1-CHLORODODECANE:
A 96-HOUR TOXICITY TEST WITH THE FRESHWATER ALGA
(*Pseudokirchneriella subcapitata*)

FINAL REPORT

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 289A-165

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.5400

ASTM E 1218-97a

OECD Guideline 201

and

EU Directive 92/69/EEC, Method C.3

AUTHORS :

Sean P. Gallagher
Timothy Z. Kendall, M.S.
Henry O. Krueger, Ph.D.

STUDY INITIATION DATE: May 16, 2008

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SUBMITTED TO :

Lonza Inc.
90 Boroline Road
Allendale, NJ 07401

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

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

SPONSOR: Lonza Inc.

TITLE: 1-Chlorododecane: A 96-Hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*)

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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 for potential contaminants were not performed in compliance with Good Laboratory Practice Standards, but were performed using a certified laboratory and standard U.S. EPA analytical methods.

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

STUDY DIRECTOR:

Sean P. Gallagher — 9/12/08
Sean P. Gallagher
Senior Biologist
Date

LABORATORY APPROVAL:

Henry O. Krueger, Ph.D. — 9/12/08
Henry O. Krueger, Ph.D.
Director of Aquatic, Plant and Insect Toxicology
Date

SPONSOR/APPLICANT APPROVAL:

Robert J. Sloan — 9/12/2008
Applicant/Submitter
Robert J. Sloan
Senior Regulatory Services Associate - Registrations
Lonza Inc.
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 Laboratory Management were as follows:

ACTIVITY:	DATE CONDUCTED:	DATE REPORTED TO: STUDY DIRECTOR:	MANAGEMENT:
Protocol	April 21, 2008	April 21, 2008	April 23, 2008
Test Substance Preparation	May 19, 2008	May 19, 2008	May 21, 2008
pH Measurements	May 23, 2008	May 23, 2008	June 3, 2008
Analytical Data and Draft Report	July 17, 2008	July 17, 2008	July 18, 2008
Biological Data and Draft Report	August 3-6, 2008	August 6, 2008	August 12, 2008
Final Report	September 12, 2008	September 12, 2008	September 12, 2008

All inspections are study-based unless otherwise noted.

Linda R. Mitchell
Director of Regulatory and Ecotox Operations

12 SEPTEMBER 2008
Date

REPORT APPROVAL

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STUDY DIRECTOR:

Sean P. Gallagher
Senior Biologist

Date 9/12/08

PRINCIPAL INVESTIGATOR:

Timothy Z. Kendall, M.S.
Supervisor

Date 9-12-08

WILDLIFE INTERNATIONAL, LTD. MANAGEMENT:

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

Date 9/12/08

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

Date 9/12/08

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SUMMARY

SPONSOR: Lonza Inc.

TITLE: 1-Chlorododecane: A 96-Hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*)

WILDLIFE INTERNATIONAL, LTD. PROJECT NUMBER: 289A-165

TEST DATES:	Study Initiation:	May 16, 2008
	Experimental Start (OECD):	May 19, 2008
	Experimental Start (EPA):	May 19, 2008
	Exposure Termination:	May 23, 2008
	Experimental Termination:	May 28, 2008

LENGTH OF EXPOSURE: 96 Hours

TEST ORGANISMS: Freshwater Alga (*Pseudokirchneriella subcapitata*)

SOURCE OF TEST ORGANISMS: Wildlife International, Ltd. Cultures
Easton, Maryland 21601

TEST CONCENTRATIONS:

Nominal	Geometric Mean Measured
Negative Control	< LOQ
Solvent Control	< LOQ
0.016 mg a.i./L	0.0055 mg a.i./L
0.031 mg a.i./L	0.0076 mg a.i./L
0.063 mg a.i./L	0.012 mg a.i./L
0.13 mg a.i./L	0.018 mg a.i./L
0.25 mg a.i./L	0.024 mg a.i./L
0.50 mg a.i./L	0.034 mg a.i./L

RESULTS (based on geometric mean measured concentrations):

CELL DENSITY:

72-Hour EC50:	0.029 mg a.i./L
95% Confidence Limits:	0.024 and 0.034 mg a.i./L
72-Hour NOAEC:	0.0076 mg a.i./L
96-Hour EC50:	>0.034 mg a.i./L
95% Confidence Limits:	Not calculable
96-Hour NOAEC:	0.0076 mg a.i./L

GROWTH RATE:

72-Hour E_rC50:	>0.034 mg a.i./L
95% Confidence Limits:	Not calculable
72-Hour NOAEC:	0.0076 mg a.i./L
96-Hour E_rC50:	>0.034 mg a.i./L
95% Confidence Limits:	Not calculable
96-Hour NOAEC:	0.012 mg a.i./L

SUMMARY
(continued)

AREA UNDER THE GROWTH CURVE:

72-Hour E_bC50:	0.027 mg a.i./L
95% Confidence Limits:	0.023 and 0.032 mg a.i./L
72-Hour NOAEC:	0.0076 mg a.i./L
96-Hour E_bC50:	0.036 mg a.i./L
95% Confidence Limits:	0.030 and 0.042 mg a.i./L
96-Hour NOAEC:	0.0076 mg a.i./L

INTRODUCTION

This study was conducted by Wildlife International, Ltd. for Lonza Inc. at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The in-life phase of the test was conducted from May 19 to 23, 2008. Raw data generated by Wildlife International, Ltd. and the original final report are filed by project number in archives located on the Wildlife International, Ltd. site.

OBJECTIVE

The objective of this study was to determine the toxicity of 1-Chlorododecane to the freshwater green alga (*Pseudokirchneriella subcapitata*) during a 96-hour exposure period.

EXPERIMENTAL DESIGN

The green alga (*Pseudokirchneriella subcapitata*) was exposed to six test concentrations, a negative control (culture medium) and a solvent control (0.1 mL/L dimethyl formamide) for 96 hours. Three replicate test chambers in each control and each treatment group were maintained. Nominal test concentrations were selected in consultation with the Sponsor and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 0.016, 0.031, 0.063, 0.13, 0.25 and 0.50 milligrams 1-Chlorododecane active ingredient per liter (mg a.i./L). Measured test concentrations were determined from samples of test medium collected from each treatment and control group at the beginning of the test and at test termination. All results were based on the geometric means of the measured test concentrations.

At test initiation an inoculum of the algal cells was added to each test chamber to achieve a nominal concentration of approximately 10,000 *Pseudokirchneriella* cells/mL. Samples were collected from each replicate test chamber at approximately 24-hour intervals during the test to determine cell densities, which were subsequently used to calculate areas under the growth curve (biomass) and growth rates. Cell densities, biomass and growth rates were used to calculate percent inhibition values relative to the pooled control over the 96-hour exposure period and were analyzed statistically to estimate EC50 values, (i.e., the theoretical test concentrations that would produce a 50% reduction in cell density (EC50), growth rate (E_rC50) and area under the growth curve (E_bC50) in comparison to the pooled control) and the corresponding 95% confidence limits for each 24-hour exposure interval. The no-observed-adverse-effect-concentration (NOAEC) was determined based

upon an evaluation of the statistical results, as well as examination of the concentration-response pattern. At the end of the 96-hour exposure period, none of the treatment groups were maximally affected. Therefore, no attempt was made to differentiate algistatic effects from algicidal effects.

MATERIALS AND METHODS

The study was conducted based on procedures outlined in the protocol, "1-Chlorododecane: A 96-Hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*)". The protocol was based on procedures outlined in U.S. Environmental Protection Agency Series 850 – Ecological Effects Test Guidelines OPPTS, Number 850.5400 (1); ASTM E 1218-97a, Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae (2); OECD Guidelines for Testing of Chemicals, 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test* (3); and Official Journal of the European Communities No. L383, Method C.3: *Algal Inhibition Test* (4).

Test Substance

The test substance used to prepare the test solutions, analytical calibration standards and the analytical matrix fortification samples for the study was received from Lonza Inc. on May 24, 2007. It was assigned Wildlife International, Ltd. identification number 8134 upon receipt and was stored under ambient conditions. The test substance, a liquid, was identified as: Barchlor 12s; CAS No. 112-52-7; Batch number N6227945. The test substance had a reported purity of 97.42% 1-Chlorododecane, and an expiration date of June 29, 2009. A copy of the test substance Certificate of Analysis is presented in Appendix 1.

Test Organism

The freshwater green alga (*Pseudokirchneriella subcapitata*) was selected as the test species for this study. The species is representative of an important group of freshwater algae, and was selected for use in the test based upon a past history of use, and ease of culturing in the laboratory. Original algal cultures were obtained from the University of Toronto Culture Collection, and had been maintained in culture medium at Wildlife International, Ltd., Easton, Maryland. Algal cells used in this test were obtained from Wildlife International, Ltd. cultures that had been actively growing in culture medium for at least two weeks prior to test initiation. Algal cells for this study were taken from a culture that had been transferred to fresh media three days prior to test initiation. The negative control organisms were expected to exhibit exponential growth over the 96-hour

exposure period. Exponential growth phase, defined as the period of growth where the algal cells are dividing at a constant rate, is indicated by the linear section of the growth curve (Figure 1).

Culture Medium

The algal cells were cultured and tested in freshwater AAP algal medium (1-3). Stock nutrient solutions were prepared by adding reagent-grade chemicals to purified Wildlife International, Ltd. well water. The test medium then was prepared by adding appropriate volumes of the stock nutrient solutions to purified well water (Appendix 2). The pH was 7.5 and the medium was sterilized by filtration (0.22 µm) prior to use. The results of the most recent analyses performed to measure the concentrations of selected contaminants in the well water used to prepare the algal medium are presented in Appendix 3.

Test Apparatus

Test chambers were sterile, 250-mL Erlenmeyer flasks plugged with foam stoppers and contained 100 mL of test or control medium. The test flasks were labeled with the project number, concentration and replicate, and were indiscriminately positioned daily on a mechanical shaker in an environmental chamber designed to maintain the desired test temperature throughout the test. The test flasks were shaken continuously at 100 rpm.

Preparation of Test Concentrations

A primary stock solution was prepared by dissolving 0.0513 g of 1-Chlorododecane in 10 mL of dimethylformamide (DMF) for a nominal concentration of 5.0 mg a.i./mL. The primary stock was inverted at least 20 times to mix, and was clear and colorless with foam on the surface. The primary stock was serially diluted with dimethylformamide (DMF) to prepare the five secondary (working) stock solutions at nominal concentrations of 0.16, 0.31, 0.63, 1.25 and 2.5 mg a.i./mL. The working stock solutions were diluted with algal medium to prepare the test solutions at nominal concentrations of 0.016, 0.031, 0.063, 0.13, 0.25 and 0.50 mg a.i./L. The solvent control solution was prepared by diluting DMF in algal medium. The concentration of DMF in the solvent control and all treatment groups was 0.1 mL/L. Test concentrations were corrected to 100% active ingredient based upon a test substance purity of 97.42%. All test solutions appeared clear and colorless.

Analytical Sampling

Samples of the test solutions were collected at approximately 0 and 96 hours to measure concentrations of the test substance. At test initiation, samples were collected from batch test solution of each treatment and control group prior to distribution into test chambers. At test termination, the replicates from each respective treatment and control group were pooled and then sampled. All samples were collected in glass vials and analyzed immediately without storage.

Analytical Method

The method used for the analysis of 1-Chlorododecane in freshwater algal medium samples was based upon methodology developed by Wildlife International, Ltd. The analytical method consisted of extracting the freshwater algal medium samples with ethyl acetate and analyzing by gas chromatography with mass selective detection.

Concentrations of 1-Chlorododecane in the samples were determined using a Hewlett-Packard Model 5890 Gas Chromatograph (GC) with a Hewlett-Packard Model 5971 Mass Selective Detector operated in the Selective Ion Monitoring (SIM) mode. Chromatographic separations were achieved using a ZB-5 column (30 m x 0.25 mm ID, 0.25 µm). The method flowchart is provided in Appendix 4.1 and the instrument parameters are summarized in Appendix 4.2.

Calibration standards of 1-Chlorododecane, ranging in concentration from 0.0150 to 0.200 mg a.i./L, were prepared using stock solutions of 1-Chlorododecane in acetone (Appendix 4.3). Calibration standards were 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 1-Chlorododecane 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 4.4.

The method limit of quantitation (LOQ) for these analyses was defined as 0.00750 mg a.i./L, calculated as the product of the concentration of the lowest calibration standard (0.0150 mg a.i./L) and the dilution factor of the matrix blank samples (0.500). Two matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses (Appendix 4.5).

Samples of freshwater algal medium were fortified at 0.0160, 0.100 and 0.500 mg a.i./L using stock solutions of 1-Chlorododecane in acetone (Appendix 4.3), and were analyzed concurrently with each test sample set. The measured concentrations for the matrix fortification samples ranged from 96.4 to 120% of nominal concentrations (Appendix 4.5).

A representative calibration curve is presented in Appendix 4.6. Representative chromatograms of low and high-level calibration standards are presented in Appendices 4.7 and 4.8, respectively. Representative chromatograms of a matrix blank sample and a matrix fortification sample are presented in Appendices 4.9 and 4.10, respectively. A representative chromatogram of a test sample is presented in Appendix 4.11.

Inoculation of Test Chambers

Prior to test initiation, an inoculum of the algal cells was prepared in freshwater algal medium at a concentration of approximately 1.0 X 10^6 cells/mL. The concentration of algal cells was verified using a hemacytometer and microscope, and 1.0 mL of the inoculum was added to each test chamber to achieve a nominal concentration of approximately 10,000 cells/mL at test initiation.

Environmental Conditions

Test chambers were held in an environmental chamber at a temperature of 24 ± 2°C. The temperature of a container of water adjacent to the test chambers in the environmental chamber was recorded twice daily during the test using a liquid-in-glass thermometer. In addition, temperature in the environmental chamber was measured continuously with a minimum/maximum thermometer. The algae were held under continuous cool-white fluorescent lighting throughout the test. Light bulbs in the environmental chamber were replaced two weeks prior to test initiation. The target light intensity was 4300 ± 10% lux. Light intensity was measured at five locations surrounding the test flasks on the shaker table at test initiation. Light intensity was measured using a SPER Scientific 840006C light meter. The pH of the medium in each treatment and control group was measured at test initiation and at test termination using a Thermo Orion Model 525 Aplus pH meter. At test initiation, pH was measured in the individual batches of test solution prepared for each treatment and the control group. At test termination, pH was measured in pooled samples of test solution collected from each of the replicates in each treatment and control group.

Algal Growth Measurements and Observations

Test medium samples were collected from each replicate of the treatment and control group for the determination of algal cell densities. Samples were collected at approximately 24-hour intervals during the 96-hour exposure and were held for a maximum of eight days under refrigerated conditions sufficient to inhibit growth until cell counts could be performed. Cell counts were performed using an electronic particle counter (Coulter Electronics, Inc.). Prior to conducting cell counts, the linearity of the instrument response was determined at settings previously established for *Pseudokirchneriella subcapitata*. A primary counting standard containing *Pseudokirchneriella subcapitata* cells was prepared, the density was verified using a hemacytometer and a microscope, and the standard was subsequently diluted to provide a series of seven counting standards for the determination of instrument linearity. Theoretical densities were assigned to each secondary counting standard based upon the verified density of the primary counting standard and the dilution ratio. The cell densities of the counting standards were measured using the electronic particle counter and were compared to the theoretical densities by performing a least squares regression analysis. Cell counts for samples collected during the test were conducted once instrument linearity was demonstrated (i.e., the R-squared value obtained through the regression analysis was 0.998). A single aliquot of each sample collected during the test was diluted with an electrolyte solution (Isoton® II). Three 0.5-mL volumes of the diluted sample were counted, and the resulting counts were averaged. The cell density of the sample was determined by adjusting the mean cell count (cells/mL) obtained using the particle counter, based upon the Y intercept and slope calculated through the regression analysis, and the dilution factor. The following equation was used:

$$\text{Cell Density of Sample (cells/mL)} = \frac{\text{Mean Cell Count (cells/mL) - Y Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

Microscopic examination of the inoculum culture prior to initiation of the test and of a pooled sample from each test concentration and the control at the end of the test was performed to assess whether the algae were normal in appearance (e.g. cell morphology, cell flocculation or aggregation and adherence of cells to the test chamber).

Statistical Analyses

The calculation of cell densities, areas under the growth curve (biomass), growth rates and percent inhibition values, as well as all statistical analyses, were conducted using "The SAS System for Windows," Version 8.2 (5). Area under the growth curve was calculated for each replicate of the control and treatment groups using the following formula:

$$A = ((N_1-N_o)/2)(t_1)+((N_1+N_2-2N_o)/2)(t_2-t_1) +...+((N_{n-1}+N_n-2N_o)/2)(t_n-t_{n-1})$$

where:
- A = Area under the growth curve
- N_o = Mean nominal number of cells/mL at t_o
- N_1 = Mean measured number of cells/mL at t_1
- N_2 = Mean measured number of cells/mL at t_2
- N_n = Mean measured number of cells/mL at t_n
- t_1 = Time of first measurement after beginning of test (hours)
- t_2 = Time of second measurement after beginning of test (hours)
- t_n = Time of nth measurement after beginning of test (hours)

Specific growth rate was calculated for each replicate of the control and treatment groups using the following formula:

$$\text{Specific Growth Rate } (\mu) = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

where:
- μ = Average specific growth rate
- N_o = Nominal cell density (cells/mL) at t_o
- N_n = Measured cell density (cells/mL) at t_n
- t_n = Time after beginning of test (hours)
- t_o = Time of beginning of test (hours)

Inhibition values were calculated for each treatment group as the percent reduction in cell density, area under the growth curve and growth rate relative to the pooled control replicates using the following formula:

$$\text{Percent Inhibition} = \frac{\text{Mean Control Response - Mean Treatment Response}}{\text{Mean Control Response}} \times 100$$

Cell densities and growth rates were analyzed statistically to estimate EC50 values, (i.e., the theoretical test concentrations that would produce a 50% reduction in cell density (EC50, growth rate

(E_rC50) and area under the growth curve (E_bC50) in comparison to the pooled control) and the corresponding 95% confidence limits for each 24-hour exposure interval. Data from the negative and solvent control groups for each parameter were compared using a t-test. Since no differences were detected between the control groups ($p > 0.05$), the control data were pooled for comparison to the treatment groups. EC50 values were calculated by non-linear regression (6). The cell density, area under the growth curve and growth rate data were evaluated for normality and homogeneity of variance (p=0.01) using the Shapiro-Wilk's and Levene's tests, respectively. With the exception of the Day 4 growth rate data, the data were normal with homogeneous variances. A log transformation of the Day 4 data was unsuccessful in correcting the unequal error variances for the growth rate. Therefore, analysis of the Day 4 growth rate data was performed on the untransformed data. All treatment groups were compared to the pooled control using ANOVA and Dunnett's test (p=0.05). The results of the statistical analyses, as well as an evaluation of the concentration-response pattern, were used to determine the NOAEC relative to each parameter at 72 and 96 hours.

RESULTS AND DISCUSSION

Conditions for the Validity of the Test

1) The mean cell density in the control flasks increased by a factor greater than 16 within three days.

2) The coefficient of variation of average specific growth rate in the control replicates during the whole test period did not exceed 7%.

3) The mean percent coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3 and 3-4) did not exceed 35%.

Measurement of Test Concentrations

Results of analyses to measure concentrations of 1-Chlorododecane in test solution samples collected during the test are presented in Table 1. Nominal concentrations selected for use in this study were 0.016, 0.031, 0.063, 0.13, 0.25 and 0.50 mg a.i./L. Samples collected on Day 0 had recoveries of 51, 50, 61, 67, 60 and 61% of nominal concentrations, respectively. Samples collected on Day 4 had recoveries below the limit of quantitation (LOQ) for all test concentrations. Efforts to determine the cause of the low measured concentrations on Day 0 were unsuccessful. 1-Chlorododecane has very low water solubility (Wildlife International, Ltd. Project No.: 289C-203) and it was assumed that the analytical results in the algae medium were affected by solubility. This

conclusion was supported by the lack of maximum inhibition at increasing concentrations. It was concluded that conduct of the study at the specified concentrations adequately represented the potential toxicity of 1-Chlorododecane to algae. Therefore, the study was conducted as described and the results of the study were based on the geometric mean measured concentrations. For values <LOQ, a value of one-half the LOQ was used to calculate the geometric mean concentration. Geometric mean measured concentrations were 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L, representing 34, 25, 19, 14, 10, and 7% of nominal concentrations, respectively. The results of the study were based on geometric mean measured concentrations.

Observations and Measurements

Measurements of temperature, pH and light intensity are presented in Tables 2, 3 and 4, respectively. Temperatures ranged from 23.2 to 23.9°C and were within the 24 ± 2°C range established for the test. Measurements of pH were 7.6 in the test solutions at the beginning of the test period and ranged from 8.2 to 8.3 at test termination. The pH tended to increase relative to increases in algal densities, which is typical for tests conducted with *Pseudokirchneriella subcapitata*. The light intensity ranged from 3930 to 4500 lux, which was within the desired range of 4,300 ± 10% lux.

The toxicity of 1-Chlorododecane to *Pseudokirchneriella subcapitata* was determined by evaluating changes in cell density over a 96-hour exposure period. Cell densities were used to calculate areas under the growth curve and growth rates for each 24-hour interval of exposure. Mean cell densities, areas under the growth curve and growth rates with the corresponding percent inhibition values are presented in Tables 5, 6 and 7, respectively. EC50, E_rC50 and E_bC50 values and the corresponding 95% confidence limits for cell density, growth rate and area under the growth curve, respectively, are presented in Table 8. Individual replicate data for each growth parameter are presented in Appendices 5, 6 and 7, while mean cell densities are illustrated graphically in Figure 1 for the pooled control and for the pooled control and each test concentration in Figure 2. Changes in mean cell density in the control replicates over the 96-hour exposure period indicated that exponential growth of cells occurred in those replicates (Figure 1). Appendix 8 provides section by section and overall specific growth rates and coefficients of variation for the control cultures to illustrate compliance with the second and third criteria for validity of the test.

After 72 hours of exposure, inhibition of cell density in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was 0.52, 0.67, 23, 27, 39 and 58%, respectively, relative to the pooled control. Inhibition of area under the growth curve in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was 1.8, -1.8, 25, 28, 42 and 61%, respectively, relative to the pooled control. Inhibition of growth rate in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was 0.19, 0.34, 5.4, 6.5, 10 and 19%, respectively, relative to the pooled control. When compared to the pooled control group, there were statistically significant ($p<0.05$) reductions in area under the growth curve, cell density and growth rate in the 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups. Consequently, the 72-hour EC50, E_rC50 and E_bC50 values were 0.029, >0.034 and 0.027 mg a.i./L, respectively. The 72-hour NOAEC for area under the growth curve, cell density and growth rate was 0.0076 mg a.i./L.

After 96 hours of exposure, inhibition of cell density in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was –3.4, 1.6, 13, 14, 22 and 39%, respectively, relative to the pooled control. Inhibition of area under the growth curve in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was –1.4, 0.56, 18, 20, 30 and 48%, respectively, relative to the pooled control. Inhibition of growth rate in the 0.0055, 0.0076, 0.012, 0.018, 0.024 and 0.034 mg a.i./L treatment groups was –0.57, 0.29, 2.3, 2.6, 4.2 and 8.6%, respectively, relative to the pooled control. When compared to the pooled group, there were statistically significant ($p<0.05$) reductions in cell density, area under the growth curve and growth rate in the 0.018, 0.024 and 0.034 mg a.i./L treatment groups. There were also statistically significant ($p<0.05$) reductions in cell density and area under the growth curve in the 0.012 mg a.i./L treatment group. Consequently, the 96-hour EC50, E_rC50 and E_bC50 values were >0.034, >0.034 and 0.036 mg a.i./L, respectively. The 96-hour NOAEC for cell density and area under the growth curve was 0.0076 mg a.i./L, and the 96-hour NOAEC for growth rate was 0.012 mg a.i./L.

At test initiation algal cells appeared normal. After 96-hours of exposure there were no noticeable changes in cell morphology at any of the concentrations tested. There was also no flocculation or aggregation of cells, or adherence of cells to test chambers noted in the controls or treatment groups.

CONCLUSIONS

Pseudokirchneriella subcapitata were exposed to six concentrations of 1-Chlorododecane and evaluated for effects on cell density, area under the growth curve (biomass) and growth rate. Cell density and area under the growth curve were the most sensitive endpoints, as defined by the lowest 96-hour NOAEC values (0.0076 mg a.i./L for both endpoints). The 96-hour NOAEC based on growth rate was 0.012 mg a.i./L. The 96-hour EC50 and E_rC50 were both >0.034 mg a.i./L, the highest geometric mean measured concentration. The 96-hour E_bC50 value was 0.036 mg a.i./L.

REFERENCES

1 **U.S. Environmental Protection Agency.** 1996. Series 850 – Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II.*

2 **ASTM.** 1997. *Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae*. American society for Testing and Materials. Guideline E 1218-97a.

3 **Organisation for Economic Cooperation and Development**. 2006. OECD Guidelines for Testing of Chemicals, 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test*. Adopted 23 March 2006.

4 **Official Journal of the European Communities.** 1992. No. L383. Method C.3. *Algal Inhibition Test.*

5 **The SAS System for Windows.** 1999 – 2001. Version 8.2. SAS Institute, Inc., Cary, NC,.

6 **Bruce, Robert D. and Donald J. Versteeg.** 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. *Environmental Toxicology and Chemistry*. 11: 1485-1494.

Table 1

Measured Concentrations of 1-Chlorododecane in Samples of Freshwater Algal Medium

Nominal Test Concentration (mg a.i./L)	Sample Number (289A-165-)	Sampling Time (Hours)	Measured Concentration (mg a.i./L)[1]	Percent of Nominal[2]	Geometric Mean Measured Concentration[3] (mg a.i./L)	Mean Percent of Nominal
Negative Control	1	0	< LOQ	--	--	--
(0.0)	9	96	< LOQ	--		
Solvent Control	2	0	< LOQ	--	--	--
(0.0)	10	96	< LOQ	--		
0.016	3	0	0.00810	50.6	0.0055	34
	11	96	< LOQ	--		
0.031	4	0	0.0156	50.3	0.0076	25
	12	96	< LOQ	--		
0.063	5*	0	0.0386	61.3	0.012	19
	13	96	< LOQ	--		
0.13	6	0	0.0869	66.8	0.018	14
	14	96	< LOQ	--		
0.25	7	0	0.149	59.5	0.024	10
	15	96	< LOQ	--		
0.50	8	0	0.306	61.2	0.034	7
	16	96	< LOQ	--		

[1] The limit of quantitation (LOQ) was 0.00750 mg a.i./L, calculated as the product of the concentration of the lowest calibration standard (0.0150 mg a.i./L) and the dilution factor of the matrix blank samples (0.500).
[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[3] Geometric mean = square root (0 hour x 96 hour). For the calculation of geometric mean, values below the LOQ were assigned a value of ½ of the LOQ.
* Sample reanalyzed.

Table 2

Temperature Measurements

Time (Days)	Temperature (°C) [1]	
	Measurement 1	Measurement 2
0	23.5	23.6
1	23.4	23.5
2	23.2	23.6
3	23.7	23.8
4	23.8	23.9

[1] Temperature measured twice daily in a container of water located adjacent to the test chambers using a calibrated hand-held liquid-in-glass thermometer. Measurements typically are taken no less than 4 hours apart. The minimum and maximum temperatures during the test period (as recorded by a separate thermometer) were 23.8 and 25.2 °C, respectively.

Table 3

pH Measurements

Geometric Mean Measured Test Concentration mg a.i./L	pH Measurements	
	Day 0[1]	Day 4[2]
Negative Control	7.6	8.2
Solvent Control	7.6	8.2
0.0055	7.6	8.3
0.0076	7.6	8.3
0.012	7.6	8.3
0.018	7.6	8.3
0.024	7.6	8.3
0.034	7.6	8.2

[1] Day 0 samples were collected from the individual batches of test solution prepared for each treatment and control group at test initiation.
[2] Day 4 samples were composites of test solution collected from each of the replicates per treatment and control group at test termination.

Table 4

Light Intensity Measurements

Time	Light Intensity (lux) [1]				
Day 0	4440	4460	4500	4140	3930

[1] Measurements were taken at five locations surrounding the test flasks on the shaker table.

Table 5

Mean Cell Density and Percent Inhibition Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	24 Hours		48 Hours		72 Hours		96 Hours	
	Mean Cell Density [1] (cells/mL)	Percent Inhibition [1,2]	Mean Cell Density [1] (cells/mL)	Percent Inhibition [1,2]	Mean Cell Density [1] (cells/mL)	Percent Inhibition [1,2]	Mean Cell Density [1,] (cells/mL)	Percent Inhibition [1,2]
Pooled Control	42,053	--	225,082	--	1,217,813	--	3,746,909	--
0.0055	39,547	6.0	215,217	4.4	1,211,490	0.52	3,875,371	-3.4
0.0076	43,628	-3.7	243,005	-8.0	1,209,664	0.67	3,686,911	1.6
0.012	27,509	35	169,384	25	933,628*	23	3,269,336*	13
0.018	29,039	31	164,451	27	888,190*	27	3,211,928*	14
0.024	21,559	49	123,903	45	739,770*	39	2,926,717*	22
0.034	18,976	55	87,895	61	507,719*	58	2,269,103*	39

[1] Calculations were performed using SAS Version 8.2. Manual calculations may differ slightly.
[2] Inhibition was calculated relative to the pooled control mean.
* Treatment group mean is significantly different ($p<0.05$, Dunnett's test) from the pooled control mean at the 72 or 96-hour test intervals.

Table 6

Mean Area Under the Growth Curve and Percent Inhibition Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	0-24 Hours		0-48 Hours		0-72 Hours		0-96 Hours	
	Mean Area[1]	Percent Inhibition [1,2]	Mean Area[1]	Percent Inhibition [1,2]	Mean Area[1]	Percent Inhibition [1,2]	Mean Area[1]	Percent Inhibition [1,2]
Pooled Control	384,632	--	3,350,250	--	20,424,994	--	79,761,664	--
0.0055	354,560	7.8	3,171,728	5.3	20,052,216	1.8	80,854,544	-1.4
0.0076	403,532	-4.9	3,603,124	-7.5	20,795,152	-1.8	79,314,048	0.56
0.012	210,112	45	2,332,828	30	15,328,972*	25	65,524,548*	18
0.018	228,468	41	2,310,348	31	14,702,044*	28	63,663,460*	20
0.024	138,712	64	1,644,260	51	11,768,340*	42	55,526,184*	30
0.034	107,716	72	1,150,168	66	8,057,532*	61	41,139,392*	48

[1] Calculations were performed using SAS Version 8.2. Manual calculations may differ slightly.
[2] Inhibition was calculated relative to the negative control mean.
* Treatment group mean is significantly different ($p<0.05$, Dunnett's test) from the pooled control mean at the 72 or 96-hour test intervals.

Table 7

Mean Growth Rate and Percent Inhibition Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	0-24 Hours		0-48 Hours		0-72 Hours		0-96 Hours	
	Mean Growth Rate[1]	Percent Inhibition [1,2]	Mean Growth Rate[1]	Percent Inhibition [1,2]	Mean Growth Rate[1]	Percent Inhibition [1,2]	Mean Growth Rate[1]	Percent Inhibition [1,2]
Pooled Control	0.0592	--	0.0648	--	0.0666	--	0.0617	--
0.0055	0.0567	4.3	0.0637	1.7	0.0665	0.19	0.0621	-0.57
0.0076	0.0613	-3.6	0.0660	-1.8	0.0664	0.34	0.0615	0.29
0.012	0.0419	29	0.0589	9.1	0.0630*	5.4	0.0603	2.3
0.018	0.0443	25	0.0582	10	0.0623*	6.5	0.0601*	2.6
0.024	0.0315	47	0.0522	19	0.0598*	10	0.0592*	4.2
0.034	0.0241	59	0.0451	30	0.0543*	19	0.0564*	8.6

[1] Calculations were performed using SAS Version 8.2. Manual calculations may differ slightly.
[2] Inhibition was calculated relative to the negative control mean.
*Treatment group mean is significantly different ($p<0.05$, Dunnett's test) from the pooled control mean at the 72 or 96-hour test intervals.

Table 8

EC50, E_rC50 and E_bC50 Values Over the 96-Hour Exposure Period

	Cell Density		Growth Rate		Area Under the Growth Curve	
Time	EC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	E_rC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)	E_bC50 (mg a.i./L)	95% Confidence Interval (mg a.i./L)
24 Hours	0.027	0.020 and 0.037	0.027	0.020 and 0.036	0.019	0.013 and 0.027
48 Hours	0.027	0.022 and 0.032	>0.034	Not calculable	0.024	0.020 and 0.029
72 Hours	0.029	0.024 and 0.034	>0.034	Not calculable	0.027	0.023 and 0.032
96 Hours	>0.034	Not calculable	>0.034	Not calculable	0.036	0.030 and 0.042

Figure 1

Growth of *Pseudokirchneriella subcapitata* in the Pooled Control
Over the 96-Hour Exposure Period

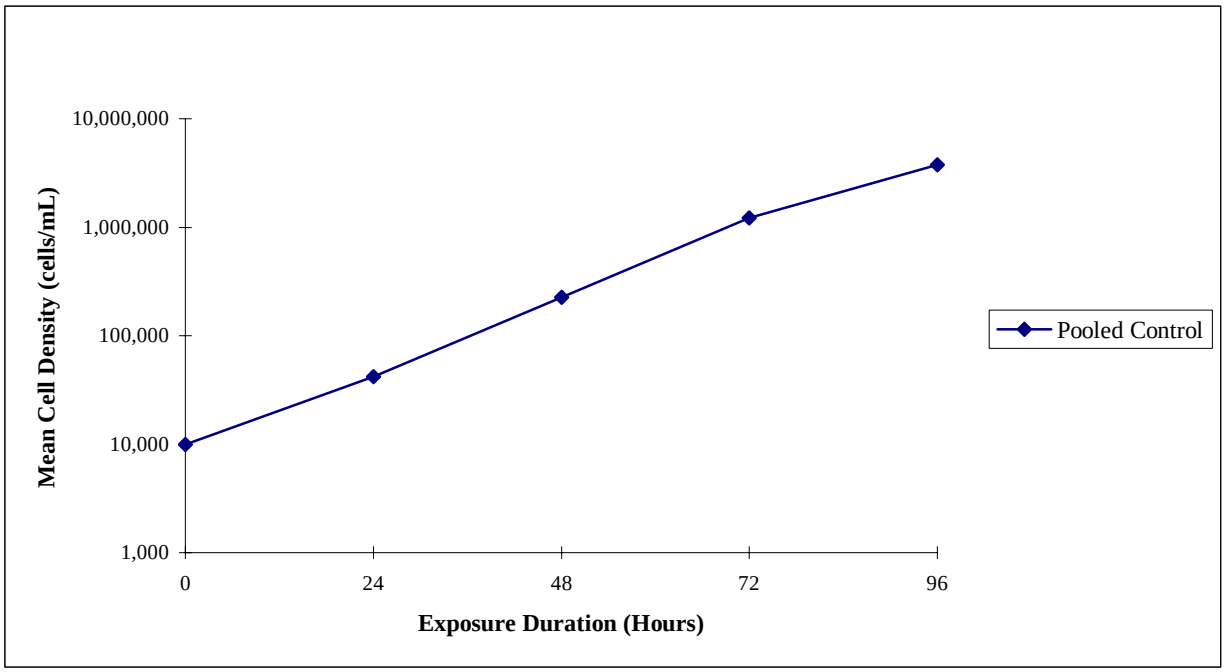
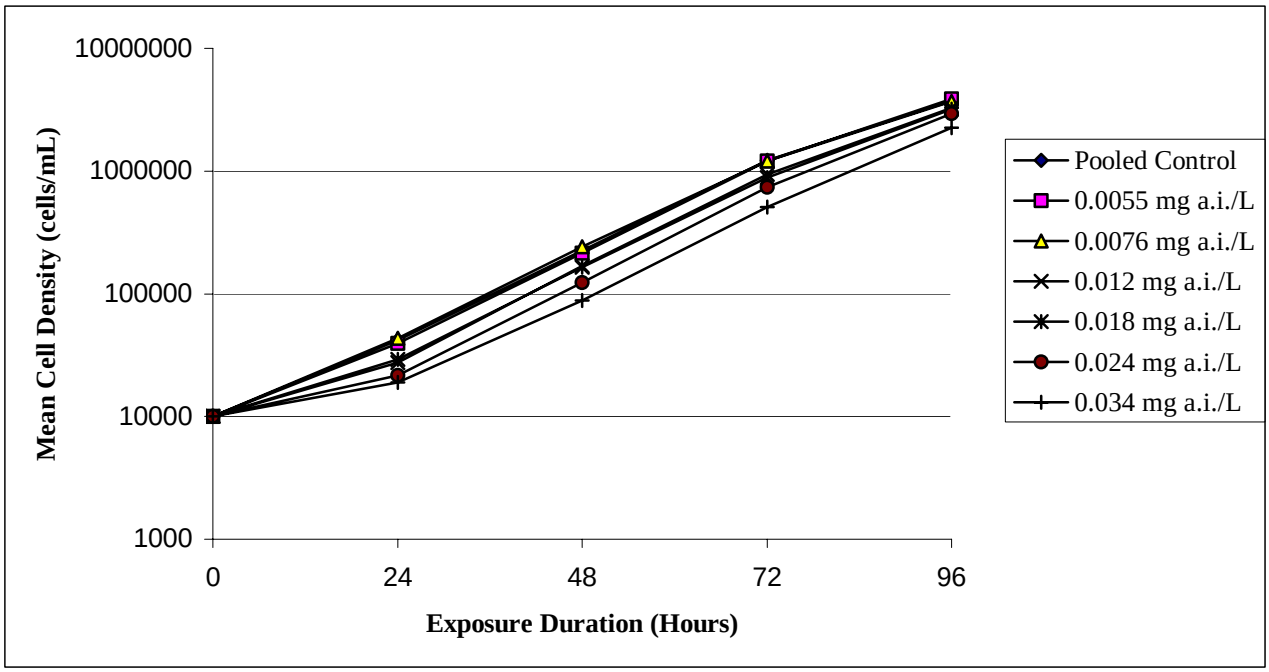


Figure 2

Concentration-Response Curve for *Pseudokirchneriella subcapitata*
Exposed to 1-Chlorododecane for 96 Hours



Appendix 1

Certificate of Analysis

CERTIFICATE OF ANALYSIS

Product: 1-Chlorododecane

Batch #: N6227945

PSL Reference No.: 070416-3R

Date of Analysis: June 29, 2007

Result:

1-Chlorododecane 97.42%

Approval: John M. Sheehy, B.A.. Date 8/16/07
Analytical Services
Product Safety Labs

QA Release: Rhonda S. Krick Date 08/17/07
Rhonda Krick, B.S.
Quality Assurance
Product Safety Labs

PSL GLP Study # 22018

Appendix 2

Freshwater Algal Medium

Compound	Nominal Concentration [1]	
$MgCl_2 \cdot 6H_2O$	12.16	mg/L
$CaCl_2 \cdot 2H_2O$	4.410	mg/L
H_3BO_3	0.1855	mg/L
$MnCl_2 \cdot 4H_2O$	0.4154	mg/L
$ZnCl_2$	3.27	µg/L
$FeCl_3 \cdot 6H_2O$	0.1598	mg/L
$CoCl_2 \cdot 6H_2O$	1.428	µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26	µg/L
$CuCl_2 \cdot 2H_2O$	0.012	µg/L
$Na_2EDTA \cdot 2H_2O$	0.300	mg/L
$NaNO_3$	25.50	mg/L
$MgSO_4 \cdot 7H_2O$	14.70	mg/L
K_2HPO_4	1.044	mg/L
$NaHCO_3$	15.00	mg/L

[1] The pH was adjusted to 7.5 using 0.1 N NaOH and 10% HCl.

Appendix 3

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

Pesticides and Organics [1]			
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 3 (Continued)

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

Metals [1]			
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 4

The Analysis of 1-Chlorododecane in Freshwater Algal Medium

Appendix 4.1

Analytical Method Flowchart for the Analysis of 1-Chlorododecane in Freshwater Algal Medium

METHOD OUTLINE FOR THE ANALYSIS OF
1-CHLORODODECANE IN FRESHWATER ALGAL MEDIUM

Centrifuge Day 4 samples at 10,000 rpm for 10 minutes.

↓

Rinse separatory funnels with ethyl acetate.

↓

Transfer the requisite volume of freshwater algal medium or test sample to the separatory funnels. Fortify recovery samples with the appropriate stock solution(s).

↓

Using a volumetric pipette, add the requisite volume of ethyl acetate to each sample. Stopper and Shake each sample (with venting) for approximately one minute. Allow the organic and aqueous layers to separate. Drain the aqueous phase to waste.

↓

Using a disposable pipette, transfer an aliquot of the ethyl acetate extract to a scintillation vial containing a few grains of sodium sulfate. Swirl the extract to ensure removal of residual water.

↓

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

Appendix 4.2

Typical Gas Chromatograph (GC) Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 5890 Gas Chromatograph (GC)
DETECTOR:	Hewlett-Packard Model 5971 Mass Selective Detector operated in Selective Ion Monitoring (SIM) mode
ANALYTICAL COLUMN:	ZB-5 column (30 m x 0.25 mm ID, 0.25 μm film thickness)
INJECTOR TEMPERATURE:	250ºC
OVEN:	Initial temperature: 45°C Initial hold time: 1.00 minute Ramp: 25.00°C/minute Final temperature: 320°C Final hold time: 2.00 minute
DETECTOR TEMPERATURE:	300°C
CARRIER GAS:	Helium
HEAD PRESSURE:	12 psi
INJECTION VOLUME:	1 μL
ION MONITORED:	91.00[1]
APPROXIMATE 1-CHLORODODECANE RETENTION TIME:	7.6 minutes

[1] The fragment ion 91 m/z (C_4H_8C1+) was selected to maintain sensitivity and selectivity, provided by its relatively high mass.

Appendix 4.3

Analytical Stocks and Standards Preparation

A primary stock solution of 1-Chlorododecane was prepared by weighing 0.1027 grams (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 was brought to volume using acetone. The primary stock solution (1.00 mg a.i./mL) was serially diluted in acetone to prepare 0.100, 0.0100 and 0.00100 mg a.i./mL stock solutions. The 0.100, 0.0100 and 0.00100 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 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)
0.0100	150	100	0.0150
0.0100	500	100	0.0500
0.0100	800	100	0.0800
0.0100	1200	100	0.120
0.1000	1500	100	0.150
0.100	200	100	0.200

Appendix 4.4

Example Calculations for a Representative Sample

The analytical result and percent recovery for sample number 289A-165-3, with a nominal concentration of 0.016 mg a.i./L, were calculated using the following equations:

$$\text{Concentration of 1-Chlorododecane in sample (mg a.i./L)} = \frac{\text{peak area - (y-intercept)}}{\text{slope}} \text{ x dilution factor}$$

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

Peak Area = 11878
Y-Intercept = -8316.06560
Slope = 1247286.70470
Dilution Factor = 0.500

$$\text{Concentration of 1-Chlorododecane in sample (mg a.i./L)} = \frac{11878 + 8316.06560}{1247286.70470} \text{ x } 0.500$$

Concentration of 1-Chlorododecane in sample (mg a.i./L) = 0.00810

$$\text{Percent of nominal concentration} = \frac{0.00810 \text{ mg a.i./L}}{0.016 \text{ mg a.i./L}} \text{ x } 100$$

Percent of nominal concentration = 50.6*

*Calculations were performed in Excel 2000 in full precision mode. Manual calculations may differ slightly.

Appendix 4.5

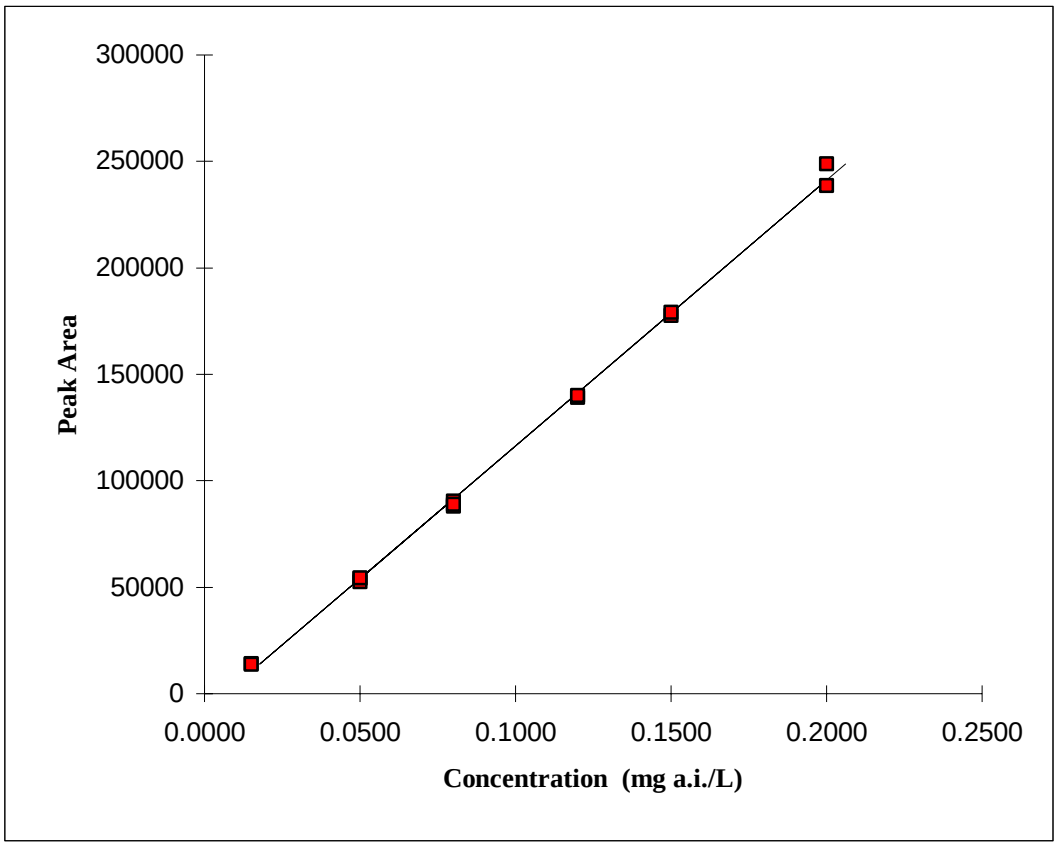
Quality Control Samples of 1-Chlorododecane in Freshwater Algal Medium

Sample Number (289A-165-)	Sample Type	Concentration of 1-Chlorododecane Fortified (mg a.i./L)	Measured[1] (mg a.i./L)	Percent Recovery[2]
MAB-1	Matrix Blank	0.0	< LOQ	--
MAB-2	Matrix Blank	0.0	< LOQ	--
MAS-1	Matrix Fortification	0.0160	0.0180	112
MAS-2	Matrix Fortification	0.100	0.103	103
MAS-4[3]	Matrix Fortification	0.500	0.482	96.4
MAS-5	Matrix Fortification	0.0160	0.0193	120
MAS-6	Matrix Fortification	0.100	0.111	111
MAS-7	Matrix Fortification	0.500	0.488	97.6
			Mean =	107
			Standard Deviation =	9.23
			CV =	8.63%

[1] The limit of quantitation (LOQ) was 0.00750 mg a.i./L, calculated as the product of the concentration of the lowest calibration standard (0.0150 mg a.i./L) and the dilution factor of the matrix blank samples (0.500).
[2] Results were generated using Excel 2000 in full precision mode. Manual calculations may differ slightly.
[3] Results from original sample (MAS-3) not reported due to suspected processing error. Fresh sample prepared and analyzed.

Appendix 4.6

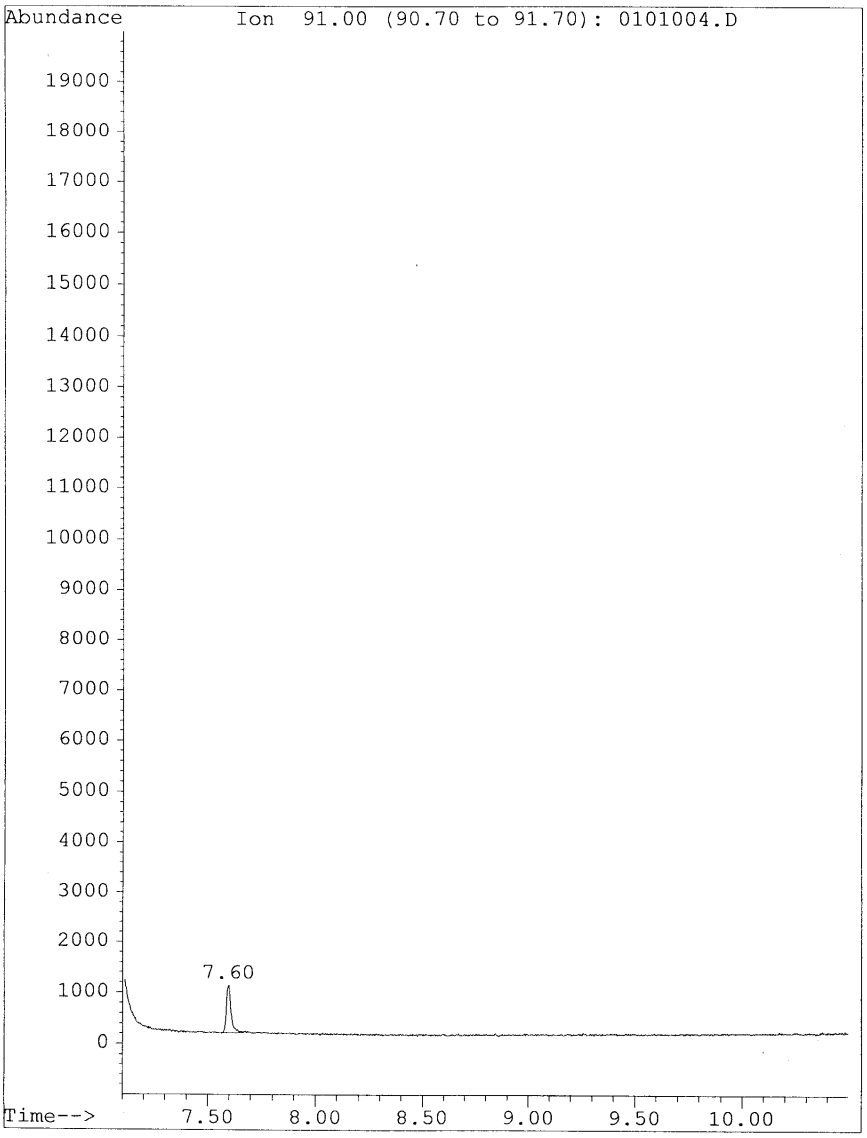
Representative Calibration Curve for 1-Chlorododecane



Slope = 1247286.70470; Y-Intercept = -8316.06560; R^2 = 0.99845

Appendix 4.7

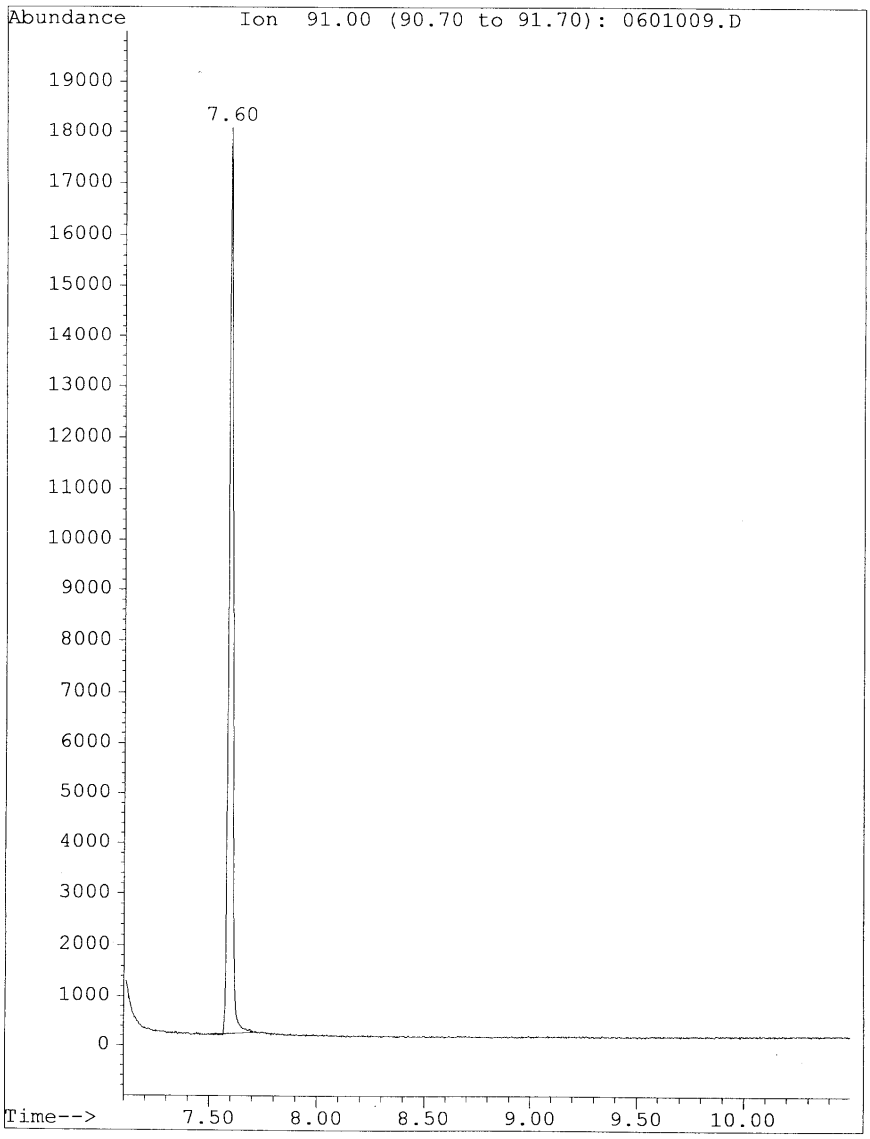
Representative Chromatogram of a Low-level 1-Chlorododecane Calibration Standard



Nominal concentration: 0.0150 mg a.i./L

Appendix 4.8

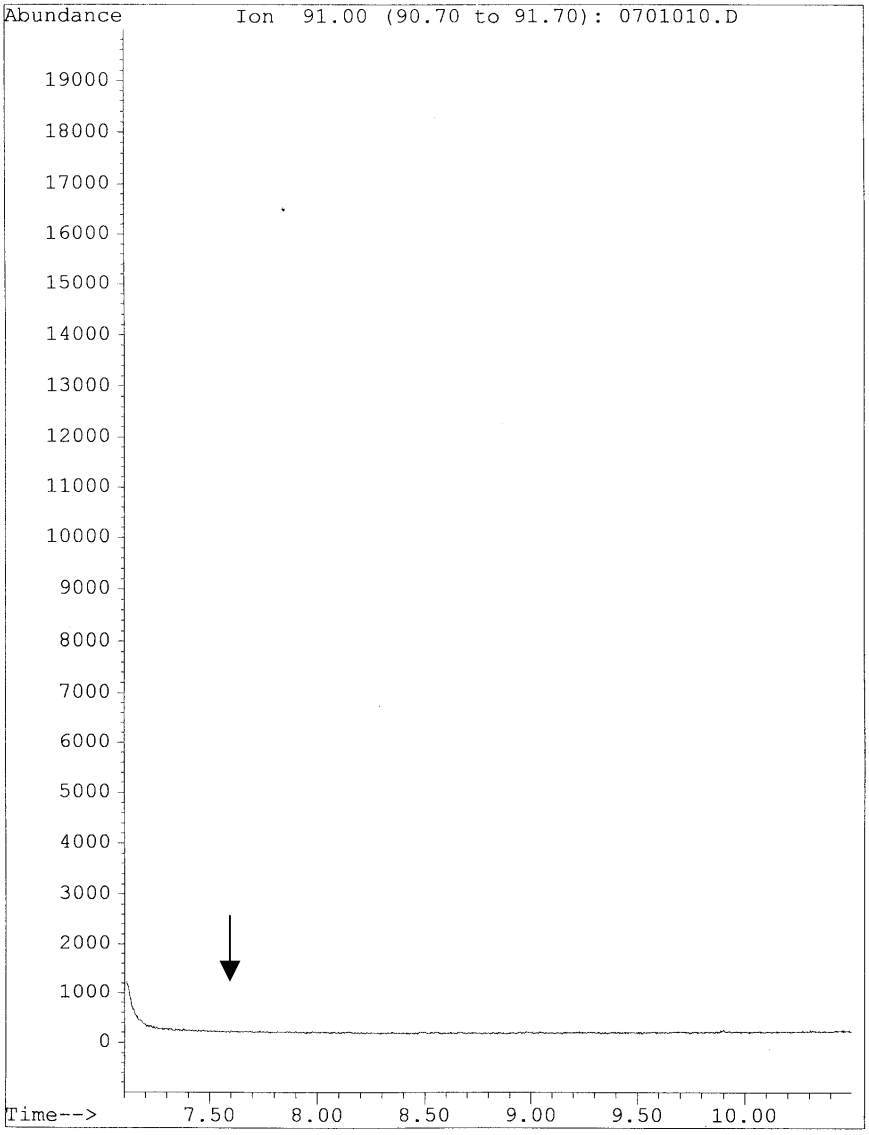
Representative Chromatogram of a High-level 1-Chlorododecane Calibration Standard



Nominal concentration: 0.200 mg a.i./L

Appendix 4.9

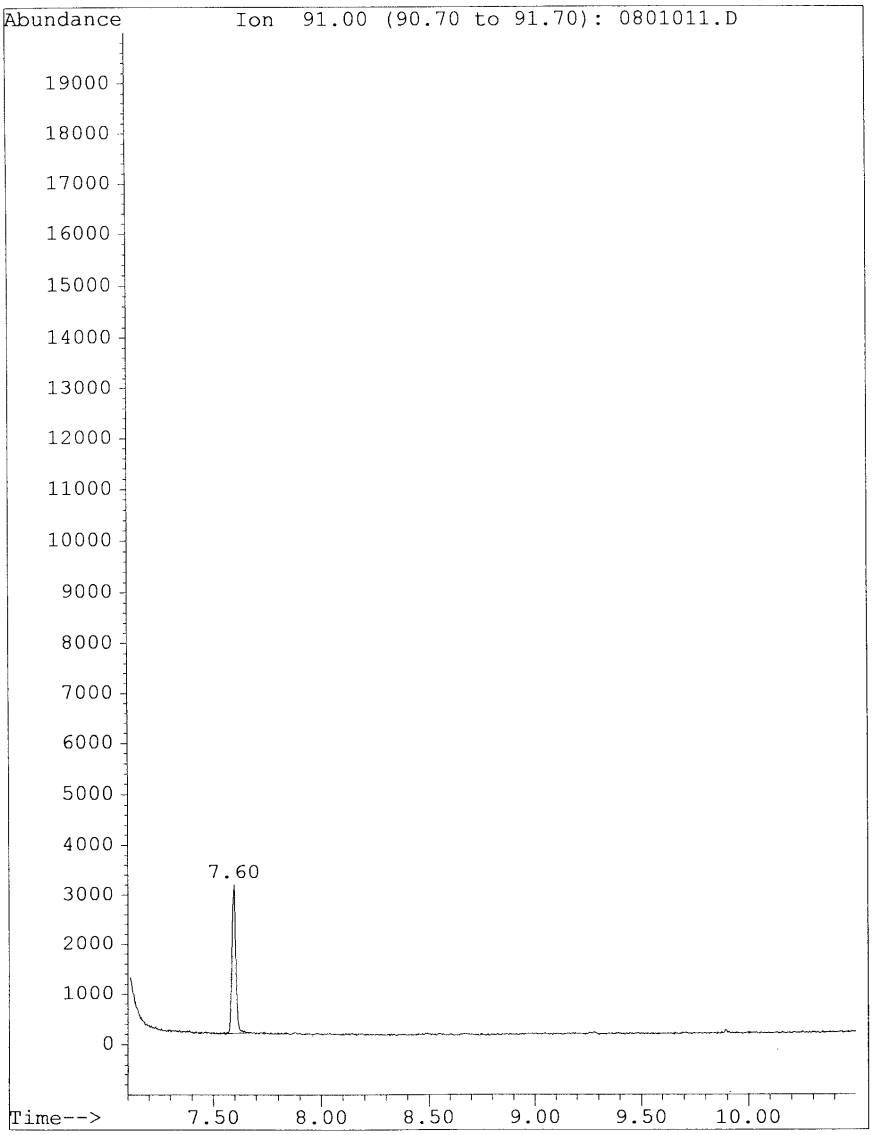
Representative Chromatogram of a Matrix Blank Sample



Sample number 289A-165-MAB-1; Day 0. The arrow indicates the approximate retention time of 1-Chlorododecane.

Appendix 4.10

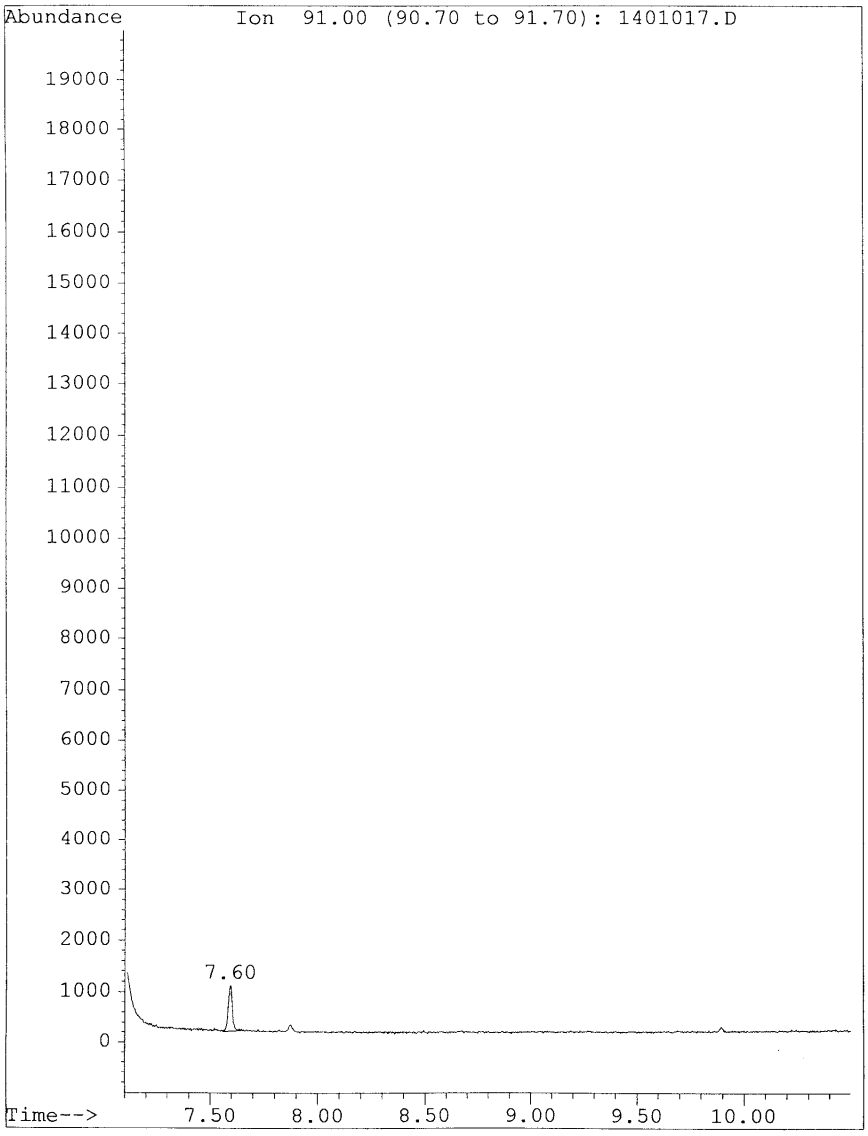
Representative Chromatogram of a Matrix Fortification Sample



Sample number 289A-165-MAS-1; Nominal concentration: 0.0160 mg a.i./L

Appendix 4.11

Representative Chromatogram of a Test Sample



Sample number 289A-165-3; Day 0; Nominal concentration: 0.016 mg a.i./L

Appendix 5

Cell Density By Replicate Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	Replicate	Cell Density (cells/mL) [1]			
		24 Hours	48 Hours	72 Hours	96 Hours
Negative Control	A	39465	242463	1322532	3783450
	B	50453	222729	1132104	3725944
	C	47409	235780	1354052	3864087
Solvent Control	A	28832	201306	1036610	3540259
	B	44901	231539	1327146	4023029
	C	41256	216676	1134435	3544687
0.0055	A	30918	180034	992121	3647172
	B	40968	253731	1348634	4064688
	C	46754	211887	1293715	3914252
0.0076	A	41297	209189	1018059	3532089
	B	42419	203485	1102681	3518548
	C	47167	316341	1508252	4010095
0.012	A	31404	174878	964807	3347650
	B	24739	168912	882877	3158061
	C	26385	164361	953201	3302298
0.018	A	31457	164682	906858	3300433
	B	28908	182604	896615	3165496
	C	26752	146067	861098	3169854
0.024	A	17444	99345	742882	2971758
	B	21671	130003	734468	2869191
	C	25563	142361	741961	2939201
0.034	A	27815	90052	650546	2489874
	B	11638	71589	404651	1809405
	C	17476	102043	467960	2508029

[1]The initial cell density of the stock culture was determined and an inoculum volume administered to each test chamber to yield a cell density of approximately 10,000 cells/mL at test initiation (0 Hours).

Appendix 6

Area Under the Growth Curve By Replicate Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	Replicate	Cumulative Area Under the Growth Curve			
		0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours
Negative Control	A	353580	3496716	22036656	83068440
	B	485436	3523620	19541616	77598192
	C	448908	3607176	22445160	84822828
Solvent Control	A	225984	2747640	17362632	72045060
	B	418812	3496092	21960312	85922412
	C	375072	3230256	19203588	75113052
0.0055	A	251016	2542440	16368300	71799816
	B	371616	3668004	22656384	87376248
	C	441048	3304740	21131964	83387568
0.0076	A	375564	3141396	17628372	71990148
	B	389028	3099876	18533868	73748616
	C	446004	4568100	26223216	92203380
0.012	A	256848	2492232	15928452	67437936
	B	176868	2260680	14642148	62893404
	C	196620	2245572	15416316	66242304
0.018	A	257484	2371152	14989632	65237124
	B	226896	2525040	15235668	63741000
	C	201024	2034852	13880832	62012256
0.024	A	89328	1250796	11117520	55453200
	B	140052	1720140	11853792	54857700
	C	186756	1961844	12333708	56267652
0.034	A	213780	1388184	10035360	47480400
	B	19656	778380	6253260	32581932
	C	89712	1283940	7883976	43355844

Appendix 7

Growth Rate By Replicate Over the 96-Hour Exposure Period

Geometric Mean Measured Test Concentration (mg a.i./L)	Replicate	Growth Rate			
		0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours
Negative Control	A	0.0572	0.0664	0.0678	0.0618
	B	0.0674	0.0647	0.0657	0.0617
	C	0.0648	0.0658	0.0682	0.0621
Solvent Control	A	0.0441	0.0625	0.0645	0.0611
	B	0.0626	0.0655	0.0679	0.0625
	C	0.0591	0.0641	0.0657	0.0612
0.0055	A	0.0470	0.0602	0.0639	0.0614
	B	0.0588	0.0674	0.0681	0.0626
	C	0.0643	0.0636	0.0675	0.0622
0.0076	A	0.0591	0.0633	0.0642	0.0611
	B	0.0602	0.0628	0.0653	0.0611
	C	0.0646	0.0720	0.0697	0.0624
0.012	A	0.0477	0.0596	0.0635	0.0606
	B	0.0377	0.0589	0.0622	0.0599
	C	0.0404	0.0583	0.0633	0.0604
0.018	A	0.0478	0.0584	0.0626	0.0604
	B	0.0442	0.0605	0.0624	0.0600
	C	0.0410	0.0559	0.0619	0.0600
0.024	A	0.0232	0.0478	0.0598	0.0593
	B	0.0322	0.0534	0.0597	0.0590
	C	0.0391	0.0553	0.0598	0.0592
0.034	A	0.0426	0.0458	0.0580	0.0575
	B	0.0063	0.0410	0.0514	0.0541
	C	0.0233	0.0484	0.0534	0.0575

Appendix 8

Specific Growth Rate of the Pooled Control Group

Section by Section Growth Rate for the Pooled Control:

Pooled Control Replicate[1]	0-24 Hour Growth Rate	24-48 Hour Growth Rate	48-72 Hour Growth Rate	72-96 Hour Growth Rate	% Coefficient of Variation[2]
A	0.0572	0.0756	0.0707	0.0438	23.17
B	0.0674	0.0619	0.0677	0.0496	13.73
C	0.0648	0.0668	0.0728	0.0437	20.47
D	0.0441	0.0810	0.0683	0.0512	27.26
E	0.0626	0.0683	0.0728	0.0462	18.59
F	0.0591	0.0691	0.0690	0.0475	16.79
					Mean: 20%

[1] Pooled control group consists of negative control (Replicates A-C) and solvent control (Replicates D-F) combined.
[2]Coefficient of Variation = S.D./mean *100
Results were generated using Excel 2000 in the full precision mode. Manual calculations may differ slightly.

Average Specific Growth Rate During the Whole Test Period:

Pooled Control Replicate	0-96 Hour Growth Rate
A	0.0618
B	0.0617
C	0.0621
D	0.0611
E	0.0625
F	0.0612
	% Coefficient of Variation: 0.84

Appendix 9

Study Protocol and Protocol Amendments

P R O T O C O L

1-CHLORODODECANE: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Pseudokirchneriella subcapitata*)

U.S. Environmental Protection Agency
Series 850 – Ecological Effects Test Guidelines
OPPTS Number 850.5400

ASTM E 1218-97a

OECD Guideline 201

and

EU Directive 92/69/EEC, Method C.3

Wildlife International, Ltd. Project Number: 289A-165

Submitted to

Lonza Inc.
90 Boroline Road
Allendale, NJ 07401

Wildlife International, Ltd.

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

February 15, 2008

Wildlife International, Ltd.

- 2 -

1-CHLORODODECANE: A 96-HOUR TOXICITY TEST
WITH THE FRESHWATER ALGA (*Pseudokirchneriella subcapitata*)

SPONSOR:	Lonza Inc. 90 Boroline Road Allendale, NJ 07401
SPONSOR'S REPRESENTATIVE:	John P. Van Miller, Ph.D., DABT Toxicology/Regulatory Services, Inc. 2365 Hunters Way Charlottesville, VA 22911
TESTING FACILITY:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601
STUDY DIRECTOR:	Sean P. Gallagher, Senior Biologist Wildlife International, Ltd.
LABORATORY MANAGEMENT:	Henry O. Krueger, Ph.D. Director of Aquatic Toxicology/Terrestrial Plants & Insects

FOR LABORATORY USE ONLY

Proposed Dates:

Experimental Start Date: 5/19/08 Experimental Termination Date: 5/23/08

Project No.: 289A-165

Test Concentrations: Neg. Control, Sol. control, 0.031, 0.063, 0.13, 0.25 and 0.5 mg ai/L

Test Substance No.: 8134 Reference Substance No. (if applicable): ⟶

PROTOCOL APPROVAL

Sean P. Gallagher — 5/16/08
STUDY DIRECTOR — DATE

[illegible] — 5/16/08
LABORATORY MANAGEMENT — DATE

[illegible] — 28 Feb 2008
SPONSOR'S REPRESENTATIVE — DATE

PROTOCOL NO.: 289/021508/PSEUDO96/OECD-OPPTS/100P-621

INTRODUCTION

Wildlife International, Ltd. will conduct an algal toxicity test with the freshwater alga, *Pseudokirchneriella subcapitata*, for the Sponsor at the Wildlife International, Ltd. aquatic toxicology facility in Easton, Maryland. The study will be performed based on procedures outlined in U.S. Environmental Protection Agency Series 850 - Ecological Effects Test Guidelines OPPTS Number 850.5400 (1); ASTM E 1218-97a *Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae* (2); OECD Guidelines for Testing of Chemicals 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test* (3); and Official Journal of the European Communities No. L383 Method C.3 *Algal Inhibition Test* (4), when possible. 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 toxicity of 1-Chlorododecane to the freshwater green alga, *Pseudokirchneriella subcapitata*.

EXPERIMENTAL DESIGN

The green alga, *Pseudokirchneriella subcapitata*, will be exposed to a geometric series of at least five test concentrations, a negative (culture medium) control and if necessary, a solvent control for 96 hours. Test concentrations will be selected in consultation with the Sponsor and will be based on information such as the results of rangefinding toxicity data, known toxicity data, physical/chemical properties of the test substance or other relevant information. Target test concentrations need not exceed 120 mg/L or the solubility limit of the test substance in water (whichever is lower). Generally, each concentration of test material used in the definitive test will be at least 50% of the next higher treatment, unless information concerning the concentration-effect curve indicates that a different dilution factor is more appropriate. The lowest test concentration tested should have no observed effect on the growth of the algae. The highest concentrations tested should inhibit growth by at least 50% relative to the control and, preferably stop growth altogether.

Test solutions will be inoculated with approximately 10,000 cells/mL. Three replicates per experimental group will be tested. In order to control bias, the position of the flasks will be determined

by indiscriminate draw daily during the exposure period. No other potential sources of bias are expected to affect the results of the study.

The response of the algae will be measured in terms of changes in cell density, area under the growth curve (biomass) and growth rate. EC50 and E_rC50 values (based on the theoretical test concentration that produces a 50% reduction in cell density and growth rate, respectively) will be calculated at 24, 48, 72, and 96 hours, when possible. The no observed adverse effect concentration (NOAEC) will be selected based upon evaluation of the statistical results and the concentration-response pattern of growth inhibition. At the end of the 96-hour exposure, algistatic effects will be differentiated from algicidal effects in those treatments which are maximally inhibited.

MATERIALS AND METHODS

Test Substance

Information on the characterization of test substance, 1-Chlorododecane, is required by Good Laboratory Practice (GLP) Standards and Principles. Specific information regarding the test substance used in this study will be recorded in the raw data and summarized in the report. 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. If verification of GLP test substance characterization is not provided to Wildlife International, Ltd., it will be noted in the compliance statement of the final report.

The Sponsor is responsible for all information related to the test substance and agrees to accept any unused test substance and/or test substance containers that cannot be appropriately disposed of by the laboratory. The laboratory will not dispose of any unused test substance without the Sponsor's authorization.

Test Solution Preparation

The test substance will be administered to the test organism in algal medium. This route of administration was selected because it represents the most likely route of exposure to algae, which exist suspended in a water column.

The desired concentrations of the test material will be obtained by preparing a single stock solution in medium and diluting the appropriate volume of stock solution with medium. The test solutions may also be prepared using secondary dilutions of the primary stock, based upon the concentration desired. All stock solutions will be prepared on the basis of active ingredient (a.i.) rather than in terms of the test substance as received from the Sponsor.

If the solubility of the test substance in water is too low, the stock solution may be prepared in an organic solvent [e.g., reagent grade (or better) acetone, or N,N-dimethylformamide]. The stock will be serially diluted with the desired solvent to provide one stock solution for each treatment group. The ratio between the stock solutions will be equivalent to the desired ratio between the test concentrations (e.g., 1.5 to 2.0). The test solutions will be prepared by diluting the desired volume of the appropriate stock with culture medium. Using this method, the solvent concentration is equivalent in all treatment groups. The solvent concentration in the final test solutions will not exceed 0.1 mL/L, when possible. If an organic solvent is necessary, an appropriate solvent control which contains the same concentration as the test solutions will be included in the test in addition to the negative (culture medium) control.

Test Organism

The freshwater green alga, *Pseudokirchneriella subcapitata*, will be used in this test. This species is representative of an important group of algae and was selected for use in the test based upon past use and ease of handling in the laboratory. Stock cultures, obtained from the University of Toronto Culture Collection or another supplier, will be maintained in culture medium at Wildlife International, Ltd. for a minimum of two weeks prior to use in a toxicity test and will be transferred periodically to fresh media to ensure growth. Algae used in toxicity tests will be in exponential growth phase, which is defined as the period of growth when algal cells are dividing at a constant rate.

Just prior to beginning the test, an inoculum of the stock culture will be prepared so that each milliliter of inoculum contains enough cells to provide an initial cell density of approximately 10,000 cells/mL in each replicate. Inoculum used for the test will be from a stock culture transferred to fresh media 3-7 days prior to the test.

Test and Culture Medium

AAP medium prepared according to Wildlife International, Ltd. Standard Operating Procedures will be used as dilution water. The medium components and concentrations are presented in Table 1 (2, 4). Stock nutrient solutions will be prepared by adding reagent-grade or better chemicals to purified Wildlife International, Ltd. well water. Appropriate volumes of the stock nutrient solutions will then be diluted with purified well water to prepare the medium. The medium will be filter sterilized (0.2 µm) or autoclaved prior to use. The final pH of the medium should be 7.5 ± 0.1. 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.

Specifications for acceptable levels of contaminants have not been established for AAP medium. However, there are no levels of contaminants reasonably expected to be present in the medium that are considered to interfere with the purpose or conduct of the study.

Test Apparatus

Test chambers will be sterile, 250-mL Erlenmeyer flasks plugged with foam stoppers and containing 100 mL of test solution. Test chambers will be indiscriminately positioned on a mechanical shaker table in an environmental chamber and will be shaken continuously at approximately 90 to 100 rpm. Test chambers will be labeled with the project number, test concentration, and replicate.

Environmental Conditions

Test flasks will be held at 24 ± 2°C under continuous fluorescent lighting (e.g., cool white light tubes) at an intensity of approximately 4300 ± 10% lux. Light bulbs will be replaced no more than two months prior to test initiation. Light intensity will be measured at five locations on the shaker table on Day 0. Temperature will be measured twice daily in the environmental chamber or in a flask of water next to test chambers using a calibrated hand-held liquid-in-glass thermometer. In addition, temperature in the environmental chamber will be measured continuously using a min/max thermometer.

The pH of each treatment and control will be measured at test initiation and termination using a Thermo Orion Model 720 pH meter or equivalent. Samples will be collected from the batch of test solution prepared at test initiation for each treatment and control group for the measurement of pH. At

test termination, pH will be measured in test solution samples collected from each replicate of the treatment and control groups, and pooled by treatment.

Biological Measurements

Cell densities will be monitored during the test by conducting cell counts using either a hemacytometer and a microscope or an electronic particle counter (Coulter Z Series, Coulter Corporation). One sample will be collected from each replicate of the treatment and control groups at approximately 24-hour intervals during the exposure period. The samples will be evaluated immediately or will be held in the dark under refrigeration until cell counts can be performed. Each sample will be diluted using an electrolyte solution (Isoton®), as needed, to maintain counting accuracy. If counts are performed with a hemacytometer, a small amount of each sample will be loaded onto the hemacytometer and the total number of cells in 10 grids will be counted. The cell density of the sample will be calculated based on the mean number of cells per grid. If an electronic particle counter is used, the samples will be diluted as necessary, using Isoton®, and three, 0.5-mL volumes of the sample will be counted. In this case, the cell density of each sample will be the mean of the three counts.

Samples of test solutions will be collected from each replicate per treatment and control group at the end of the test. Samples will be pooled within their respective treatments, and subsamples will be removed and examined microscopically for atypical cell morphology (e.g., changes in shape, size or color). Growth of cells in the flasks also will be assessed for aggregations or flocculations of cells and adherence of the cells to the test chamber. These observations are qualitative and descriptive, and are not used in end-point calculations.

At the end of the test, algistatic effects, which result in the inhibition of cell growth, will be differentiated from algicidal effects, which result in the death of cells. Aliquots (0.5 mL) of test solution will be taken from each replicate of those treatment groups where growth is maximally inhibited. Maximally inhibited treatments are those in which it cannot be visually determined if live algal cells are present (e.g., absence of color to the test solution). These aliquots will be pooled by concentration and diluted with untreated culture medium to a concentration of the test substance that theoretically should not affect growth. A negative control will be prepared by diluting a 0.5-mL aliquot from one negative control replicate to 100 mL with culture medium. Growth of these subcultures will be monitored for up to an additional 9 days to determine whether inhibition of growth observed during the test is reversible.

Samples will be collected for cell counts at recovery phase initiation, termination and at approximately 3-day intervals between initiation and termination. The recovery phase may be terminated once algal growth is sufficient to indicate that the algal cells collected from the treatment groups have fully recovered from effects due to the test substance. This will be determined through visual examination of the recovery test solutions.

Sampling for Analytical Measurements

Test solution samples will be collected from each treatment level at the beginning of the test and from the pooled replicates of each treatment level at the end of the test to determine concentrations of the test substance. Samples collected on Day 4 may be centrifuged or filtered to remove algal cells prior to analysis. Samples will be processed immediately for analysis, or placed in an appropriate storage container (e.g., glass, polypropylene or polyethylene bottle) and stored under the appropriate condition (e.g., refrigeration or ambient) until analyzed. The sample scheme is summarized below:

PROPOSED NUMBERS OF VERIFICATION SAMPLES

Experimental Group	Day 0	Day 4
Control	1	1
Solvent Control (if needed)	1	1
Level 1-Low Concentration	1	1
Level 2	1	1
Level 3	1	1
Level 4	1	1
Level 5-High Concentration	1	1
Totals	7	7

Total Number of Verification Samples = 14

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

Conditions for the Validity of the Test

The following criteria will be used to judge the validity of the test:

1) the mean cell density in the control flasks must increase by a factor of at least 16 within 3 days,

2) the coefficient of variation of average specific growth rates in the control replicates during the whole test period should not exceed 7%.

3) the mean percent coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3) must not exceed 35%.

Data Analysis

Growth rates will be calculated for the treatment and control groups at 24, 48, 72 and 96 hours using the following formula:

$$\mu = \frac{\ln N_n - \ln N_o}{t_n - t_o}$$

Where:

μ = Average specific growth rate
N_0 = Nominal cell density (cells/mL) at t_o
N_n = Measured cell density (cells/mL) at t_n
t_n = Time after beginning of test (hours)
t_o = Time of beginning of test (hours)

Percent inhibition of cell density and growth rate will be calculated for each treatment group using the following formula:

$$\text{Percent Inhibition} = \frac{\text{Mean Control Response - Mean Treatment Response}}{\text{Mean Control Response}} \times 100$$

EC50 and E_rC50 values will be determined at 24, 48, 72 and 96 hours, when possible, using non-linear regression (5) or other suitable techniques with treatment response (cell density, biomass and growth rate) and exposure concentration data.

A no-observed-adverse-effect concentration (NOAEC) will be selected at 72 and 96 hours based on an evaluation of the concentration-response pattern and the results of the statistical analyses of the cell density, biomass and growth rate data at 72 and 96 hours. Statistically significant differences between the control and the treatment groups will be identified using analysis of variance (ANOVA) and a test to compare treatment mean responses to the control response (e.g., Dunnett's test or Bonferroni's t-test). Data will be assessed for normality and homogeneity of variances prior to performing the ANOVA. Transformations will be used to correct any condition of non-normality or unequal variances, when possible. If a solvent control is used in addition to the negative control, their responses will be compared using Student's t-test. Data from the two control groups will be pooled for treatment level analyses if no statistical differences exist. Otherwise, all comparisons will be made to the solvent control group. Statistical comparisons for the ANOVA and the comparison of mean responses will be carried out using SAS System for Windows software (6) or equivalent. Additional analyses of the data may be conducted if deemed appropriate by the Study Director. The results of the analyses will be documented in the raw data and summarized in the final report.

RECORDS TO BE MAINTAINED

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

1. The original signed protocol and protocol amendments and/or deviations (if any).
2. Identification and characterization of the test substance, if provided by Sponsor.
3. Dates of initiation and termination of the study.
4. Source of algae.
5. Culture conditions.
6. Growth measurements.
7. Calculation and preparation of test concentrations.
8. Observations.

9. The methods used to analyze test substance concentrations and the results of analytical measurements.
10. Statistical calculations.
11. Test conditions and physical/chemical measurements.
12. Results of exploratory or rangefinding tests, if applicable.
13. The original final report.

FINAL REPORT

A final report formatted to meet U.S. EPA's PR Notice 86-5 reporting requirements will be prepared by Wildlife International, Ltd. A draft report will be provided to the Sponsor's Representative for review prior to issuance of a final report. The report will include, but will 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, including strain (if available), age or life stage, 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 conditions and 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. A graph plotting the concentration response curve, if available.
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.
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 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 amendment, and will be signed by the Study Director.
19. A copy of the final protocol and protocol amendments (if any).

CHANGING OF PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director following approval by the Sponsor's Representative. The amendment will clearly identify the part(s) of the protocol to be amended, the reasons for the alteration(s) and the impact that the alteration(s) will have on the study. Amendments will be considered as part of the protocol and will be attached to the final protocol. Unplanned changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All deviations 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 Parts 160 and 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,

Wildlife International, Ltd.

Ltd. Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. A statement of compliance with Good Laboratory Practices will be prepared for all portions of the study conducted by Wildlife International, Ltd. Raw data for all work performed at Wildlife International, Ltd. and the original 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. **U.S. Environmental Protection Agency.** 1996. Series 850- Ecological Effects Test Guidelines (*draft*), OPPTS Number 850.5400: *Algal Toxicity, Tiers I and II.*

2. **ASTM.** 1997. *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae.* American Society for Testing and Materials. Guideline E 1218-97a.

3. **Organisation for Economic Cooperation and Development.** 2006. OECD Guidelines for Testing of Chemicals, Guideline 201: *Freshwater Alga and Cyanobacteria, Growth Inhibition Test.* Adopted 23 March 2006.

4. **Official Journal of the European Communities.** 1992. No. L383. Method C.3. *Algal Inhibition Test.*

5. **Bruce, Robert D. and Donald J. Versteeg**. 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. *Environmental Toxicology and Chemistry*. 11: 1485-1494.

6. **The SAS System for Windows**. 1999. Version Eight. SAS Institute Inc., Cary, North Carolina.

Wildlife International, Ltd.

TABLE 1

TABLE 1

FRESHWATER ALGAL MEDIUM CONSTITUENTS

Component	Nominal Concentration
$MgCl_2 \cdot 6H_2O$	12.16 mg/L
$CaCl_2 \cdot 2H_2O$	4.410 mg/L
H_3BO_3	0.1855 mg/L
$MnCl_2 \cdot 4H_2O$	0.4154 mg/L
$ZnCl_2$	3.27 µg/L
$FeCl_3 \cdot 6H_2O$	0.1598 mg/L
$CoCl_2 \cdot 6H_2O$	1.428 µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 µg/L
$CuCl_2 \cdot 2H_2O$	0.012 µg/L
$Na_2EDTA \cdot 2H_2O$	0.300 mg/L
$NaNO_3$	25.50 mg/L
$MgSO_4 \cdot 7H_2O$	14.70 mg/L
K_2HPO_4	1.044 mg/L
$NaHCO_3$	15.00 mg/L

The pH of the medium will be adjusted, as necessary, to 7.5 ± 0.1 using 0.1 N NaOH or 10% HCl.

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: 1-Chlorododecane: A 96-Hour Toxicity Test with the Freshwater Alga (*Pseudokirchneriella subcapitata*)

PROTOCOL NO.: 289/021508/PSEUDO96/OECD-OPPTS/100P-621 **AMENDMENT NO.:** 1

SPONSOR: Lonza Inc. **PROJECT NO.:** 289A-165

EFFECTIVE DATE: May 16, 2008

AMENDMENT: Final Report Section, Page 11

Replace: A final report formatted to meet U.S. EPA's PR Notice 86-5 reporting requirements will be prepared by Wildlife International, Ltd.

With: A final report will be prepared by Wildlife International, Ltd.

REASON: The final report is not required to comply with EPA's PR Notice 86-5 format for the intended study submission(s).

IMPACT: This change has no impact on the integrity and validity of the study.

STUDY DIRECTOR	7/7/08 DATE
LABORATORY MANAGEMENT	7/7/08 DATE
SPONSOR'S REPRESENTATIVE	3 July 2008 DATE

AMENDMENT TO STUDY PROTOCOL

STUDY TITLE: 1-Chlorododecane: A 96-Hour Toxicity Test with the Freshwater Alga (*Pseudokircheriella subcapitata*)

PROTOCOL NO.: 289/021508/PSEUDO96/OECD-OPPTS/100P-621 **AMENDMENT NO.:** 2

SPONSOR: Lonza Inc. **PROJECT NO.:** 289A-165

EFFECTIVE DATE: May 16, 2008

AMENDMENT: Test concentrations, Page 2

ADD: 0.016 mg a.i./L

REASON: The 0.016 mg a.i./L test concentration was requested by the Sponsor's Representative prior to experimental start and was tested, but was inadvertently omitted from the protocol at the time of initiation.

AMENDMENT: Page 4, Experimental Design, Change the second sentence of the first full paragraph

FROM: EC50 and E_rC50 values (based on the theoretical test concentration that produces a 50% reduction in cell density and growth rate, respectively) will be calculated at 24, 48, 72, and 96 hours, when possible.

TO: EC50, E_rC50 and E_bC50 values (based on the theoretical test concentration that produces a 50% reduction in cell density, growth rate and area under the growth curve, respectively) will be calculated at 24, 48, 72 and 96 hours, when possible.

REASON: The protocol is being clarified to indicate that E_bC50 values will be calculated for the area under the growth curve (biomass).

AMENDMENT: Page 9, under Conditions for the Validity of the Test, change criterion 3 to:

The mean percent coefficient of variation for section-by-section growth rates (days 0-1, 1-2, 2-3 and 3-4) must not exceed 35%.

REASON: This validity criterion is being corrected to include growth rates for days 3-4 of the test.

AMENDMENT: Page 10, Data Analysis, Change the first sentence of the page to:

FROM: EC50 and E_rC50 values will be determined at 24, 48, 72 and 96 hours, when possible, using nonlinear regression (5) or other suitable techniques with treatment response (cell density, biomass and growth rate) and exposure concentration data.

TO: EC50, E_rC50 and E_bC50 values will be determined at 24, 48, 72 and 96 hours, when possible, using non-linear regression (5) or other suitable techniques with treatment response (cell density, growth rate and biomass) and exposure concentration data.

and

ADD: Area under the growth curve will be calculated for the treatment and control groups at 24, 48, 72 and 96 hours using the following formula:

$$A = ((N_1-N_o)/2)(t_1)+((N_1+N_2-2N_o)/2)(t_2-t_1) +...+((N_{n-1}+N_n-2N_o)/2)(t_n-t_{n-1})$$

where:

A	=	Area under the growth curve
N_o	=	Mean nominal number of cells/mL at t_o
N_1	=	Mean measured number of cells/mL at t_1
N_2	=	Mean measured number of cells/mL at t_2
N_n	=	Mean measured number of cells/mL at t_n
t_1	=	Time of first measurement after beginning of test (hours)
t_2	=	Time of second measurement after beginning of test (hours)
t_n	=	Time of nth measurement after beginning of test (hours)

REASON: The protocol is being clarified to indicate that E_bC50 values will be calculated for the area under the growth curve (biomass), and to indicate the formula used to calculate the area under the growth curve.

IMPACT: These amendments document the additional test concentration used in the study, clarify the validity criterion for determination of section-by-section growth rate during the test, and clarify the how the data will be analyzed and evaluated for the study, and have no adverse impact on the integrity and validity of the study.

Sean P. Gallagher — 9/12/08
STUDY DIRECTOR — DATE

K. Kim — 11 Sept 08
LABORATORY MANAGEMENT — DATE

P.V. — 11 Sept 2008
SPONSOR'S REPRESENTATIVE — DATE

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, Supervisor
4. John R. Porch, Supervisor of Terrestrial Plant and Insect Studies
5. Sean P. Gallagher, Senior Biologist
6. Allison M. Jacobs, Biologist
7. Josh Arnie, Biologist