H; AN	AN	1J; AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
Negative Control	A	10	AN	AN	AN	AN	AN	AN	AN	2J; AN	AN	AN	AN	AN	AN	AN
	B	10	1J; AN	AN	AN	AN	AN	3H; AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	1J; AN	AN	AN
	D	10	AN	AN	AN	1G; AN	AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

Appendix 7
Page 2
Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
0.65	A	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	1J; AN	AN	AN	1J; AN	AN
	B	10	AN	AN	AN	AN	AN	AN	1H; AN	AN	AN	1J; AN	AN	AN	AN	1J; AN
	C	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	1J; AN	1J; AN	AN	AN	AN
	D	10	AN	AN	1H; AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	1J; AN
	E	10	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	1J; AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	1J; AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
0.65	A	10	AN	AN	1G; AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	1G; AN	AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	1G; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; G = observed on the surface of the sediment.

Appendix 7
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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
1.1	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	1H; AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	1J; AN
	E	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	1H; AN	AN	1J; AN	1J; AN	AN	AN	1J; AN	1J; AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	2J; AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
1.1	A	10	AN	AN	AN	1J; AN	AN	AN	AN	AN	1G; AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; J = observed outside the test compartment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
1.6	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN	1J; AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN	2J; AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN	AN	1J; AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN

[1]Observations: AN = appear normal; J = observed outside the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
1.6	A	10	1J; AN	AN	1J; AN	AN	AN	1H; AN	AN	AN	1J; AN	AN	1J; AN	AN	AN	AN
	B	10	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	1J; AN	AN	2J; AN	AN	AN	AN	2J; AN	AN
	D	10	AN	AN	1J; AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN
	H	10	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	1J; AN	AN	1J; AN	AN	AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
4.8	A	10	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	1J; AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	2J; AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1H; AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
4.8	A	10	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	1G; AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
8.9	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	1J; AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	2J; AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	2J; AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; J = observed on the outside of the test compartment.

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
8.9	A	10	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	1G; AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	1J; AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; J = observed on the outside of the test compartment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Rep.	Number Exposed	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
17	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	1J; AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	AN	AN	AN	AN	AN	AN	1G; AN	AN	2J; AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; J = observed on the outside of the test compartment.

Mean Measured Concentration (mg/Kg)	Rep.	Number Exposed	Day 14	15	16	17	18	19	20	21	22	23	24	25	26	27
17	A	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	B	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	C	10	AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	D	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	E	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	F	10	AN	AN	1H; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	G	10	1J; AN	AN	AN	1J; AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN
	H	10	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN	AN

[1]Observations: AN = appear normal; H = observed climbing the walls of the test compartment or on the screens; J = observed outside the test compartment.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
Negative Control	A	0	1J; AN	23
	B	0	AN	15
	C	0	AN	22
	D	0	AN	21
	E	0	AN	16
	F	0	AN	13
	G	0	AN	17
	H	0	AN	11
			Mean (± Stdev)	17.3 (± 4.4)
0.65	A	0	AN	15
	B	0	AN	21
	C	0	AN	28
	D	0	AN	13
	E	0	AN	19
	F	1	AN	9
	G	0	AN	14
	H	3 MAD	AN	7
			Mean (± Stdev)	15.8 (± 6.8)

[1]Observations: AN = appear normal; J = observed on the outside of the test compartment; MAD = missing and assumed dead.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
1.1	A	0	AN	13
	B	0	AN	12
	C	0	AN	14
	D	0	AN	13
	E	0	AN	16
	F	0	AN	17
	G	0	AN	17
	H	0	1J; AN	19
			Mean (± Stdev)	15.1 (± 2.5)
1.6	A	0	AN	14
	B	0	AN	10
	C	0	AN	12
	D	0	AN	16
	E	5 MAD	AN	5
	F	0	AN	10
	G	0	AN	13
	H	0	AN	14
			Mean (± Stdev)	11.8 (± 3.4)

[1]Observations: AN = appear normal; J = observed on the outside of the test compartment; MAD = missing and assumed dead.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
4.8	A	0	1J; AN	12
	B	0	AN	12
	C	0	AN	10
	D	2 MAD	AN	8
	E	0	1J; AN	12
	F	0	AN	10
	G	0	AN	11
	H	0	AN	17
			Mean (± Stdev)	11.5 (± 2.6)
8.9	A	1 MAD	AN	9
	B	0	AN	12
	C	0	AN	14
	D	4 MAD	AN	6
	E	6 MAD	AN	4
	F	3 MAD	AN	7
	G	0	AN	11
	H	0	AN	10
			Mean (± Stdev)	9.1 (± 3.3)

[1]Observations: AN = appear normal; J = observed on the outside of the test compartment; MAD = missing and assumed dead.

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Observations of Mortality, Reproduction and Effects by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

Mean Measured Concentration (mg/Kg)	Replicate	Day 28		
		No. Dead	Obs.	No. Survivors
17	A	2 MAD	AN	8
	B	0	AN	10
	C	0	AN	11
	D	1 MAD	AN	9
	E	2 MAD	AN	8
	F	0	AN	12
	G	0	AN	12
	H	2 MAD	AN	8
			Mean (± Stdev)	9.8 (± 1.8)

[1]Observations: AN = appear normal; G = observed on the surface of the sediment; MAD = missing and assumed dead.

Appendix 8

Worm Dry Weights by Replicate in a Prolonged Sediment Toxicity Test with the Oligochaete (*Lumbriculus variegatus*)

	Average Individual Dry Weight (mg) by Mean Measured Concentration						
Replicate	Negative Control	0.65 mg/Kg	1.1 mg/Kg	1.6 mg/Kg	4.8 mg/Kg	8.9 mg/Kg	17 mg/Kg
A	1.18	1.53	0.92	0.93	0.93	1.43	0.79
B	1.10	1.19	0.66	1.40	1.16	0.83	0.87
C	1.96	1.09	1.04	0.99	1.05	0.96	2.18
D	1.77	1.52	1.08	0.70	0.97	1.30	1.48
E	1.19	1.52	0.96	1.80	0.74	2.12	0.55
F	1.62	1.86	0.86	1.30	1.38	0.89	1.78
G	1.12	1.31	0.84	1.20	1.10	1.02	0.72
H	0.77	1.80	0.93	0.86	0.54	5.43	3.41
Mean ± Std. Dev.	1.34 ± 0.40	1.48 ± 0.27	0.91 ± 0.13	1.15 ± 0.35	0.98 ± 0.26	1.75 ± 1.55	1.47 ± 0.97

On Day 0, a subset of 20 organisms were impartially removed from the culture and used to determine dry weight. The average individual dry weight was determined to be 0.59 mg.

Appendix 9

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, Plant and Insect Toxicology
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Timothy Z. Kendall, M.S., Supervisor
4. Timothy Ross B.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist

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OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-114

OECD Guideline 218

AUTHORS:

Susan T. Thomas, B.S.
Henry O. Krueger, Ph.D.
Timothy Z. Kendall, M.S.

STUDY INITIATION DATE: April 21, 2009

STUDY COMPLETION DATE: October 13, 2009

SUBMITTED TO:

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

Wildlife International, Ltd.
8598 Commerce Drive
Easton, Maryland 21601 USA
1 (410) 822-8600

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS NO. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-114

STUDY COMPLETION: October 13, 2009

This study was conducted in compliance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999), with the following exceptions:

Periodic analyses of well water and sediment for potential contaminants were not performed according to Good Laboratory Practice Standards, but were performed using a certified laboratory and standard U.S. EPA analytical methods.

STUDY DIRECTOR:

Susan Thomas — 10/13/09

Susan Thomas, B.S.
Biologist

Date

SPONSOR APPROVAL:

Sponsor Representative — 10/20/09

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 Parts 160 and 792, 17 August 1989); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM (98)17); and Japan MAFF, 11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999. 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	October 1, 2008	October 1, 2008	October 6, 2008
Dosing	April 25, 2009	April 25, 2009	April 27, 2009
Matrix Fortifications	May 26, 2009	May 26, 2009	June 15, 2009
pH Measurements	May 26, 2009	May 26, 2009	June 15, 2009
Analytical Data and Draft Report	August 28 and 29, 2009	August 31, 2009	September 10, 2009
Biological Data and Draft Report	August 29 and 30, 2009	August 31, 2009	September 10, 2009
Final Report	October 13, 2009	October 13, 2009	October 13, 2009

All inspections were study-based unless otherwise noted.

Marshall T. Hynson
Quality Assurance Program Supervisor

10/13/2009
Date

REPORT APPROVAL

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS NO. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-114

STUDY DIRECTOR:

Susan Thomas — 10/13/09

Susan T. Thomas, B.S.
Biologist
Date

PRINCIPAL INVESTIGATOR:

Tim Kendall — 10/13/09

Timothy Z. Kendall, M.S.
Supervisor
Date

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

10/13/09

Henry O. Krueger, Ph.D.
Director of Aquatic, Plant and Insect Toxicology
Date

Willard B. Nixon — 10/13/09

Willard B. Nixon, Ph.D.
Director of Chemistry
Date

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SUMMARY

SPONSOR: Silicones Environmental, Health and Safety Council

TITLE: Octamethyltrisiloxane (L3; CAS NO. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 570A-114

TEST SUBSTANCE: Octamethyltrisiloxane (L3; CAS NO. 107-51-7), supplied as Dow Corning® 200 Fluid, 1 cst.; (Batch No. 0002680862; 99.9%)

TEST DATES:

Experimental Start (OECD):	April 25, 2009
Experimental Start (EPA):	April 28, 2009
Biological Termination:	May 26, 2009
Experimental Termination:	May 27, 2009

LENGTH OF EXPOSURE: 28 Days

TEST ORGANISM: Midge (*Chironomus riparius*)

AGE OF TEST ORGANISMS: 1 - 3 Days

SOURCE OF TEST ORGANISMS: Environmental Consulting and Testing
1423 N. 8th St. Suite 118
Superior, WI 54880

TEST CONCENTRATIONS:

Nominal	Mean Measured
Negative Control	<LOQ
31 mg/Kg	7.4 mg /Kg
63 mg/Kg	14 mg/Kg
125 mg/Kg	39 mg/Kg
250 mg/Kg	84 mg/Kg
500 mg/Kg	210 mg/Kg
1000 mg/Kg	435 mg/Kg

RESULTS: Based on mean measured test concentrations

28-Day LC50:	166 mg/Kg with 95% confidence limits of 84 and 435 mg/Kg
NOEC:	39 mg/Kg
LOEC:	84 mg/Kg, based on development rate

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Silicones Environmental, Health and Safety Council at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the definitive test was conducted from April 28, 2009 to May 26, 2009. Raw data generated by Wildlife International, Ltd., and a copy of the final report are filed under Project Number 570A-114 in archives located on the Wildlife International, Ltd. site. The original final report is archived at Silicones Environmental, Health and Safety Council.

OBJECTIVE

The objective of this study was to determine the effects of sediment-incorporated test substance on the midge, *Chironomus riparius*, during a 28-day exposure period under semi-static test conditions. The measured endpoints of the test were emergence ratio (percent emergence), development rate and the development time.

EXPERIMENTAL DESIGN

Groups of midges (first instar chironomid larvae) were exposed to a series of six nominal test concentrations in sediment (31, 63, 125, 250, 500 and 1000 mg/Kg, dry weight) and a negative control for 28 days under semi-static test conditions. Four replicate test chambers were maintained in each treatment and control group, with 20 midges in each test chamber, for a total of 80 midges per test concentration. Each test chamber contained a quantity of sediment and overlying water. Three additional replicates were added in each treatment and control group for analytical sampling of sediment and one additional replicate was added to each treatment and control group for Day 0 water quality measurements. The "analytical" and "water quality" replicates sampled on Day 0 contained no midges, while midges were added at test initiation to the "analytical" replicates sampled on Days 7 and 28. Biological observations were not made for Day 7 and 28 "analytical" replicates.

The results of the study are based on the arithmetic mean measured test concentrations in sediment. Sediment samples were collected and analyzed from the "analytical replicates" of each concentration. The collection and analyses were done after the addition of test organisms to the test system on Day 0, on Day 7 and at the end of the test.

Test chambers were arranged in a randomized pattern in a temperature controlled environmental chamber approximately 49 hours prior to test initiation to condition the sediment prior to introduction of organisms. Midges were impartially assigned to exposure chambers at test initiation. Observations of mortality and abnormal behavior were made daily during the test. The total number of adults emerged at the end of the test period was recorded.

MATERIALS AND METHODS

The study was conducted according to the procedures outlined in the protocol, "Octamethyltrisiloxane (L3; CAS NO. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment" (Appendix 1). The protocol was based on the OECD Guideline 218: *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment* (1).

Test Substance

The test substance used to prepare the dosed sediment, matrix fortifications and analytical calibration standards was received from Dow Corning Corporation on August 19, 2008. It was assigned Wildlife International, Ltd. identification number 8690 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as Dow Corning 200® Fluid, 1 cSt; Batch No. 0002680862; Chemical Abstract (CAS) number 107-51-7. The test substance contained 99.9% active ingredient by analysis and had an expiration date of May 27, 2010 (Appendix 2).

Test Sediment

The sediment used in the study was a formulated sediment based on the recommendations of OECD Guideline 218 (1). The sediment was composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand (Appendix 4). The sand and clay were mixed in a PK Twinshell mixer for 20 minutes without the peat, since the peat was added later. The targeted organic carbon content of the final mixture was 5.0 ± 1.0%. The dry soil was stored under ambient conditions until used. The final pH of the sediment was 7.0. The percent organic carbon of the sediment was found to be 2.2 as determined by Agvise Laboratories (Northwood, ND) (Appendix 6). Analyses were performed to determine the concentrations of selected organic and inorganic constituents in a representative sample of sediment similar to that which was used in the study and the results of the analyses are summarized in Appendix 7.

A 28-day ration of food (280 mg Tetramin Flake food) was dry mixed into the test chambers after the sediment was added, but immediately before adding the water to the test chambers, and 49 hours prior to adding the organisms. The percent organic carbon of the sediment is based solely on peat as the source of organic carbon. The food added to the test system during the test was not included in the measurement of organic carbon.

Preparation of Test Concentrations

The test substance was administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment dwelling organisms.

Nominal test concentrations (31, 63, 125, 250, 500 and 1000 mg/Kg) were prepared on a dry weight basis, (i.e., mg test substance/Kg dry sediment) using a two-step process. First, neat test material was added to a predetermined amount of conditioned and air-dried peat. The peat and test material were mixed overnight in a Nalgene bottle on a rotary mixer. Second, a predetermined amount of formulated sediment was added to the peat and test material in the nalgene bottle and mixed on a rotary mixer until appearing homogenous (approximately 30 - 40 minutes).

After mixing, dosed formulated sediment was transferred to eight replicate test beakers (2000 mL glass beakers) until reaching a depth of 2 cm. The transfer time was kept to a minimum (approximately 10-20 minutes) by completely processing one concentration before the other to prevent potential for volatilization of the test substance. After all 8 beakers had approximately 2 cm of sediment added, a 28-day ration of food (approximately 280 mg of Tetramin flake food) was added to each beaker that was to contain organisms and was gently stirred into the dry sediment. Finally, approximately 8 cm of UV sterilized well water was added to the beakers. After the water was added to the beakers, the beakers were placed in the environmental test chamber that was used for the study.

Of the eight replicate test chambers prepared for each treatment and control group, four test chambers were used for the evaluation of survival and emergence, three chambers were used for analytical confirmation and one test chamber was used for water quality measurements on Day 0. The sediment/water mixtures were allowed to acclimate for approximately 49 hours prior to the introduction of the test organisms. At test initiation the overlying water in all test chambers appeared slightly cloudy tan. At test termination the overlying water in the negative control, 31, 63, 125, 250 and 500 mg/Kg

treatment groups appeared slightly cloudy and tan and the overlying water in the 1000 mg/Kg treatment group appeared clear and tan.

Test Organism

The midge, *Chironomus riparius*, was selected as the test species for this study. *Chironomus riparius* are representative of an important group of aquatic invertebrates and were selected for use in the study based upon past history of use and ease of culturing in the laboratory. Egg masses were obtained from Environmental Consulting and Testing, Superior, Wisconsin. The supplier verified the identity of the species. The organisms were held for five days prior to the start of the test at approximately the same temperature and in water from the same source as the water used during the test.

At test initiation, the midges were collected from the culture and impartially added one and two at a time to test chambers. All transfers were made below the air/water interface using wide-bore pipettes. Six replicate test chambers in each treatment and control group contained organisms (four test chambers for the evaluation of effects plus two chambers for Day 7 and Day 28 analytical purposes), while two test chambers per group were prepared without organisms, one for Day 0 analytical purposes and one for Day 0 water quality measurements.

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 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 again was filtered (0.45 µm) and passed through an ultraviolet sterilizer to remove microorganisms and particles. The results of periodic analyses performed to measure the concentrations of selected contaminants in the well water used by Wildlife International, Ltd. are presented in Appendix 5. Hardness, alkalinity, pH and specific conductance were measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week period preceding the test are summarized in Appendix 3.

Test Apparatus

Test chambers consisted of 2000-mL glass beakers containing approximately 2 cm of sediment and 8 cm of overlying water. Loose plastic covers were placed over each test chamber during the test. Aeration was applied to each test chamber through a glass pipette that did not extend to a depth closer than 2 cm from the surface of the sediment. Air was bubbled into the test chamber at a rate greater than 1 bubble per second but not so great as to disturb the sediment. Test chambers were arranged in a randomized block order in an environmental chamber. Test chambers were labeled with project number, test concentration and replicate.

Environmental Conditions

Lighting used to illuminate the culture and test chambers during holding and testing was provided by fluorescent tubes that emitted wavelengths similar to natural sunlight (Colortone® 50). 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 when lights went on and off to avoid sudden changes in light intensity. Light intensity at test initiation was 414 lux at the surface of the water over one representative test chamber.

The target test temperature during the study was 20 ± 2°C. Temperature was measured using a liquid-in-glass thermometer in the overlying water of alternating replicate test chambers of each treatment and control group daily during the study. Temperature also was measured continuously in a beaker of water adjacent to the test chambers using a Fulscope ER/C Recorder. The continuous recorder was verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen measurements were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group daily during the test. Measurements of pH and ammonia were made on samples of overlying water collected from one alternating replicate test chamber of each treatment and control group at test initiation, weekly during the test and at test termination. Hardness, alkalinity and specific conductance were measured in a sample of overlying water from the control group and the highest concentration treatment group (1000 mg/Kg) at the beginning and end of the test.

Light intensity was measured using a SPER Scientific Model 840006C light meter. 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. Hardness and alkalinity measurements were made by titration (2). Specific conductance was measured using an Acorn Series Model CON6 Conductivity-Temperature meter. Ammonia was measured using a Thermo Orion Model 720Aplus pH/ISE meter.

Observations

The test chambers were observed daily during the test to determine the number of mortalities and the number of individuals exhibiting signs of toxicity or abnormal behavior. During the period of expected emergence, the sex and number of fully emerged midges was recorded on a daily basis. After identification, the midges were removed from the test chambers. If the total number of emerged midges at the end of the test were fewer than what was initially placed in each replicate, then the number of midges not accounted for were considered dead. The numbers of emerged midges by day are found in Appendix 8.

Statistical Analyses

The 28-Day LC50 was calculated using the computer software of C.E. Stephan (4). The program is designed to calculate the LC50 value and 95% confidence interval by probit analysis, the moving average method, or binomial probability with nonlinear interpolation (5, 6, 7). In this study, the binomial method was used to calculate the LC50 value. The LC50 value was calculated using the mortality data collected at the end of the test. The no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC) were determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence ratios and development rates.

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, was calculated for each replicate of each treatment and control groups. The emergence ratio (rate) and development rate were calculated for each replicate of each control and treatment group using the SAS System for Windows Version 8.2 (3). The emergence ratio, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_e}{n_a}$$

where: ER = emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The emergence ratio data were arcsine transformed using the following formula to normalize the data prior to statistical analyses:

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_e}{n_a}}\right)$$

where: ERarc = arcsin-transformed emergence ratio
n_e = sum of midges emerged per test chamber
n_a = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day. Development rate was calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where: i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment (= $\sum f_i$)
x_i = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

where: day_i = inspection day (days since application).

All statistical procedures were performed using SAS version 8.2 (3) and Stephan version 1.1 (4). The data were analyzed using Dunnett's test, as appropriate, to identify those treatment levels that were statistically different ($p<0.05$) from the negative control group. An ANOVA procedure looking at the interaction between sexes was used to evaluate sensitivity between sexes for development rates and total number emerged. There were no statistically significant interactions found between sex and treatments for development rate and therefore the data for each sex were pooled for this variable. There is no

feasible way to determine the sex of one – four day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence ratio are not meaningful because calculations require knowing how many individuals of each sex are initially exposed. However, since there were no statistically significant interactions found between sex and treatment for the total number emerged, the emergence ratio data for both sexes were pooled for analysis.

Analytical Sampling

Sediment samples were collected from the analytical replicates from each test concentration and control shortly after the introduction of the organisms on Day 0, on Day 7 and at test termination on Day 28.

Analytical Method

The analytical method used for the analysis of L3 was developed by Wildlife International, Ltd. Sediment was sampled from the test beakers and approximately 5 g of sediment was added to each French square bottle. To each sample, approximately 50 mL of ethyl acetate was added. Samples were sonic disrupted for five minutes and the sediment was allowed to settle for approximately two minutes. An aliquot of the extract was removed and centrifuged at 14,500 for approximately 10 minutes. Secondary dilutions were made in ethyl acetate, as necessary and the extracts were transferred into autosampler vials and submitted for analysis.

Concentrations of L3 in the samples were determined using either an Agilent Model 5890 or 6890 Gas Chromatograph (GC) with either an Agilent Model 5873 or 5975 Mass Selective Detector operated in SIM mode. Chromatographic separations were achieved using a DB-5 column (30 m x 0.25 mm, 0.25 µm film thickness) or a ZB-5 column (30 m x 0.25 mm, 0.25 µm film thickness). A method flowchart for the analysis of L3 is presented in Appendix 8.1 and typical instrumental parameters are summarized in Appendix 8.2.

Calibration standards of L3 analytical standard, ranging in concentration from 0.500 to 15.0 mg a.i./L were prepared in ethyl acetate using a stock solution of L3 in acetone. Calibration standards were prepared and analyzed with each sample set. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of L3 in the samples was determined by substituting 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 8.4.

The LOQ was 5.00 mg/Kg, calculated as the product of the low calibration standard (0.500 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).

Sediment samples were fortified at 30.0, 100 and 1000 mg/Kg using stock solutions of L3 in acetone (Appendix 8.3), and were analyzed concurrently with the samples. The measured concentrations for the matrix fortification samples ranged from 65.9 to 96.3% of nominal concentrations (Appendix 8.5).

A representative calibration curve is presented in Appendix 8.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 8.7 and 8.8, respectively. Representative chromatograms of a matrix blank and matrix fortification sample are presented in Appendices 8.9 and 8.10, respectively. A representative chromatogram of a test sample is presented in Appendix 8.11.

RESULTS AND DISCUSSION

Confirmation of Test Concentrations

Measured concentrations of L3 in the sediment samples collected during the definitive test are presented in Table 1. Measured concentrations of L3 in the sediment samples collected on Day 0 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were 6.48, 9.81, 27.5, 100, 170 and 395 mg/Kg, respectively. Measured concentrations of L3 in the sediment samples collected on Day 7 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were 8.30, 23.3, 51.0, 68.0, 188 and 619 mg/Kg, respectively. Measured concentrations of L3 in the sediment samples collected on Day 28 from the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were 7.38, 9.70, 39.7, 84.0, 273 and 290 mg/Kg, respectively. Measured concentrations of the test substance in the negative control sediment samples collected on Days 0, 7 and 28 were below the limit of quantification (<5.00 mg/Kg dry sediment). Arithmetic mean measured concentrations of L3 in the sediment, for the duration of the study, for the 31, 63, 125, 250, 500 and 1000 mg/Kg treatment groups were determined to be 7.4, 14, 39, 84, 210 and 435 mg/Kg, respectively, which are equivalent to 24, 23, 32, 34, 42 and 44% of nominal, respectively.

Observations and Measurements

Measurements of temperature, dissolved oxygen and pH of the overlying water in the test chambers are presented in Tables 2, 3 and 4, respectively. Temperatures measured in the test chambers were within the 20 ± 2°C range established for the test. Dissolved oxygen concentrations remained ≥ 7.1 mg/L (79% of saturation) throughout the test, and measurements of pH ranged from 8.1 to 8.6. On Day 22, aeration was found not functioning properly in replicates 500 mg/Kg replicate B and 1000 mg/Kg replicate B. A sample of the overlying water was collected from the two replicates and the dissolved oxygen levels were 7.8 and 7.9 mg/L, respectively. Aeration was restored to the two replicates and dissolved oxygen was re-measured at the end of the work day and found to be 9.0 and 9.0 mg/L, respectively.

Measurements of ammonia in the overlying water are presented in Table 4. Ammonia levels exceeded 4.0 mg/L in the overlying water of some replicates on Days 7 and 14; therefore, the overlying water was partially renewed on those days in each replicate to prevent toxicity caused by high levels of ammonia. Measurements of hardness, alkalinity and conductivity of the overlying water in the test chambers are presented in Table 5 and were consistent with typical water quality of Wildlife International, Ltd well water.

Observations of emergence in individual replicates at each observation interval are presented in Appendix 9. Adults that emerged appeared normal. Emergence was first noted on Day 13 of the test. Mortality at test termination in the negative control, 7.4, 14, 39, 84, 210 and 435 mg/Kg treatment groups was 13, 10, 6.3, 8.8, 29, 58 and 96%, respectively (Table 6). The LC50 value based on mortality of midges (*Chironomus riparius*) exposed to sediment-incorporated L3 was 166 mg/Kg with 95% confidence limits of 84 and 435 mg/Kg.

Mean development time in the negative control group was 17.7 days (Table 7). Mean development times in the 7.4, 14, 39, 84, 210 and 435 mg/Kg treatment groups were 18.0, 17.4, 18.9, 20.3, 21.8 and 23.8 days, respectively. There were statistically significant differences ($p<0.05$) in development times between the negative control group and the 210 and 435 mg/Kg treatment groups, using a two-tailed Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for development time was determined to be 84 mg/Kg and the LOEC for development time was determined to be 210 mg/Kg.

The mean emergence ratios in the negative control, 7.4, 14, 39, 84, 210 and 435 mg/Kg treatment groups were 0.88, 0.90, 0.94, 0.91, 0.71, 0.43 and 0.04, respectively (Table 8). There were statistically significant reductions ($p<0.05$) in emergence ratios between the negative control and the 210 and 435 mg/Kg treatment groups, using a Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for emergence ratios was determined to be 84 mg/Kg and the LOEC for emergence ratio was determined to be 210 mg/Kg (Table 8).

Mean development rates (defined as the portion of larval development which takes place per day) in the negative control, 7.4, 14, 39, 84, 210 and 435 mg/Kg treatment groups were 0.0604, 0.0588, 0.0608, 0.0558, 0.0519, 0.0486 and 0.0431, respectively (Table 8). There were statistically significant reductions ($p<0.05$) in development rates between the negative control and the 84, 210 and 435 mg/Kg treatment groups, using a Dunnett's t-test, that were considered to be treatment-related. Therefore, the NOEC for development rate was determined to be 39 mg/Kg and the LOEC for development rate was determined to be 84 mg/Kg (Table 8).

CONCLUSIONS

The 28-Day LC50 value based on mortality of midges (*Chironomus riparius*) exposed to sediment-incorporated Octamethyltrisiloxane (L3; CAS NO. 107-51-7) was 166 mg/Kg with 95% confidence limits of 84 and 435 mg/Kg. There were treatment-related effects observed on development times, mean emergence ratios and development rates in the 210 and 435 mg/Kg treatment groups. There was also a treatment-related effect observed on development rate in the 84 mg/Kg treatment group, making it the most sensitive endpoint. Therefore, the 28-Day LOEC, based on development rate, was 84 mg/Kg and the 28-Day NOEC was 39 mg/Kg.

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Table 1

Measured Concentrations of L3 in Sediment Samples from a Prolonged Sediment Toxicity Test with *Chironomus riparius*

Nominal Test Concentration (mg/Kg)	Sample Number (570A-114-)	Sampling Time (Days)	Measured Concentration (mg/Kg)[1,2]	Percent of Nominal[3]	Arithmetic Mean Measured Concentration (mg/Kg)[3]	Mean Percent of Nominal[3]
Negative Control	1	0	< LOQ	--	--	--
	8	7	< LOQ	--		
	15	28	< LOQ	--		
31	2*	0	6.48	20.9	7.4	24
	9	7	8.30	26.8		
	16*	28	7.38	23.8		
63	3	0	9.81	15.6	14	23
	10	7	23.3	37.0		
	17	28	9.70	15.4		
125	4	0	27.5	22.0	39	32
	11	7	51.0	40.8		
	18	28	39.7	31.8		
250	5	0	100	40.2	84	34
	12	7	68.0	27.2		
	19	28	84.0	33.6		
500	6	0	170	34.1	210	42
	13	7	188	37.7		
	20	28	273	54.6		
1000	7	0	395	39.5	435	44
	14	7	619	61.9		
	21	28	290	29.0		

[1] The limit of quantitation (LOQ) was 5.00 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.500 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).
[2] Analytical results were generated using wet weights. The tabulated values are reported on a dry weight basis.
[3] Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.
* Values were extrapolated.

Table 2

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Temperature (°C) [1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep.	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Neg. Control	20.3	20.3	20.3	20.4	20.4	20.2	20.2	20.2	20.3	20.3	20.3	20.3	20.2	20.3
7.4	20.2	20.2	20.1	20.2	20.3	20.1	20.1	20.1	20.2	20.2	20.2	20.2	20.2	20.1
14	20.2	20.3	20.2	20.2	20.2	20.1	20.1	20.1	20.2	20.3	20.2	20.2	20.2	20.1
39	20.1	20.2	20.2	20.3	20.2	20.1	20.0	20.1	20.3	20.2	20.2	20.3	20.2	20.1
84	20.1	20.1	20.2	20.3	20.3	20.1	20.0	20.0	20.2	20.2	20.2	20.2	20.1	20.1
210	20.2	20.2	20.2	20.3	20.3	20.1	20.1	20.2	20.3	20.1	20.1	20.4	20.3	20.2
435	20.2	20.1	20.2	20.2	20.2	20.1	20.0	20.0	20.2	20.2	20.1	20.2	20.2	20.2

[1] Temperature measured continuously during the test ranged from approximately 19.5 to 20.5°C.

Table 2 (Continued)

Temperature of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Rep.	Temperature (°C)														
		Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
		C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Neg. Control		20.3	20.3	20.3	20.5	20.3	20.2	20.2	20.3	20.3	20.3	20.3	20.2	20.3	20.3	20.2
7.4		20.2	20.1	20.2	20.2	20.2	20.2	20.0	20.2	20.1	20.1	20.2	20.2	20.2	20.2	20.1
14		20.2	20.2	20.2	20.3	20.2	20.2	20.2	20.1	20.1	20.1	20.2	20.1	20.2	20.2	20.1
39		20.1	20.2	20.2	20.3	20.2	20.2	20.2	20.1	20.1	20.1	20.2	20.1	20.2	20.2	20.1
84		20.1	20.2	20.2	20.3	20.2	20.2	20.0	20.1	20.2	20.2	20.1	20.2	20.2	20.2	20.1
210		20.1	20.2	20.2	20.3	20.2	20.3	20.0	20.1	20.2	20.1	20.3	20.2	20.2	20.2	20.2
435		20.1	20.2	20.2	20.3	20.2	20.1	20.2	20.1	20.1	20.0	20.2	20.2	20.2	20.1	20.1

Table 3

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Dissolved Oxygen (mg/L)[1]													
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Rep.	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Neg. Control	8.5	8.8	8.5	7.6	8.5	8.3	8.2	7.5	8.2	8.0	8.2	7.8	8.3	8.3
7.4	8.4	8.2	8.5	8.3	8.5	8.4	8.2	8.2	8.2	8.4	7.8	8.2	8.3	8.4
14	8.4	8.6	8.4	7.9	7.9	8.4	7.9	7.8	7.1	8.0	8.0	7.8	7.7	8.3
39	8.5	8.6	8.5	8.1	8.1	8.5	8.1	7.8	7.1	8.4	8.0	7.7	8.1	8.3
84	7.7	8.1	8.4	8.5	8.2	8.5	7.4	8.3	8.2	8.3	8.2	8.0	8.1	7.9
210	8.3	8.3	8.4	8.4	8.3	8.1	8.2	8.3	8.1	7.8	8.1	8.1	8.0	7.5
435	8.4	8.3	8.5	8.3	8.3	8.7	8.0	8.8	8.2	8.1	8.1	8.0	7.9	7.9

[1] A dissolved oxygen concentration of 5.4 mg/L represents 60% saturation at 20°C in freshwater.

Table 3 (Continued)

Dissolved Oxygen in Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Dissolved Oxygen (mg/L)														
	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28
Rep.	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A
Neg. Control	8.4	8.0	7.9	8.1	8.7	8.6	8.9	8.8	8.8	8.6	8.3	8.2	8.3	8.3	8.5
7.4	8.0	8.2	8.1	8.1	8.4	8.6	9.0	8.8	8.6	8.6	8.2	8.0	8.0	8.1	8.5
14	8.0	8.1	7.2	7.7	8.5	8.2	8.7	8.7	8.7	8.4	8.3	8.1	8.0	8.0	8.4
39	8.1	8.0	7.6	7.9	8.4	8.3	8.8	8.8	8.7	8.5	8.3	8.1	8.2	8.0	8.4
84	8.0	8.2	7.7	7.8	8.1	8.3	8.8	8.7	8.4	8.6	8.1	8.0	8.1	8.0	8.4
210	8.0	8.1	7.9	7.7	8.2	8.4	8.9	8.1	8.5	8.3	8.2	8.0	8.1	8.0	8.4
435	8.1	8.1	7.9	7.9	8.3	8.5	8.7	8.4	8.4	8.5	8.1	7.9	8.0	8.1	8.2

Table 4

pH and Ammonia (mg/L) of Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day									
	0		7		14		21		28	
	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia	pH	Ammonia
Rep:	A	H	D		C		B		A	
Negative Control	8.4	0.777	8.3	6.28	8.4	2.05	8.5	<0.17[1]	8.6	<0.17
7.4	8.5	0.797	8.5	5.49	8.4	3.01	8.6	<0.17	8.6	<0.17
14	8.2	0.836	8.4	4.38	8.4	4.19	8.6	<0.17	8.6	<0.17
39	8.4	0.961	8.4	4.80	8.5	3.83	8.6	<0.17	8.6	<0.17
84	8.1	0.945	8.5	4.51	8.5	5.54	8.6	<0.17	8.6	<0.17
210	8.4	0.941	8.6	4.41	8.4	0.221	8.5	<0.17	8.6	<0.17
435	8.5	0.914	8.5	4.57	8.5	2.77	8.5	<0.17	8.5	<0.17

[1] The lowest standard used for ammonia calibration was 0.17 mg/L.

Table 5

Hardness, Alkalinity and Conductivity of the Overlying Water in the Test Chambers

Mean Measured Concentration (mg/Kg)	Day 0 Hardness (mg/L as $CaCO_3$)	Day 0 Alkalinity (mg/L as $CaCO_3$)	Day 0 Conductivity (µmhos/em)	Day 28 Hardness (mg/L as $CaCO_3$)	Day 28 Alkalinity (mg/L as $CaCO_3$)	Day 28 Conductivity (µmhos/cm)
Replicate:		H			A	
Negative Control	136	176	366	136	172	320
435	142	182	377	140	180	340

Table 6

Percent Emergence and Mortality of Midges (*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Number Exposed	Number of Emerged Midges - Day 28			Percent Emergence[1]	Percent Mortality[2]
			Males	Females	Total		
Negative Control	A	20	10	6	16	80	20
	B	20	7	10	17	85	15
	C	20	11	7	18	90	10
	D	20	12	7	19	95	5
	Group	80	40	30	70	88	13
7.4	A	20	9	10	19	95	5
	B	20	10	7	17	85	15
	C	20	12	6	18	90	10
	D	20	8	10	18	90	10
	Group	80	39	33	72	90	10
14	A	20	13	6	19	95	5
	B	20	11	8	19	95	5
	C	20	5	15	20	100	0
	D	20	8	9	17	85	15
	Group	80	37	38	75	94	6.3
39	A	20	16	4	20	100	0
	B	20	10	7	17	85	15
	C	20	7	10	17	85	15
	D	20	8	11	19	95	5
	Group	80	41	32	73	91	8.8
84	A	20	11	4	15	75	25
	B	20	12	7	19	95	5
	C	20	5	2	7	35	65
	D	20	9	7	16	80	20
	Group	80	37	20	57	71	29
210	A	20	8	6	14	70	30
	B	20	5	2	7	35	65
	C	20	5	5	10	50	50
	D	20	1	2	3	15	85
	Group	80	19	15	34	43*	58
435	A	20	0	0	0	0	100
	B	20	2	0	2	10	90
	C	20	1	0	1	5	95
	D	20	0	0	0	0	100
	Group	80	3	0	3	4*	96

[1] The LC50 was calculated to be 167 mg/Kg with 95% confidence limits of 84 and 435 mg/Kg.
[2] The percent mortality is calculated based on the number of organisms that did not emerge as well as those that died in the pupae or larval stage.
* There was a statistically significant difference ($p<0.05$) from the negative control using Dunnett's t-test.

Table 7

Mean Development Time of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Mean Development Time	
		Replicate Mean (days)	Group Mean[1,2] (days)
Negative Control	A	18.1	17.7
	B	18.2	
	C	18.0	
	D	16.3	
7.4	A	17.5	18.0
	B	18.2	
	C	17.7	
	D	18.7	
14	A	16.6	17.4
	B	16.1	
	C	18.1	
	D	18.9	
39	A	18.5	18.9
	B	17.9	
	C	19.5	
	D	19.7	
84	A	18.1	20.3
	B	19.7	
	C	22.1	
	D	21.4	
210	A	20.1	21.8*
	B	19.9	
	C	21.2	
	D	26.0	
435	A	--	23.8*
	B	24.5	
	C	23.0	
	D	--	

[1] Calculated using Excel 2000. Manual calculations may differ slightly.
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
* There was a statistically significant difference from the negative control ($p<0.05$) using two-tailed Dunnett's t-test.
-- = No emergence occurred, therefore a value could not be calculated.

Table 8

Emergence Ratio and Development Rate of Midges (*Chironomus riparius*)
During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)	Replicate	Emergence Ratio		Development Rate[1]	
		Replicate	Mean[2]	Replicate	Mean[2]
Negative Control	A	0.80	0.88	0.0589	0.0604
	B	0.85		0.0590	
	C	0.90		0.0594	
	D	0.95		0.0642	
7.4	A	0.95	0.90	0.0601	0.0588
	B	0.85		0.0581	
	C	0.90		0.0596	
	D	0.90		0.0572	
14	A	0.95	0.94	0.0635	0.0608
	B	0.95		0.0649	
	C	1.00		0.0590	
	D	0.85		0.0558	
39	A	1.00	0.91	0.0566	0.0558
	B	0.85		0.0585	
	C	0.85		0.0542	
	D	0.95		0.0537	
84	A	0.75	0.71	0.0574	0.0519*
	B	0.95		0.0531	
	C	0.35		0.0476	
	D	0.80		0.0497	
210	A	0.70	0.43*	0.0525	0.0486*
	B	0.35		0.0528	
	C	0.50		0.0496	
	D	0.15		0.0393	
435	A	--	0.04*	--	0.0431*
	B	0.10		0.0417	
	C	0.05		0.0444	
	D	--		--	

[1] The development rate represents that portion of larval development which takes place per day.
[2] Calculated using the SAS Systems for Windows. Manual calculations may differ slightly.
[3] There was a statistically significant reduction from the negative control ($p < 0.05$), using Dunnett's t-test.

Appendix 1

Study Protocol and Deviation

PROTOCOL

OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

Guideline:

OECD Guideline 218

WILDLIFE INTERNATIONAL LTD. STUDY NUMBER:
570A-114

SPONSOR

Silicones Environmental, Health and Safety Council
2325 Dulles Corner Boulevard, Suite 500
Herndon, Virginia 20171

PERFORMING LABORATORY

Wildlife International, Ltd.

8598 Commerce Drive
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September 11, 2008

Wildlife International, Ltd.

OCTAMETHYLTRISILOXANE (L3; CAS NO. 107-51-7): A PROLONGED SEDIMENT TOXICITY TEST WITH *CHIRONOMUS RIPARIUS* USING SPIKED SEDIMENT

SPONSOR:	Silicones Environmental, Health and Safety Council 2325 Dulles Corner Boulevard, Suite 500 Herndon, Virginia 20171
SPONSOR'S REPRESENTATIVE:	Kurt R. Drottar Dow Corning Corporation Health and Environmental Sciences 2200 West Salzburg Road Auburn, Michigan 48611
TESTING FACILITY:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601 USA
STUDY DIRECTOR:	Susan T. Thomas, Biologist Wildlife International, Ltd.
LABORATORY MANAGEMENT:	Henry O. Krueger Ph.D. Director of Aquatic, Plant and Insect Toxicology
LABORATORY QUALITY ASSURANCE MANAGER:	Linda R. Mitchell Director of Regulatory and Ecotox Operations Wildlife International, Ltd.

PROTOCOL APPROVAL

Susan Thomas STUDY DIRECTOR	4/21/09 DATE
LABORATORY MANAGEMENT	4/21/09 DATE
SPONSOR'S REPRESENTATIVE	9/12/08 DATE

PROTOCOL NO.: 570/091108/CHI-SED/SUB570

Wildlife International, Ltd.

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: April 27, 2009 Experimental Termination Date: May 25, 2009

Project No.: 570A-114

Test Concentrations: Negative Control, 31, 63, 125, 250, 500 and 1000 mg/kg

Test Substance No.: 8690 Reference Substance No. (if applicable): NA

PROTOCOL NO.: 570/091108/CHI-SED/SUB570

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INTRODUCTION

Wildlife International, Ltd. will conduct a prolonged sediment toxicity test with the midge, *Chironomus riparius*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be based upon the OECD Guideline 218: Sediment-Water Chironomid Toxicity Test Using Spiked Sediment (1). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

OBJECTIVE

The objective of this study is to determine the effects of a sediment-incorporated test substance on the midge, *Chironomus riparius*, during a 20 to 28 day exposure period under static or semistatic test conditions. The measured endpoints of the test are emergence ratio (percent emergence), development rate, and the development time.

EXPERIMENTAL DESIGN

Groups of midges will be exposed to a geometric series of at least five test concentrations, a negative (untreated sediment) control, and if necessary, a solvent control for approximately 20 to 28 days. Four replicate test chambers will be maintained in each treatment and control group, with 20 midges in each chamber for a total of 80 individuals per test concentration. Each test chamber will contain a quantity of sediment and overlying water. Additional replicate test chambers may be included for analytical sampling of sediment and for water quality measurements. No midges will be placed in "analytical" or "water quality" replicate(s) sampled at the beginning of the test. However, replicate(s) sampled on Day 7 and at the end of the test will contain midges. Test concentrations in the sediment will be prepared on a mg/kg dry weight basis.

The formulated sediment will use peat as its source of organic matter in order to achieve an organic content of approximately 4 to 5%. Test substance will first be mixed with peat and then dry components of the formulated sediment (sand and clay). After mixing, dry formulated sediment will be transferred to each test chamber and a 28-day ration of food (tetramin) will be dry mixed into each test chamber. Water will then be added to each chamber approximately 48 hours before adding organisms. Partial renewals of overlying water will occur if weekly ammonia concentrations approach 4 mg/L.

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Test concentrations in the sediment will be prepared on a dry weight basis (i.e., mg/Kg dry sediment). Nominal test concentrations will be selected in consultation with the Sponsor, and will be based upon information such as the results of exploratory range-finding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Generally, each test substance concentration used in the definitive test will be at least 60% of the next higher concentration unless information concerning the concentration-effect curve indicates that a different dilution factor would be more appropriate.

The water solubility of Octamethyltrisiloxane (L3; CAS No. 107-51-7) is 34.49 µg/L (2). Preliminary trials have shown that the test material is stable in sediment. Reporting values for pore water and overlying water does not seem meaningful due to the low solubility of Octamethyltrisiloxane in water. Therefore, only sediment samples from the analytical sampling test compartments will be collected from each control and treatment group at specified intervals for analysis of the test substance. Results of the analyses will be used to verify the exposure over time. The results of the study will be based on the nominal test concentrations in sediment, when appropriate.

To control bias, midges will be impartially assigned to exposure chambers at test initiation (e.g. 4 groups of 20 larvae per chamber). No other potential sources of bias are expected to affect the results of the study. The total number of adults emerged at the end of the test period (approximately 28 days) will be recorded. Percent emergence in the treatment groups will be used to calculate, when possible, the EC50. The dose-response pattern and appropriate statistical analyses will be used to define the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC).

MATERIALS AND METHODS

Test Substance

Information on the characterization of test, control or reference substances is required by Good Laboratory Practice (GLP) Standards and Principles. The Sponsor is responsible for providing Wildlife International, Ltd. verification that the test substance has been characterized according to

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GLPs prior to its use in the study. GLP characterization of the test substance has been conducted by the Sponsor and is documented at Dow Corning.

Identification:	Octamethyltrisiloxane (L3) (Supplied as Dow Corning® 200 Fluid, 1 cst.)
Lot No.:	0002680862
Expiration Date:	May 27, 2010
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS No.:	107-51-7
Physical Description:	Colorless Liquid
Chemical Stability:	Stable according to the MSDS
Purity:	99.9 ± 0.03 area % purity
Solubility:	34.49 µg/L
Storage Conditions:	Room Temperature
Archive Requirements:	A reserve sample was not retained.

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.

Preparation of Test Concentrations

The test substance will be administered to the test organism in sediment. This route of administration was selected because it represents the most likely route of exposure to sediment-dwelling organisms.

Neat test substance will first be incorporated into peat. The amount of peat used in the mixture will be the same amount required to bring the organic carbon content of a batch of sediment to 5%. The peat and test substance will be mixed overnight on a rotary mixer. The treated peat will then be added to dry sediment that has all ingredients with the exception of peat and the test substance. The mixing time will be as short as possible (approximately 20 - 30 minutes) or until the mixture appears to be uniformly mixed into the sediment. The exact mixing time will be recorded in the data.

After mixing, sediment will be placed in the bottom of the test chambers to a depth of approximately 2 cm (approximately 150 ml). The weight of the sediment added to each chamber will be measured using a top-loading balance and recorded. A pre-weighed aliquot of Tetramin Flake Food (280 mg) will be added to the sediment prior to adding the overlying water and mixed into the sediment by hand using a glass stir rod. Approximately 8 cm of water will be slowly added into the

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test chamber in such a manner as to minimize the disturbance to the sediment. A 1:4 ratio of sediment to overlying water will be maintained throughout the study. A mark on each test chamber noting the level of the water will be made so that water lost due to evaporation can be replaced in the test chambers as needed with reverse osmosis water. The transfer time will be kept to a minimum (approximately 10 – 20 minutes) and one batch of sediment will be completely processed before the other to prevent the potential for volatilization of the test substance. After the test chambers are prepared, they will be placed in a randomized block arrangement in an environmental chamber at the target temperature and the sediment-water mixtures will be allowed to settle for approximately 48 hours before adding the test organisms. A partial renewal of the overlying water will take place if ammonia concentrations exceed 4 mg/L. The renewal will be performed in manner that will minimize the disturbance of the sediment and midges.

Test Organism

The midge, *Chironomus riparius*, has been selected as the test species for this study. Midges represent an important group of aquatic invertebrates, and have been selected for use in the test based upon past use history and ease of culturing in the laboratory. Environmental Consulting and Testing (ECT), Superior, WI or some other source will supply identified egg masses. Midges to be used in the test will be first instar larvae (approximately 1-4 days after hatching) at test initiation. Larvae will be collected from at least three separate egg masses to start the test and will be hatched in water from the same source and at approximately the same temperature as will be used during the test. Midge larvae will be fed Tetramin Flake Food or equivalent incorporated into the sediment at the beginning of the test. This ration of food is equivalent to feeding the organisms ten mg daily during the test. No other supplemental food will be added during the test.

Specifications for acceptable levels of contaminants in the food for midges have not been established. However, there are no levels of contaminants reasonably expected to be present in the diet that are considered to interfere with the purpose or conduct of the study.

Test Sediment

Formulated sediment based on the recommendations of OECD Guideline 218 will be used. The sediment will be composed of approximately 10% sphagnum peat moss, 20% silt and clay (kaolin clay) and 70% industrial quartz sand. The dry constituents of the soil will be mixed in a PK

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Twinshell or equivalent mixer without peat, since peat will be mixed with test substance and added later. Calcium carbonate will be added as needed to adjust the pH to 7.0 ± 0.5. The organic carbon content of the final mixture of sediment should be 5.0 ± 1.0 and may be adjusted by the use of appropriate amounts of peat or sand.

A 28-day ration of food (tetramin fish food or equivalent) will be dry mixed into the test chambers after sediment has been added, but immediately before adding water to the test chamber, and 48 hours before adding the organisms. The amount of food added will be documented in the raw data. The percent organic carbon is based solely on peat as the source of organic carbon. The food added to the test system during the test is not included in the measurement of organic carbon.

Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of a similar representative batch of formulated sediment and results of the analyses will be summarized in the final report. In addition, a sample of the sediment used for the study will be sent to Agvise Laboratories, Northwood, ND (or similar facility) to determine the sediment composition.

Dilution Water

Water used for the holding and testing of midges will be obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The water will be passed through a sand filter and pumped into a 37,800-L storage tank where the water will be aerated with spray nozzles. Prior to use the water will be filtered to 0.45 µm in order to remove microorganisms and fine particles and passed through an ultraviolet sterilizer. Water used for holding and testing is characterized as moderately hard. Typical values for hardness, alkalinity, pH and specific conductance are approximately:

Hardness, mg/L as $CaCO_3$	145
Alkalinity, mg/L as $CaCO_3$	190
pH	8.1
Specific Conductance, µmhos/cm	330

Hardness, alkalinity, pH and specific conductance will be measured weekly to monitor the consistency of the well water. Means and ranges of the measured parameters for the four-week

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period preceding the test will be provided in the final report. Analyses will be performed at least once annually to determine the concentrations of selected organic and inorganic constituents of the well water and results of the analyses will be summarized in the final report.

Test Apparatus

Test chambers will consist of 2-L glass beakers, or equivalent, containing approximately 2 cm of sediment and approximately 8 cm of overlying water. Loose plastic covers will be placed over each test chamber during the test. Aeration will be applied to each test chamber through a glass pipette that shall extend to a depth of not closer than 2 cm from the surface of the sediment. Air will be bubbled into the test chambers at a rate greater than 1 bubble/sec but not so great as to disturb the sediment. Test chambers will be arranged in a randomized block order in an environmental chamber. Test chambers will be labeled with the project number, test concentration and replicate. A check will be performed at least once daily to monitor the water levels in the test chambers, the temperature in the environmental chamber where the study is being conducted and the proper function of the aeration.

Environmental Conditions

Lighting used to illuminate the cultures and test chambers during culturing and testing will be provided by fluorescent tubes that emit wavelengths similar to natural sunlight (e.g., Colortone® 50). A photoperiod of 16 hours of light and 8 hours of dark will be controlled with an automatic timer. A 30-minute transition period of low light intensity will be provided when lights go on and off to avoid sudden changes in light intensity. Light intensity will be measured at test initiation with a SPER Scientific Ltd. light meter or equivalent and should fall within the range of 100 to 1000 lux.

The target test temperature will be 20 ± 2°C. Temperature will be measured using a liquid-in-glass thermometer in the overlying water in one alternating replicate test chamber of each experimental group daily during the test. Temperature also will be measured with a continuous recorder in a beaker of water placed adjacent to the test chambers. Recorder measurements will be verified with a liquid-in-glass thermometer prior to test initiation.

Dissolved oxygen will be measured in one alternating replicate of each experimental group daily during the test using a Thermo Orion Model 850Aplus dissolved oxygen meter, or equivalent.

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Measurements of pH will be made at the beginning and end of the test and at least once per week on a sample of the overlying water from each experimental group. Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent. If a treatment replicate reaches 100% mortality, dissolved oxygen, pH, and temperature measurements will be taken in that replicate at that time, then discontinued.

Ammonia measurements will be made in a sample of overlying water from one or more of the "water quality" replicates at the beginning of the test, and from one alternating biological replicate from each experimental group weekly during the test. Measurements of pH will be made using a Thermo Orion Model 720Aplus pH meter, or equivalent. Ammonia will be measured using a Thermo Orion Model 720Aplus pH/ISE meter, or equivalent. Hardness, alkalinity and specific conductance will be measured in a sample of the overlying water from one or more of the "water quality" replicates at the beginning of the test and from a biological replicate at the end of the test, from the controls and the highest treatment group. Hardness and alkalinity measurements will be made by titration using procedures based on methods in *Standard Methods for the Examination of Water and Wastewater* (3). Specific conductance will be measured using a Yellow Springs Instrument Model 33 Salinity-Conductivity-Temperature meter, or equivalent.

Test Procedures and Biological Measurements

After the settling period for the water/sediment systems, one to two first instar larvae will be sequentially added to test chambers until each chamber contains its complement of 20 individuals. After adding the test organisms to the test chambers, the test chambers will be placed in a walk-in environmental chamber and organized in a randomized block arrangement.

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Test chambers will be observed daily to make visual assessments of any abnormal behavior (e.g. leaving sediment, unusual swimming). During the period of expected emergence the sex and number of fully emerged midges will be recorded on a daily basis. After identification the midges will be removed from chambers. Egg masses deposited prior to the end of the test will be recorded and then removed to prevent re-introduction of larvae into the sediment. If the total number of individuals emerged in each replicate at the end of the test are fewer than what was initially placed in each replicate, then the number of individuals not accounted for will be considered dead. The test duration for *Chironomus riparius* is approximately 28 days. If midges emerge earlier, the study can terminate after a minimum of five days following emergence of the last adult in the control.

Sampling for Analytical Measurements

Sediment samples from the analytical sampling test chambers will be collected from selected concentrations at test initiation, on Day 7, and at the end of the test. The overlying water will be removed from the test chamber. Sediment samples will be processed immediately for analysis when possible, or placed in an appropriate storage container (e.g., glass or polypropylene bottle) and stored under the appropriate conditions (frozen, refrigeration or ambient) until analyzed. The sampling scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 7	Termination (28)
Control	1	1	1
Level 1-Low Concentration	1	1	1
Level 2	1	1	1
Level 3	1	1	1
Level 4	1	1	1
Level 5-High Concentration	1	1	1
	7	7	7

The above numbers of samples represent those collected from the test and do not include quality control (QC) samples such as matrix blanks and fortifications prepared and analyzed during the analytical chemistry phase of the study.

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Analytical Chemistry

Chemical analysis of the samples will be performed by Wildlife International, Ltd. The analytical method used will be based upon chromatographic methodology provided by the Sponsor and/or developed at Wildlife International, Ltd. The methodology used to analyze the test samples will be documented in the raw data and summarized in the final report.

Statistical Analyses

When the dose-response pattern allows calculation of an EC50 value, the data will be analyzed using the computer software 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 method, or binomial probability with nonlinear interpolation (5,6,7). The EC50 value will be calculated, when possible, using mortality data collected at the end of the test. The no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) will be determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence rates and development rates.

The mean development time, the mean time span between the application of the test substance and the emergence of the experimental cohort of midges, will be calculated for each replicate of each treatment and control group. The emergence rate and development rate will be calculated for each replicate of each control and treatment group using the SAS System for Windows Version 8.2 (8). The emergence rate, representing the number of midges emerged divided by the total number exposed, was calculated using the following formula:

$$ER = \frac{n_g}{n_e}$$

where: ER = emergence rate
n_g = sum of midges emerged per test chamber
n_e = number of larvae introduced

The emergence rate data were arcsine transformed using the following formula to normalize the data prior to statistical analyses:

$$ER_{arc} = \arcsin\left(\sqrt{\frac{n_g}{n_e}}\right)$$

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where: ERarc = arcsin-transformed emergence rate
n_e = sum of midges emerged per test chamber
n_o = number of larvae introduced

The development rate is the reciprocal of the development time and represents that portion of larval development which took place per day. Development rate will be calculated using the following formula:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where: $\bar{x}$ = development rate
i = index of inspection interval
m = maximum number of inspection intervals
f_i = number of midges emerged in the inspection interval i
n_e = total number of midges emerged until the end of experiment ($= \Sigma f_i$)
x_i = development rate of the midges emerged in interval i

$$x_i = \frac{1}{(day_i - 1/2)}$$

where: day_i = inspection day (days since application).

The data will be analyzed using Dunnett's test, as appropriate, to identify those treatment levels that are statistically different ($p<0.05$) from the negative control group (5, 6). If data cannot be pooled, the most appropriate comparisons will be made to either the negative or solvent control, or both. If other statistical procedures are used to analyze the data, the procedures will be described in the raw data and final report.

An ANOVA procedure looking at the interaction between sexes will be used to evaluate sensitivity between sexes for development rates. The data will be analyzed for significant interactions between sex and treatments for development rate to determine if data can be pooled between sexes. There is no feasible way to determine the sex of three day old larvae at the time organisms are added to test chambers. Therefore, evaluations of the sensitivity between sexes for emergence rate are not

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possible because calculations require knowing how many individuals of each sex are initially exposed. If there are no significant interactions found between sex and treatments for the number of female and male midges that emerge, the emergence rate data for both sexes will also be pooled for analysis.

RECORDS TO BE MAINTAINED

Records to be maintained for data generated by Wildlife International, Ltd. will include, but not be limited to:

1. A copy of the signed protocol.
2. Identification and characterization of the test substance, if provided by the Sponsor.
3. Dates of initiation and termination of the test.
4. *Chironimus riparius* culture records.
5. Stock solution calculation and preparation, if applicable.
6. Biological observations.
7. Water chemistry results (e.g., hardness and alkalinity).
8. Statistical calculations, if applicable.
9. Test conditions (light intensity, photoperiod, etc.).
10. Calculation and preparation of test concentrations.
11. Copy of final report.

FINAL REPORT

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

1. Name and address of the facility performing the study.
2. Dates upon which the study was initiated and completed, and the definitive experimental start and termination dates.
3. A statement of compliance signed by the Study Director addressing any exceptions to Good Laboratory Practice Standards.
4. Objectives and procedures, as stated in the approved protocol, including all changes to the protocol.
5. The test substance identification including name, chemical abstract number or code number, strength, purity, composition, and other information provided by the Sponsor.

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6. Stability and solubility of the test substance under the conditions of administration, if provided by the Sponsor.
7. A description of the methods used to conduct the test.
8. A description of the test organisms, including the source, scientific name, age or life stage, feed types, light intensity and photoperiod.
9. A description of the preparation of the test solutions.
10. The methods used to allocate organisms to test chambers and begin the test, the number of organisms and chambers per treatment, and the duration of the test.
11. A description of circumstances that may have affected the quality or integrity of the data.
12. The name of the Study Director and the names of other scientists, professionals, and supervisory personnel involved in the study.
13. A description of the transformations, calculations, and operations performed on the data, a summary and analysis of the biological data and analytical chemistry data, and a statement of the conclusions drawn from the analyses.
14. Statistical methods used to evaluate the data.
15. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
16. The location where raw data and final report are to be stored.
17. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates that any findings were reported to the Study Director and Management.
18. 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 alteration.

CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by 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.

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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 and/or Part 792); OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17); and Japan MAFF (11 NohSan, Notification No. 6283, Agricultural Production Bureau, 1 October 1999). Each study conducted by Wildlife International, Ltd. is routinely examined by the Wildlife International, Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Raw data for all work performed at Wildlife International, Ltd. and a copy of the final report will be filed by project number in archives located on the Wildlife International, Ltd. site, or at an alternative location to be specified in the final report.

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REFERENCES

1. **OECD Guideline 218.** 2004. *Sediment-Water Chironomid Toxicity Test Using Spiked Sediment.* Adopted 13 April 2004.

2. **Varaprath, S., C.L. Frye and J. Hamelink.** 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263 – 1265.

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. **Thompson, W.R.** 1947. *Bacteriological Reviews.* Vol. II, No. 2. Pp. 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.

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

8. **The SAS System for Windows.** 1999-2001. Release 8.2, (TS2M0). SAS Institute, Inc. Cary, North Carolina.

Wildlife International, Ltd.

DEVIATION TO STUDY PROTOCOL

STUDY TITLE: Octamethyltrisiloxane (L3; CAS No. 107-51-7): A Prolonged Sediment Toxicity Test with *Chironomus riparius* Using Spiked Sediment

PROTOCOL NO.: 570/091108/CHI-SED/SUB570 **DEVIATION NO.:** 1

SPONSOR: Silicones Environmental, Health and Safety Council **PROJECT NO.:** 570A-114

DATE OF DEVIATION: April 26, 2009

DEVIATION: The percent organic carbon content of the formulated sediment was 2.2 instead of 5.0 ± 1.0, as stated in the protocol.

REASON: The sediment was prepared using the composition described in the protocol. The carbon content can vary dependent upon the organic content of the peat. This deviation from the protocol had no adverse impact upon the results or interpretation of the study.

Susan Thomas
STUDY DIRECTOR

9/10/09
DATE

LABORATORY MANAGEMENT

9/10/09
DATE

Appendix 2

Certificate of Analysis

Dow Corning INTERNAL

The L3 was characterized according to GLPs [1]:

Identification:	Octamethyltrisiloxane (L3) (Supplied as Dow Corning® 200 Fluid, 1 cst.)
Batch Number:	0002680862
Expiration Date:	May 27, 2010
Source:	Dow Corning Corporation, Auburn, Michigan 48611
CAS Number:	107-51-7
Physical Description:	Colorless Liquid
Stability:	Stable according to the MSDS
Purity:	99.9 ± 0.03 area % purity
Water Solubility:	34.49 µg/L [2]
Storage Conditions:	Room Temperature
Archive:	A reserve sample was not retained.

References:

1. Stutts, D.H. 2006. Characterization of Octamethyltrisiloxane (L3) (Batch Number 0002680862). Dow Corning Report Number 2006-I0000-56652.
2. Varaprath, S., C.L. Frye and J. Hamelink. 1996. Aqueous solubility of permethylsiloxanes (silicones). Environ. Toxicol. Chem. 15:1263-1265.

Appendix 3

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

	Mean	Range
Specific Conductance (µmhos/cm)	380(N = 4)	371 - 393
Hardness (mg/L as $CaC0_3$)	140 (N = 4)	136 - 144
Alkalinity (mg/L as $CaC0_3$)	179 (N = 4)	178- 180
pH	8.1 (N = 4)	8.0 – 8.1

Appendix 4

Formulated Sediment Preparation [1]

Constituents	Weight (g)
Quartz Sand	14,000
Kaolin Clay	4,000
Peat Moss	2,000

[1]The sand and clay were mixed in a PK Twinshell® mixer for 20 minutes and the dry sediment was stored under ambient conditions until used.

[2] The peat moss was added separately to each individual batch (750 gram batch) of sediment prepared. The amount of peat moss added was adjusted for the percent moisture (70%).

The pH was measured in the negative control sediment once all ingredients were mixed together and found to be 7.0.

Appendix 5

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	<0.010	Gamma BHC – Lindane	<0.010
Alpha BHC	<0.010	Gamma Chlordane	<0.010
Alpha Chlordane	<0.010	Guthion (Azinphos-methyl)	<3.0
Beta BHC	<0.010	HCB	<0.010
Bolstar	<2.0	Heptachlor	<0.010
Chlordane	<0.51	Heptachlor Epoxide	<0.010
Coumaphos	<3.0	Kepone	<0.20
Delta BHC	<0.010	Malathion	<2.0
Demeton-O	<2.0	Merphos	<6.1
Demeton-S	<2.0	Methoxychlor	<0.10
Diazinon	<3.0	Methyl Parathion	<2.0
Dichlorvos	<3.0	Mevinphos	<3.0
Dieldrin	<0.020	Mirex	<0.033
Disulfoton	<2.0	Naled	<2.0
Dursban (Chlorpyrifos)	<2.0	o,p-DDD	<0.020
Endosulfan I	<0.010	o,p-DDE	<0.020
Endosulfan II	<0.020	o,p-DDT	<0.020
Endosulfan Sulfate	<0.020	p,p-DDD	<0.020
Endrin	<0.020	p,p-DDE	<0.020
Endrin Aldehyde	<0.10	p,p-DDT	<0.020
Endrin Ketone	<0.020	Phorate	<2.0
EPN	<2.0	Ronnel	<2.0
Ethion	<2.0	Stirophos	<2.0
Ethoprop	<3.0	Telodrin	<0.010
Ethyl Parathion	<2.0	Tokuthion	<2.0
Famphur	<2.0	Toxaphene	<3.0
Fensulfothion	<9.1	Trichloronate	<2.0
Fenthion	<2.0	Trithion	<2.0

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 5 (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	12.3
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	7.23
Calcium	32.5	Selenium	< 0.0200
Chloride	3.1	Silver	< 0.0050
Chromium	< 0.0150	Sodium	19.4
Cobalt	< 0.0050	Sulfate	5.8
Copper	< 0.0100	Thallium	< 0.0300
Fluoride	0.52	Vanadium	< 0.0050
Iron	< 0.200	Zinc	< 0.0200
Lead	< 0.0150		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 6

Agvise Soil Characterization Report

AGVISE LABORATORIES

604 Highway 15 West
P.O. Box 510
Northwood, ND 58267
(701) 587 6010
FAX (701) 587 6013
email: agvise@polarcomm.com
Homepage: www.agvise.com

AGVISE Soil Characterization Report

Submitting firm = WILDLIFE INTERNATIONAL
Protocol or Study No = VARIOUS
Sample ID. = 570A-114 NEGATIVE CONTROL
Trial ID. = BOTTOM
Date Received = 8-20-09
Date Reported = 08-26-2009

AGVISE Lab No 09-928

Percent Sand	72
Percent Silt	10
Percent Clay	18
USDA Textural Class (hydrometer method)	Sandy Loam
Bulk Density (disturbed) gm/cc	0.87
Cation Exchange Capacity (meq/100 g)	9.7
% Moisture at 1/3 Bar	17.5
% Organic Carbon --Walkley Black	2.2
% Organic Matter--Walkley Black	3.9
pH in 1:1 soil:water ratio	7.5

Base Saturation Data

Cation	Percent	ppm
Calcium	45.9	888
Magnesium	42.0	488
Sodium	1.0	23
Potassium	0.3	12
Hydrogen	10.8	10

These tests were completed in compliance of 40 CFR Part 160.

Larry Wikoff
Analytical Investigator

Date 8/27/09

Agricultural Testing

Appendix 7

Analyses of Pesticides, Organics and Metals
in Wildlife International, Ltd. Sediment

Pesticides and Organics			
Component	Measured Concentration (µg/kg)	Component	Measured Concentration (µg/kg)
Aldrin	< 0.93	Gamma BHC – Lindane	< 0.93
Alpha BHC	< 0.93	Gamma Chlordane	< 9.3
Alpha Chlordane	< 0.93	Guthion (Azinphos-methyl)	< 75
Beta BHC	< 0.93	HCB	< 0.93
Bolstar	< 75	Heptachlor	< 0.93
Chlordane	< 19	Heptachlor Epoxide	< 0.93
Coumaphos	< 75	Kepone	< 7.9
Delta BHC	< 0.93	Malathion	< 75
Demeton-O	< 75	Merphos	< 75
Demeton-S	< 75	Methoxychlor	< 9.3
Diazinon	< 75	Methyl Parathion	< 75
Dichlorvos	< 75	Mevinphos	< 75
Dieldrin	< 1.9	Mirex	< 1.9
Disulfoton	< 84	Naled	< 75
Dursban (Chlorpyrifos)	< 75	o,p-DDD	< 1.9
Endosulfan I	< 0.93	o,p-DDE	< 1.9
Endosulfan II	< 1.9	o,p-DDT	< 1.9
Endosulfan Sulfate	< 1.9	p,p-DDD	< 1.9
Endrin	< 1.9	p,p-DDE	< 1.9
Endrin Aldehyde	< 1.9	p,p-DDT	< 1.9
Endrin Ketone	< 1.9	Phorate	< 75
EPN	< 75	Ronnel	< 75
Ethion	< 75	Stirophos	< 75
Ethoprop	< 75	Telodrin	< 1.3
Ethyl Parathion	< 75	Tokuthion	< 75
Famphur	< 75	Toxaphene	< 37
Fensulfothion	< 220	Trichloronate	< 75
Fenthion	< 75	Trithion	< 75

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 7 (Continued)

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

Metals			
Component	Measured Concentration (mg/kg)	Component	Measured Concentration (mg/kg)
Aluminum	9960	Magnesium	3180
Antimony	< 2.18	Manganese	4.65
Arsenic	< 2.18	Mercury	< 0.108
Barium	14.0	Nickel	2.14
Beryllium	< 0.545	Nitrate by IC (solid)	< 1.7
Bromide by IC (solid)	< 5.6	Nitrite by IC (solid)	< 1.1
Cadmium	< 0.545	Potassium	105
Calcium	5460	Selenium	< 2.18
Chloride by IC (solid)	51.5	Silver	< 0.545
Chromium	5.60	Sodium	< 109
Cobalt	< 0.545	Sulfate by IC (solid)	78.2
Copper	< 1.09	Thallium	< 3.27
Fluoride by IC (solid)	1.6	Vanadium	2.15
Iron	396	Zinc	5.45
Lead	9.75		

[1]Analyses performed by Lancaster Laboratories on samples collected on December 22, 2008.

Appendix 8

The Analysis of L3 in Sediment

Appendix 8.1

Analytical Method Flowchart for the Analysis of L3 in Sediment

METHOD OUTLINE FOR THE ANALYSIS OF L3 IN SEDIMENT

Rinse all glassware with ethyl acetate.

↓

Sample sediment from the test beakers. Add the requisite volume of sediment (~5 g) to each 4 oz. French square bottle.

↓

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

↓

Add approximately 50 mL of ethyl acetate to each sample. Sonic disrupt the mixture for five minutes and allow the sediment to settle for approximately two minutes.

↓

Remove an aliquot of extract and centrifuge at 14,500 rpm for 10 minutes.

↓

Make secondary dilutions in ethyl acetate, as necessary.

↓

Transfer the extracts into autosampler vials and submit for analysis.

Appendix 8.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Agilent Model 6890 Gas Chromatograph (GC)
DETECTOR:	Agilent Model 5973 Mass Selective Detector operated in SIM mode
ANALYTICAL COLUMN:	DB-5 column (30 m x 0.25 mm, 0.25 µm)
INJECTOR TEMPERATURE:	325°C
RUN TIME:	14.52 minutes
OVEN:	Initial temperature: 50°C Initial hold time: 1.00 minute Ramp 1: 7.00°C/minute Final temperature 1: 100°C Final hold time 1: 0.00 minute Ramp 2: 40.00°C/minute Final temperature 2: 315°C Final hold time 2: 1.00 minute
DETECTOR TEMPERATURE:	230°C
INJECTION VOLUME:	1.00 µL
MAKE-UP GAS:	Helium
HEAD PRESSURE:	Approximately 7.6 psi
APPROXIMATE L3 RETENTION TIME:	5.5 minutes

Appendix 8.3

Analytical Stocks and Standards Preparation

Stock solutions of L3 were prepared by weighing 1.0010 g (weight corrected for purity) 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 acetone. This primary stock solution (10.0 mg a.i./mL) was diluted in acetone to produce 1.00 and 0.100 mg a.i./mL stock solutions. The 10.0 and 1.00 mg a.i./mL stock solutions were used to fortify the quality control samples. Calibration standards were prepared in ethyl acetate using the 0.100 mg a.i./mL stock solution. The following shows the dilution scheme for a set of calibration standards:

Stock Concentration (mg a.i./mL)	Aliquot (µL)	Final Volume (mL)	Standard Concentration (mg a.i./L)
0.100	50.0	10.0	0.500
0.100	300	10.0	3.00
0.100	600	10.0	6.00
0.100	900	10.0	9.00
0.100	1200	10.0	12.0
0.100	1500	10.0	15.0

Appendix 8.4

Example Calculations for a Representative Sediment Sample

The analytical result and percent recovery for sample number 570A-114-4, with a nominal concentration of 125 mg/Kg, were calculated using the following equations:

$$\text{L3 in sample (mg/Kg)} = \frac{\text{Peak Area - Y-intercept}}{\text{Slope}} \text{ X Dilution Factor} \div \text{Dry/Wet Soil Mass}$$

$$\text{Percent of nominal concentration} = \frac{\text{measured concentration of L-3 in sample (mg/Kg)}}{\text{nominal concentration of L-3 in sample (mg/Kg)}} \text{ X } 100$$

Peak Area = 1849347
Y-intercept = 70443.7980
Slope = 1160346.2531
Dilution factor = 9.94
Dry Soil Mass (g)/Wet Soil Mass (g) = 6.03 g/10.87 g = 0.554

$$\text{L3 in sample (mg/Kg)} = \frac{1849347 - 70443.7980}{1160346.2531} \text{ X } 9.94 \div 0.554$$

Concentration of L3 in sample (mg/Kg) = 27.5 mg/Kg

$$\text{Percent of nominal concentration} = \frac{27.5 \text{ mg/Kg}}{125 \text{ mg/Kg}} \text{ X } 100$$

Percent of nominal concentration = 22.0%

Appendix 8.5

Matrix Fortifications Analyzed Concurrently During Sample Analyses in Sediment

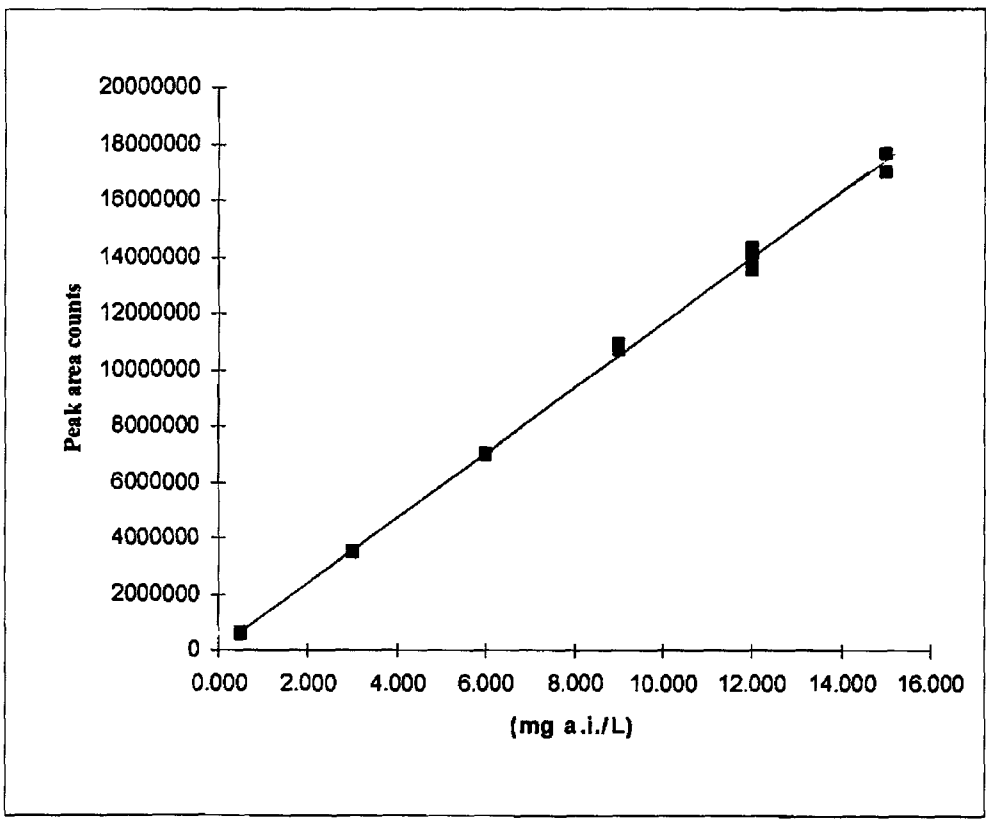
Sample Number (570A-114-)	Sample Type	Fortified Concentration (mg/Kg)	Measured Concentration (mg/Kg)[1]	Percent Recovery[2]
MAB-1	Matrix Blank	0.0	< LOQ	--
MAB-2	Matrix Blank	0.0	< LOQ	--
MAB-3	Matrix Blank	0.0	< LOQ	--
MAS-1	Matrix Fortification	30.0	24.4	81.5
MAS-2	Matrix Fortification	100	84.5	84.5
MAS-3	Matrix Fortification	1000	963	96.3
MAS-4	Matrix Fortification	30.0	23.3	77.8
MAS-5	Matrix Fortification	100	79.2	79.2
MAS-6	Matrix Fortification	1000	809	80.9
MAS-7	Matrix Fortification	30.0	19.8	65.9
MAS-8	Matrix Fortification	100	66.5	66.5
MAS-9	Matrix Fortification	1000	795	79.5
			Mean =	79.1
			Standard Deviation =	9.14
			CV =	11.6%

[1] The limit of quantitation (LOQ) was 5.00 mg/Kg, calculated as the product of the concentration of the lowest calibration standard (0.500 mg a.i./L) and the dilution factor of the matrix blank samples (10.0).

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

Appendix 8.6

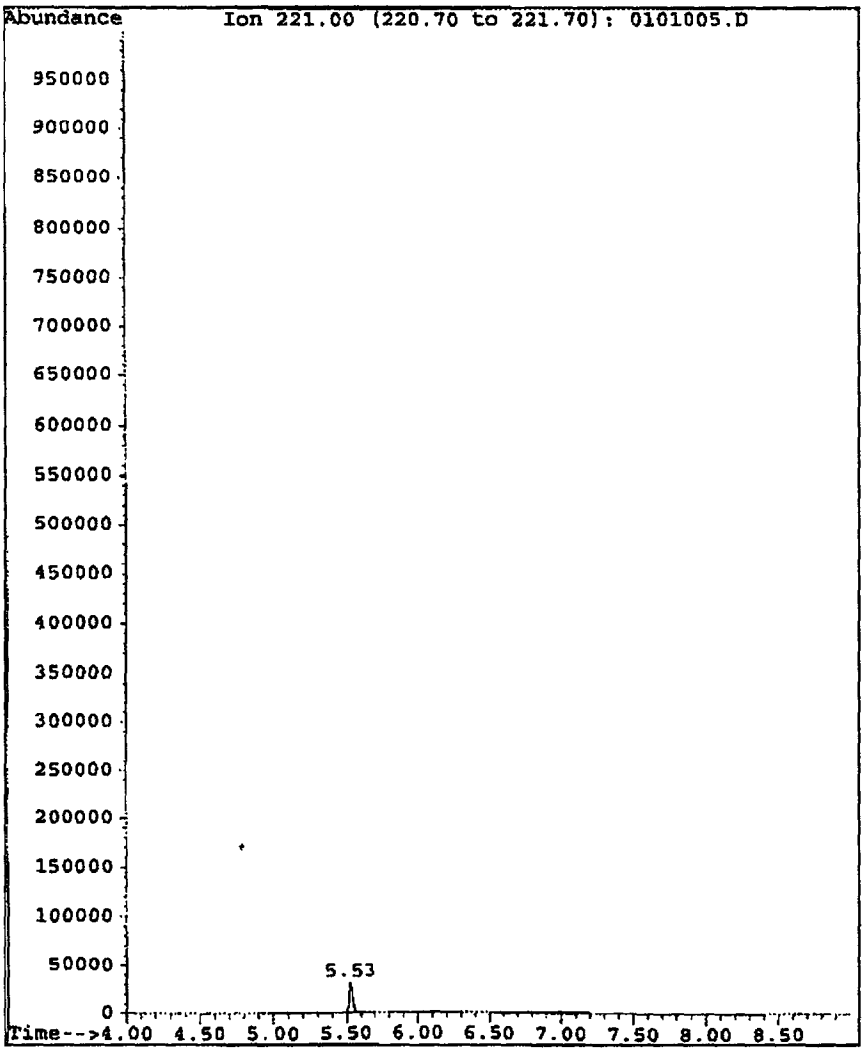
Representative Calibration Curve for L3 in Sediment



Slope = 1160346.2531; Y-Intercept = 70443.7980; $R^2 = 0.99810$

Appendix 8.7

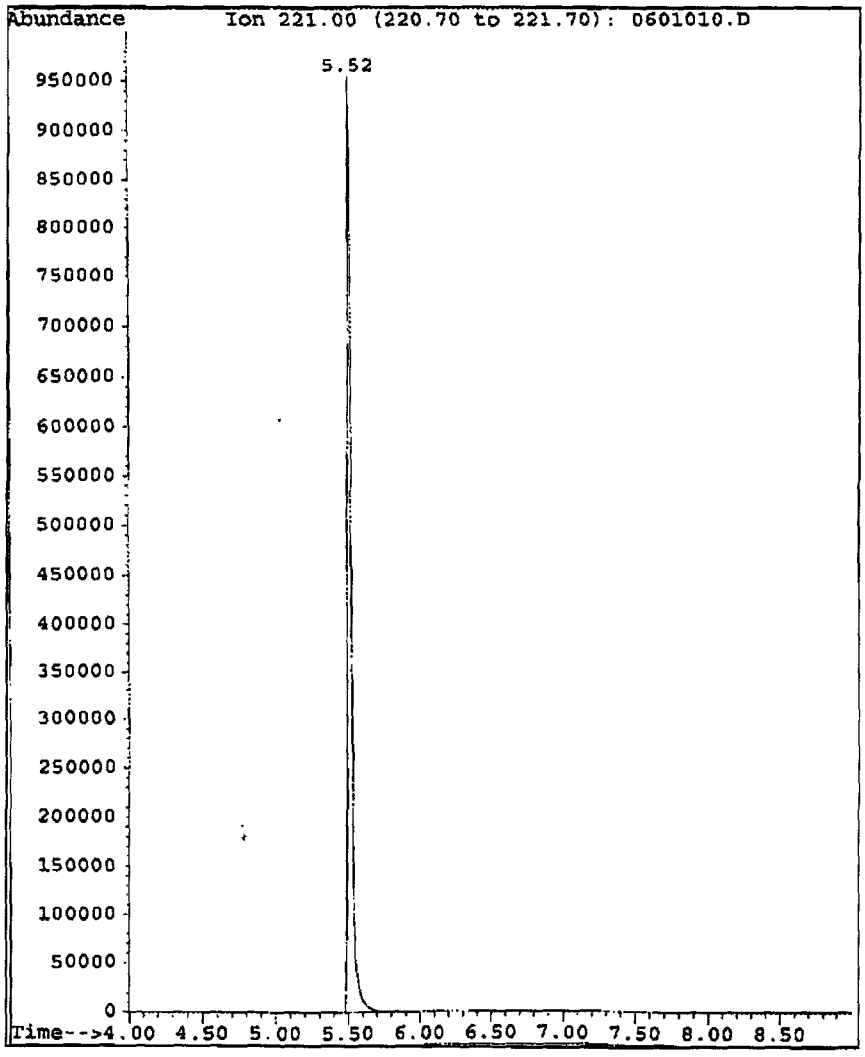
Representative Chromatogram of a Low-level L3 Calibration Standard



Nominal concentration: 0.500 mg a.i./L

Appendix 8.8

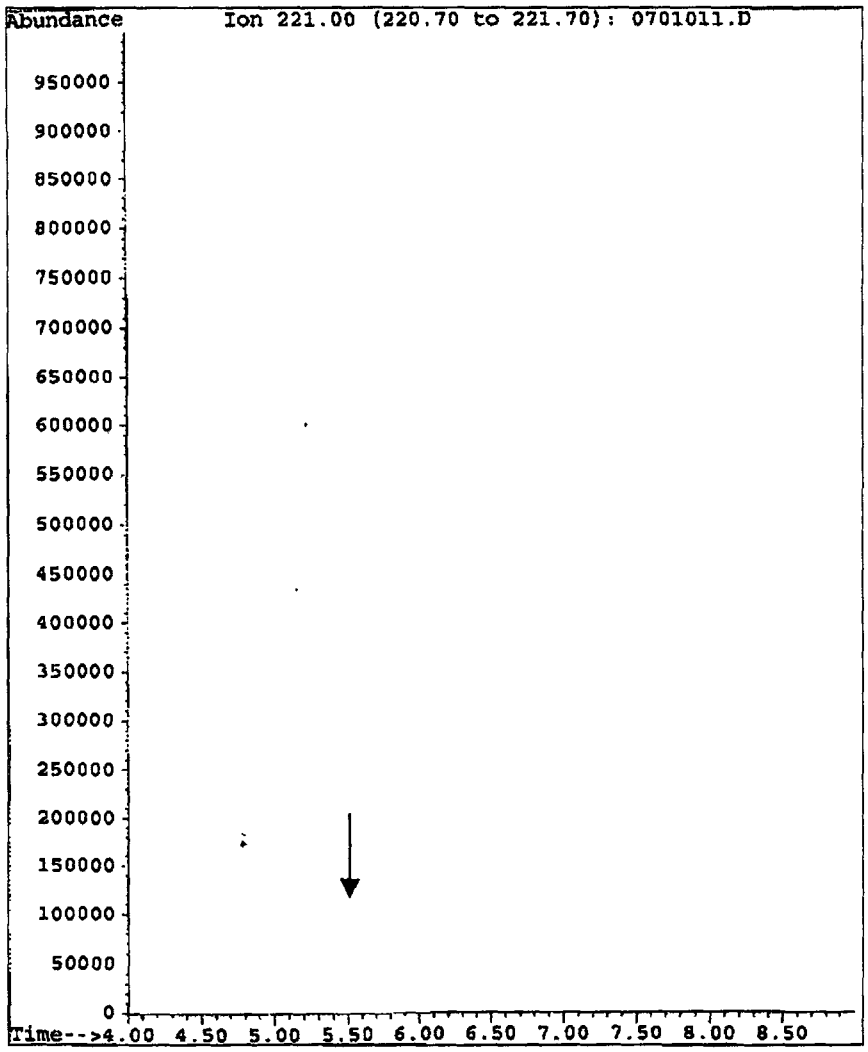
Representative Chromatogram of a High-level L3 Calibration Standard for Sediment



Nominal concentration: 15.0 mg a.i./L

Appendix 8.9

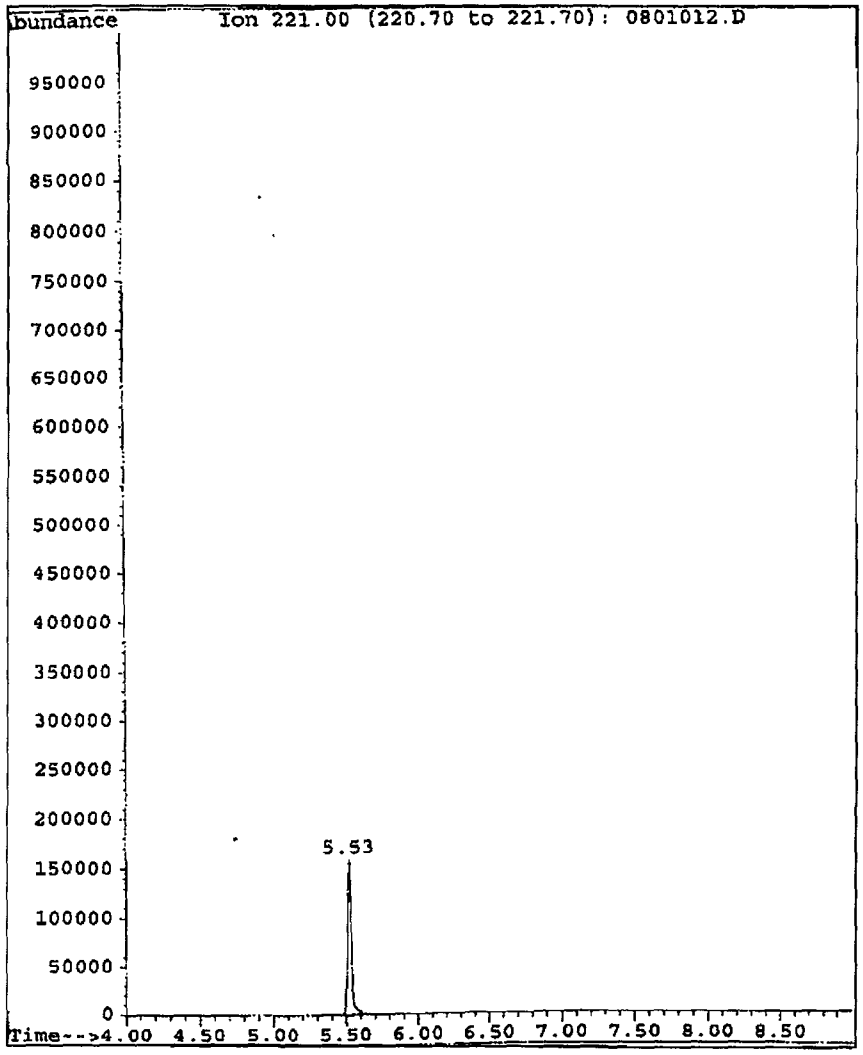
Representative Chromatogram of a Sediment Matrix Blank



Sample number 570A-114-MAB-1. The arrow indicates the approximate retention time of L3.

Appendix 8.10

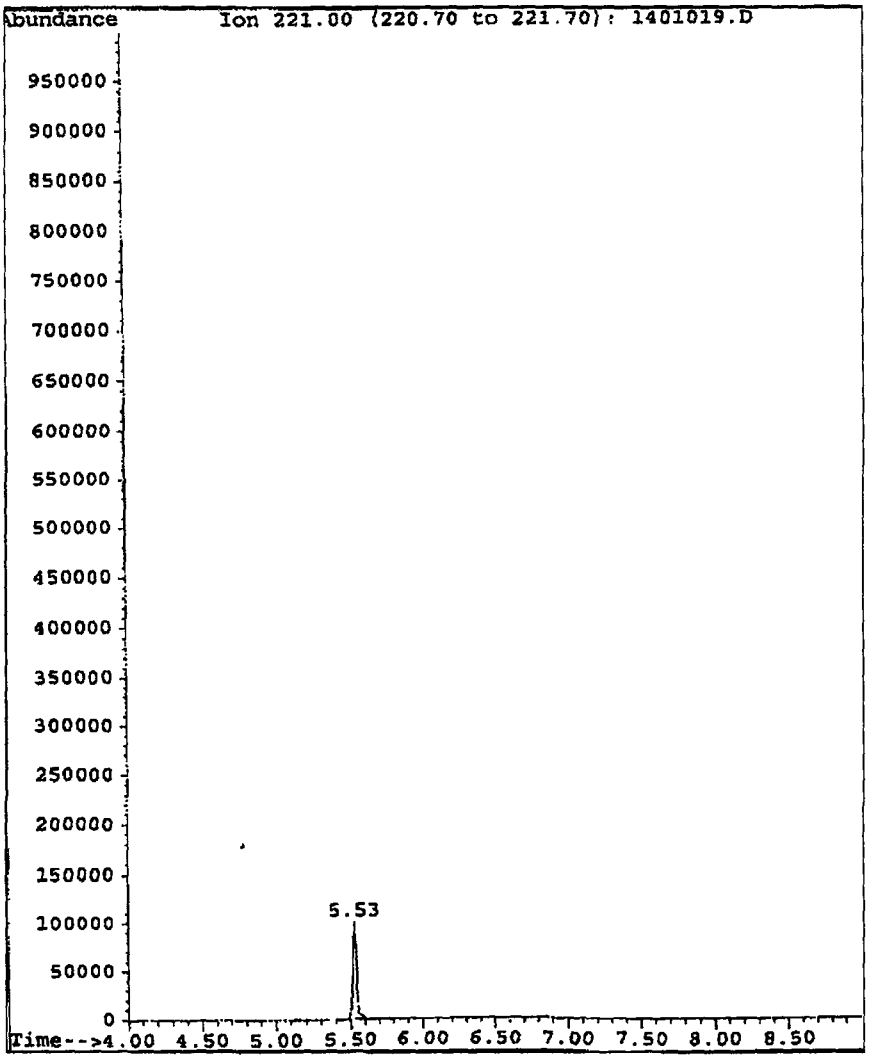
Representative Chromatogram of a Sediment Matrix Fortification



Sample number 570A-114-MAS-1; 30.0 mg a.i./Kg, nominal concentration.

Appendix 8.11

Representative Chromatogram of a Day 0 Sediment Sample



Sample number 570A-114-4; 125 mg/Kg nominal concentration

Appendix 9

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Males and Females pooled) by Day of Test															
Replicate		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	0	1	3	3	3	0	2	1	0	1	1	0	0	0	0	1
	B	0	3	2	2	2	2	1	0	3	0	0	0	1	0	0	1
	C	0	0	5	5	1	3	0	1	0	0	1	0	0	0	1	1
	D	1	2	4	5	3	2	0	1	0	1	0	0	0	0	0	0
7.4	A	0	1	4	3	3	4	1	1	0	0	0	1	1	0	0	0
	B	0	0	4	3	1	1	4	2	0	0	1	0	0	0	1	0
	C	0	1	3	4	3	1	3	1	0	0	0	0	2	0	0	0
	D	0	1	3	3	2	1	3	0	1	0	1	2	0	0	0	1
14	A	1	2	6	3	1	2	1	1	0	1	1	0	0	0	0	0
	B	0	4	5	3	2	3	1	1	0	0	0	0	0	0	0	0
	C	0	2	4	2	5	1	0	1	1	1	0	1	0	2	--	--
	D	0	1	1	1	4	2	2	1	3	0	0	1	0	0	0	1
39	A	0	0	2	2	4	3	3	2	1	2	1	--	--	--	--	--
	B	0	1	2	3	1	3	2	3	1	1	0	0	0	0	0	0
	C	0	1	0	2	2	4	1	0	3	1	1	0	0	1	1	0
	D	0	1	2	0	2	3	2	1	2	2	2	0	1	0	0	1
84	A	0	0	1	1	4	5	1	2	0	0	0	1	0	0	0	0
	B	0	0	1	0	3	4	2	3	1	2	2	0	0	0	1	0
	C	0	0	0	1	0	0	0	2	0	0	2	0	0	1	1	0
	D	0	0	0	2	0	3	1	3	1	1	0	1	0	0	2	2
210	A	0	0	1	2	0	2	1	3	1	1	0	1	1	0	0	1
	B	0	0	0	1	0	2	1	1	0	1	0	0	0	1	0	0
	C	0	0	0	1	1	0	1	2	0	1	1	2	0	0	1	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1
435	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Notes: -- = all emerged.

Appendix 9 (Continued)

Observations of Emergence by Replicate of the Midge (*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Males) by Day of Test															
Replicate		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	0	1	3	3	1	0	2	0	0	0	0	0	0	0	0	0
	B	0	3	2	1	1	0	0	0	0	0	0	0	0	0	0	0
	C	0	0	5	4	1	1	0	0	0	0	0	0	0	0	0	0
	D	1	2	4	3	1	1	0	0	0	0	0	0	0	0	0	0
7.4	A	0	1	3	3	2	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	4	3	1	0	1	1	0	0	0	0	0	0	0	0
	C	0	1	3	3	3	0	1	0	0	0	0	0	1	0	0	0
	D	0	1	3	2	1	0	1	0	0	0	0	0	0	0	0	0
14	A	1	2	6	2	0	2	0	0	0	0	0	0	0	0	0	0
	B	0	4	4	2	0	1	0	0	0	0	0	0	0	0	0	0
	C	0	2	1	1	1	0	0	0	0	0	0	0	0	0	--	--
	D	0	1	1	1	2	1	1	1	0	0	0	0	0	0	0	0
39	A	0	0	2	2	3	3	2	2	0	2	0	--	--	--	--	--
	B	0	1	2	2	0	2	1	1	0	1	0	0	0	0	0	0
	C	0	1	0	2	1	1	0	0	0	1	0	0	0	1	0	0
	D	0	1	2	0	0	2	0	1	1	1	0	0	0	0	0	0
84	A	0	0	1	1	2	4	0	2	0	0	0	1	0	0	0	0
	B	0	0	1	0	2	3	1	2	1	1	1	0	0	0	0	0
	C	0	0	0	1	0	0	0	2	0	0	1	0	0	1	0	0
	D	0	0	0	2	0	3	1	2	0	0	0	1	0	0	0	0
210	A	0	0	1	2	0	1	0	2	0	1	0	1	0	0	0	0
	B	0	0	0	1	0	2	1	0	0	1	0	0	0	0	0	0
	C	0	0	0	1	0	0	1	0	0	0	1	1	0	0	1	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
435	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Observations: -- = all emerged.

Appendix 9 (Continued)

Observations of Emergence by Replicate of the Midge
(*Chironomus riparius*) During a Prolonged Sediment Toxicity Test

Mean Measured Concentration (mg/Kg)		Numbers of Emerged Midges (Females) by Day of Test															
Replicate		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
NC	A	0	0	0	0	2	0	0	1	0	1	1	0	0	0	0	1
	B	0	0	0	1	1	2	1	0	3	0	0	0	1	0	0	1
	C	0	0	0	1	0	2	0	1	0	0	1	0	0	0	1	1
	D	0	0	0	2	2	1	0	1	0	1	0	0	0	0	0	0
7.4	A	0	0	1	0	1	4	1	1	0	0	0	1	1	0	0	0
	B	0	0	0	0	0	1	3	1	0	0	1	0	0	0	1	0
	C	0	0	0	1	0	1	2	1	0	0	0	0	1	0	0	0
	D	0	0	0	1	1	1	2	0	1	0	1	2	0	0	0	1
14	A	0	0	0	1	1	0	1	1	0	1	1	0	0	0	0	0
	B	0	0	1	1	2	2	1	1	0	0	0	0	0	0	0	0
	C	0	0	3	1	4	1	0	1	1	1	0	1	0	2	--	--
	D	0	0	0	0	2	1	1	0	3	0	0	1	0	0	0	1
39	A	0	0	0	0	1	0	1	0	1	0	1	--	--	--	--	--
	B	0	0	0	1	1	1	1	2	1	0	0	0	0	0	0	0
	C	0	0	0	0	1	3	1	0	3	0	1	0	0	0	1	0
	D	0	0	0	0	2	1	2	0	1	1	2	0	1	0	0	1
84	A	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	1	1	1	1	0	1	1	0	0	0	1	0
	C	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
	D	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	2
210	A	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1
	B	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
	C	0	0	0	0	1	0	0	2	0	1	0	1	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
435	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Observations: -- = all emerged or dead.

Appendix 10

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, Plant and Insect Toxicology
2. Willard B. Nixon, Ph.D., Director of Chemistry
3. Timothy Z. Kendall, M.S., Supervisor
4. Timothy Ross, B.S., Laboratory Supervisor
5. Susan T. Thomas, B.S., Aquatic Biologist