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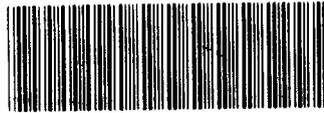
Shell Oil Company



One Shell Plaza  
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Dear Sir:

**SUBJECT: AQUATIC TOXICITY OF NEODOL® 45-13 (CAS# 68951-67-7) IN  
FATHEAD MINNOW, DAPHNIA MAGNA AND SELENASTRUM  
CAPRICORNUTUM**

The subject TSCA 8(e) submission was filed by Shell Oil Company April 7, 1994 and preliminary data transmissions were provided. The complete reports (attached) are now available and are provided as supplemental information to the original TSCA 8(e) filing.

These supplemental reports are filed to provide information EPA may find useful. In no way are they intended as a waiver of any rights or privileges belonging to Shell Oil Company as the reporting corporation, its agents or employees. The reporting corporation, its agents and employees, reserve the right to object to these reports' use or admissibility in any subsequent judicial or administrative proceeding against the corporation, its agents or employees.

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These reports contains no confidential business information.

The following person should be contacted if you have questions or a need for discussion.

11/16/94

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Manager, Product Safety and Compliance  
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P.O. Box 4320  
Houston, TX 77210  
Telephone No. 713-241-6958  
Fax. 713-241-3325

Very truly yours,

A handwritten signature in black ink, appearing to read "R. N. Shulman". The signature is fluid and cursive, with a long horizontal stroke at the end.

R. N. Shulman, General Manager  
Health, Safety, and Environment  
Shell Oil Company

THG/sjh

Attachments

**NEODOL<sup>®</sup> 45-13 - ACUTE TOXICITY TO  
FATHEAD MINNOW (*Pimephales promelas*)  
UNDER STATIC RENEWAL CONDITIONS**

**TSCA TEST GUIDELINES § 797.1400**

**Submitted to:**

**Shell Development Company  
P.O. Box 1380  
Houston, Texas 77251-1380**

**SLI Report #94-7-5358**

**Sponsor Protocol/Project No.: WRC TOX No. 1209**

**SLI Study #777.0294.6109.101**

**Study Director: Maura K. Collins**

**Springborn Laboratories, Inc.  
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790 Main Street  
Wareham, Massachusetts 02571-1075**

**Analytical Support:  
Battelle Ocean Sciences  
397 Washington Street  
Duxbury, Massachusetts 02332**

**30 August 1994**

**FINAL REPORT**

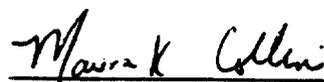
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**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The data and report presented for "NEODOL<sup>®</sup> 45-13 - Acute Toxicity to Fathead Minnow (*Pimephales promelas*) Under Static Renewal Conditions" were produced and compiled in accordance with all pertinent TSCA Good Laboratory Practice regulations with the following exception: routine water contaminant screening analyses for pesticides, PCBs and metals were conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, Pennsylvania. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc.). Stability, characterization, and verification of the test material identity and maintenance of records on the test material are the responsibility of the Study Sponsor. At the termination of the testing program, all remaining test material will be sent to the Study Sponsor. Archival of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.

  
Maura K. Collins      31 Aug. 1994  
Study Director      Date

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**SUMMARY****NEODOL<sup>®</sup> 45-13 - Acute Toxicity to Fathead Minnow  
(*Pimephales promelas*) Under Static Renewal Conditions**

**SPONSOR:** Shell Development Company  
P.O. Box 1380  
Houston, Texas 77251-1380

**PROTOCOL TITLE:** "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with Fathead Minnows Following TSCA Test Guidelines § 797.1400," Springborn Protocol #021494/TSCA/SHELL/FM-SR and Protocol Amendment #1 dated 5 April 1994.

**REPORT NUMBER:** 94-7-5358

**STUDY NUMBER:** 777.0294.6109.101

**TEST MATERIAL:** NEODOL<sup>®</sup> 45-13, CAS #68951-67-7, Lot #20944-178 (TANK TM 996), a clear liquid reported by the Study Sponsor to contain 100% active ingredient, received 17 February 1994.

**TEST DATES:** 11 to 15 April 1994

**TEST ORGANISM:** *Pimephales promelas*

Total length: Mean = 38 mm;  
range = 34 to 43 mm; N = 30  
Wet weight: Mean = 0.54 g;  
range = 0.36 to 0.79 g; N = 30  
Source: Springborn Laboratories culture facility

**DILUTION WATER:** Fortified Well Water  
(2 batches used during definitive testing)

pH: 7.1 (batch 4), 7.5 (batch 6)  
Specific conductivity: 200 and 240  $\mu$ mhos/cm,  
respectively  
Total hardness as CaCO<sub>3</sub>: 68 and 64 mg/L, respectively  
Total alkalinity as CaCO<sub>3</sub>: 44 and 43 mg/L, respectively

**TEST CONDITIONS:** 96-hour duration, 22 to 23 °C, illumination range of 537 to 859 lux (50 to 80 footcandles), photoperiod of 16 hours light and 8 hours dark

**NOMINAL TEST CONCENTRATIONS:** 0.52, 0.87, 1.4, 2.4 and 4.0 mg/L

**MEAN MEASURED CONCENTRATIONS:** 0.32, 0.62, 1.1, 1.9 and 3.4 mg/L

**EFFECT CRITERION:** Death as defined by lack of opercular movement by test fish.

**RESULTS:** The 96-hour LC50 value was estimated by nonlinear interpolation to be 1.0 mg/L (95% confidence interval calculated by binomial probability of 0.62 to 1.9 mg/L).

The 96-hour No-Observed-Effect Concentration (NOEC) was determined to be 0.62 mg/L.

## 1.0 INTRODUCTION

The purpose of this study was to estimate the acute toxicity (LC50) of NEODOL® 45-13 to fathead minnow (*Pimephales promelas*) under static renewal conditions. The LC50 is defined as the concentration of test material in dilution water which causes mortality of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of potential acute hazards resulting from release of the test material into aquatic environments. The study was initiated on 2 March 1994, 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 96-hour definitive test was conducted from 11 to 15 April 1994 at the Environmental Sciences Division of Springborn Laboratories, Inc. (SLI), in Wareham, Massachusetts. All original raw data and the final report produced during this study are stored at Shell Development Company.

## 2.0 MATERIALS AND METHODS

### 2.1 Protocol

Procedures used in this acute toxicity study followed those described in the Springborn protocol entitled "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with Fathead Minnows Following TSCA Test Guidelines § 797.1400," Springborn Protocol #021494/TSCA/SHELL/FM-SR and Protocol Amendment #1 dated 5 April 1994 (Appendix I). The methods described in this protocol meet or exceed the standard procedures described in the U.S. EPA Toxic Substances Control Act (TSCA) Test Guidelines § 797.1400 (U.S. EPA, 1992) and meet the primary technical objectives of the Shell Research Limited/Sittingbourne Research Center guidelines (SOP No. 81, Edition No. 8).

### 2.2 Test Material

The test material, NEODOL® 45-13, was received from Shell Development Company, Houston, Texas on 17 February 1994. Upon receipt at Springborn, the test material was stored at room temperature (approximately 20 °C) in a dark ventilated cabinet. Test concentrations are reported as milligrams per liter (mg/L). The following information describes the test material:

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Empirical Formula:	not available
Chemical Name:	not available
Physical Appearance:	clear liquid
Lot No.:	20944-178 (TANK TM 996)
CAS Registry No.:	68951-67-7
Purity:	100% (Appendix II)
Molecular Weight:	777 g/mole (average)
Water Solubility:	complete, may form gel
Vapor Pressure:	< 0.1 mm Hg at 75 °F

### 2.3 Test Organisms

The fathead minnow (Springborn Lot #94A39) used in this toxicity test were obtained from laboratory cultures maintained at Springborn. The culture water was "soft" water and was drawn from a 100-meter deep bedrock well into a concrete reservoir where it was aerated and supplemented with well water supplied by the Town of Wareham, Massachusetts. Prior to testing, the fish were held in a 500-L fiberglass tank under a photoperiod of 16 hours light and 8 hours darkness. Other parameters monitored in the holding tank were a pH of 6.8 to 7.1, dissolved oxygen concentration with a range of 97 to 102% of saturation and conductivity with a range of 130 to 140 micromhos per centimeter ( $\mu\text{mhos/cm}$ ). This water had total hardness and alkalinity ranges as calcium carbonate ( $\text{CaCO}_3$ ) of 36 to 40 mg/L and 19 to 25 mg/L, respectively (SLI Weekly Record of Fish Holding Water Characteristics, Vol. 6 and the SLI Gravity Feed Tank Water Quality Analysis Logbook, Vol. 9). Test fish were maintained under these conditions for a minimum of 14 days. The temperature range in the holding tank was 22 to 23 °C during this period. The fish were fed a dry commercial pelleted food, *ad libitum*, daily except during the 48-hours prior to testing. Representative samples of the food source were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix III). Food sources were considered to be of acceptable quality since the total concentration of pesticides measured was less than 0.3 mg/kg (ASTM, 1985). No mortality was observed among the test fish population during the 48-hour period prior to test initiation (SLI Daily Record of Fish Holding Conditions). A representative sample (N = 30) of fish from the test population had a mean total length of 38 mm (range 34 to 43 mm) and a mean wet weight of 0.54 g (range 0.36 to 0.79 g) (SLI Fish Length and Weight Log, Vol. II).

## 2.4 Reference Test

A copper nitrate reference test was conducted with the test organism population from 19 to 23 April 1994. The resulting 96-hour LC50 was calculated by moving average angle analysis to be 133  $\mu\text{g/L}$  (95% confidence interval of 99 to 176  $\mu\text{g/L}$ ) (SLI *Pimephales promelas* Copper Nitrate Reference Log, Vol. V). In addition to the above data, culture records document the ability of this population of *Pimephales promelas* to successfully and actively feed, grow and reproduce over a period of several generations. Based on the results of the reference test and the successful culture of *Pimephales promelas*, it was established that this population was suitable for testing.

## 2.5 Test Dilution Water

Two separate batches of dilution water were prepared for the study by reconstituting "soft" laboratory water (e.g., a mixture of unadulterated well water and untreated town well water) with various reagents to a specified hardness. The dilution water was from the same source as the culture water described above. The two batches of water had a total hardness as  $\text{CaCO}_3$  of 68 and 64 mg/L, respectively; a total alkalinity as  $\text{CaCO}_3$  of 44 and 43 mg/L, respectively; a pH of 7.1 and 7.5, respectively; and a specific conductivity of 200 and 240  $\mu\text{mhos/cm}$ , respectively (SLI Statics Modified GFT Water Quality Log Book, Vol. III). Representative samples of the dilution water source were analyzed for the presence of metals, pesticides and PCBs (Appendix IV). None of these compounds were detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM Standard Practice (ASTM, 1980). In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. Based on these analyses, the TOC concentration of the dilution water source was 0.793 mg/L for the month of April 1994 (SLI TOC Master Log). In addition, TOC concentration and total suspended solids (TSS) analyses were conducted at Springborn on the batches of dilution water used during this study. The TOC concentration of the fortified well water was determined to be 0.851 to 2.946 mg/L Batch #'s 4 and 6 respectively, for the month of April. The Total Suspended Solids (TSS) for these batches of dilution water was determined to be less than 2.0 mg/L. Several species of daphnids (a representative freshwater organism generally recognized to be sensitive to chemical challenges) maintained in water from

the same source as the dilution water utilized in this study have successfully survived and reproduced over several generations. The performance of the daphnid cultures, in combination with the previously mentioned analyses, confirms the acceptability of this dilution water for bioassays.

## 2.6 Test Conditions

Test vessels were positioned in stratified random order in a waterbath designed to maintain test solution temperatures at  $22 \pm 2$  °C. Test solutions were not aerated. The photoperiod during testing was the same as that provided in the fathead minnow culture area. Light at an intensity of 537 to 859 lux (50 to 80 footcandles) was provided at the surface of the solution. Light intensity was measured with a General Electric type 214 light meter. The culture area received a regulated photoperiod of 16 hours of light and 8 hours of darkness. Sudden transitions from light to dark and vice versa were avoided.

## 2.7 Test Concentrations

Selection of nominal NEODOL<sup>®</sup> 45-13 concentrations for the 96-hour definitive toxicity test with *Pimephales promelas* was based on toxicity information developed at Springborn through preliminary testing. The nominal concentrations selected were 0.52, 0.87, 1.4, 2.4 and 4.0 mg/L.

## 2.8 Test Procedures

The static renewal toxicity test was conducted in 18.9-L glass aquaria which contained 15 L of test solution. The exposure solution in each test vessel had a depth of 18.4 cm and a surface area of 819 cm<sup>2</sup>. Duplicate test aquaria were established for each treatment level and control. Prior to test initiation, a 10 mg/mL stock solution was prepared by heating the glass bottle containing the test material to a temperature of 50-60 °C, until the test material was completely melted. Ten grams (10.0000 g) of test material was then removed using a glass pipet, and was diluted with 1 L of distilled, deionized water. The 1-liter stock solution was stirred for several minutes. The resultant stock solution was observed to be clear and colorless with no sign of undissolved test material.

Replicate treatment level solutions with nominal concentrations of 0.52, 0.87, 1.4, 2.4 and 4.0 mg/L were prepared by diluting the appropriate amount of the stock solution with 15 L of dilution water. The exposure solutions were stirred for 30 seconds with a TAMCO Model 700 laboratory stirrer and teflon®-coated stirbar. The test solutions were observed to be clear and colorless with no sign of undissolved test material. Test solutions were renewed at 24, 48 and 72 hours of exposure following the procedure mentioned above. One set of control vessels was also established which contained the same dilution water and maintained under the same conditions as the test vessels but contained no NEODOL® 45-13. A duplicate set of exposure vessels was established to prepare renewal solutions.

Approximately 30 minutes after the test solutions were prepared, fathead minnow were impartially added to each test vessel (10 fathead minnow per replicate, 20 fathead minnow per treatment level and control). Fathead minnow were added to the test vessels no more than two at a time until all vessels contained two fish. This procedure was repeated until all replicate test vessels contained ten fish. Dead fathead minnow were removed from the test vessels at each observation interval. At each renewal period, the fathead minnow were carefully transferred from the aged (24-hours old) test solutions into their respective freshly prepared test solutions using a fine-mesh dip net. Fathead minnow were not fed during the study.

## 2.9 Test Monitoring

All aquaria were examined twice daily (Days 1, 2, 3 and 4) during the exposure period as follows: mortalities were recorded, dead fish were removed, and observations of the fish for sublethal effects (e.g. loss of equilibrium) and the physical characteristics of the test solutions were recorded. Dissolved oxygen concentration, temperature and pH were measured in all exposure solutions at test initiation and at each 24-hour interval. Water quality parameters were recorded in both the aged and freshly prepared test solutions at 24, 48 and 72 hours of exposure. Total hardness, total alkalinity, acidity and specific conductance were measured at 0-hour in each replicate of the control and test solutions. In addition, the temperature of the surrounding water in the waterbath was continuously monitored throughout the exposure period.

Total hardness concentration presented in this report was measured by the EDTA titrimetric method and total alkalinity concentration was determined by potentiometric titration to an endpoint of pH 4.5 (APHA *et al.*, 1985). Acidity was determined by potentiometric titration to an endpoint of pH 8.3. Specific conductivity was measured with a Yellow Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe. A Jenco Model 601A pH meter and combination electrode was used to measure pH. Dissolved oxygen concentration was measured with a YSI Model #57 dissolved oxygen meter and probe. Daily temperature was measured with a Fisher Scientific alcohol thermometer. Continuous temperature monitoring was performed using a Fisher Scientific Min/Max thermometer.

### **2.10 Analytical Measurements**

During the definitive exposure period, water samples were removed from each replicate solution of each treatment level and the control at 0, 24, 72 and 96 hours. A composite of the water samples (replicates A and B) for each treatment level and control were analyzed for NEODOL<sup>®</sup> 45-13 concentration. Sample containers were approximately 700-mL borosilicate glass bottles with Teflon<sup>®</sup>-lined screw caps. Samples analyzed at the 0- and 72-hour sampling intervals were removed from the freshly prepared exposure solutions. Samples analyzed at 24 and 96 hours were removed from the aged exposure solutions. In addition, a sample of the primary stock solution used to formulate the exposure solutions was collected for analysis at 0, 24, 48 and 72 hours. Each exposure solution sample was collected from the test vessel by removal of the exposure solution with a glass beaker and transferring the solution to a graduated cylinder. The measured amount of solution was then transferred to the sample container. Sample containers were completely filled to minimize headspace. On the day of collection, all samples were preserved with 1% formalin. Within 24 hours of preparation, the samples were shipped to Battelle Ocean Sciences, Duxbury, Massachusetts, for analysis using the Shell Development Company analytical method entitled "Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and HPLC/ELSD (Evaporative Light Scattering Detection) in Dilute Aqueous Solutions". This method is described in Battelle Ocean Sciences Study #SD-930119 (Appendix V). All of the glassware used in testing and sample collection was

thoroughly washed with sequential rinses of a 10% solution of nitric acid, acetone, distilled-deionized water, isopropanol and distilled-deionized water.

### **2.11 Determination of LC50 and NOEC**

The measured concentrations tested and the corresponding mortality data derived from the toxicity test were used to estimate 24-, 48-, 72- and 96-hour median lethal concentrations (LC50) and 95% confidence intervals. The LC50 is defined as the concentration of the test material in dilution water which caused mortality of 50% of the test organism population at the stated time interval. If at least one test concentration caused mortality of greater than or equal to 50% of the test population, then a computer program, modified from the program of C. Stephan (Stephan, 1982, personal communication), was used to calculate the LC50 values and 95% confidence intervals.

Three statistical methods were available in the computer program: moving average angle analysis, probit analysis, and nonlinear interpolation with 95% confidence intervals calculated by binomial probability. Moving average angle and probit analyses yield statistically sound results only if at least two concentrations produce mortality of between 0 and 100% in the test population. The selection of reported LC50 values and 95% confidence intervals was based upon an examination of the database and the results of the computer analysis. Selection criteria included the establishment of a concentration-effect relationship, the number of concentrations causing partial responses, and the span of responses bracketing the LC50 value. If two or more statistical methods produced acceptable results, then the method which yielded the smallest 95% confidence interval was selected. The No-Observed-Effect Concentration (NOEC) during the 96-hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there was no toxicant-related mortality or physical and behavioral abnormalities (e.g., lethargy, loss of equilibrium), with respect to the control organisms.

### 3.0 RESULTS

#### 3.1 Preliminary Test

Prior to initiating the definitive study, a preliminary range-finding test was conducted at Springborn from 1 to 5 March 1994 at nominal concentrations of 1.0, 4.0, 10, 40, 100 and 400 mg/L. One exposure vessel was established for each treatment level and control. At 24 hours of exposure, 100% mortality was observed among organisms exposed to  $\geq 4.0$  mg/L NEODOL<sup>®</sup> 45-13. No mortality or sublethal effects (e.g., complete loss of equilibrium, darkened pigmentation) were observed at the lowest treatment level (1.0 mg/L) or the control. Based on these results, the definitive study was conducted at the following nominal concentration range: 0.52, 0.87, 1.4, 2.4 and 4.0 mg/L.

#### 3.2 Definitive Test

**3.2.1 Evaluation of Test Conditions** - The measurements of the water quality parameters (i.e., pH, dissolved oxygen concentration, temperature) recorded during the definitive study are presented in Tables 1 and 2. Dissolved oxygen concentrations measured in the freshly prepared exposure solutions ranged from 94 to 105% of saturation throughout the 96-hour study. Dissolved oxygen concentrations in the aged solutions of the two highest treatment levels tested (2.4 and 4.0 mg/L, nominal) decreased to 45 - 53% of saturation between solution renewals. Although the dissolved oxygen concentrations briefly decreased to levels below 60% of saturation (e.g., limit established by TSCA Guideline 797.1400), the dissolved oxygen concentrations measured were sufficient for the survival and normal behavior of the exposed organisms. The temporary decrease in dissolved oxygen concentration did not contribute to the toxic-response observed at the 2.4 and 4.0 mg/L (nominal) treatment levels. Dissolved oxygen concentrations measured in the aged solutions of the three remaining treatment levels and the control ranged from 62 to 81% of saturation during the 96-hour exposure. Throughout the exposure period, the pH for the control and test solutions ranged from 6.8 to 8.4. Analysis of the control and treatment level solutions at test initiation established a total hardness (as CaCO<sub>3</sub>) ranging from 60 to 72 mg/L, a total alkalinity (as CaCO<sub>3</sub>) ranging from 44 to 48 mg/L and specific conductivity of 250  $\mu$ mhos/cm. These results demonstrate that the pH, total hardness, alkalinity and conductivity measured were unaffected by the concentrations of NEODOL<sup>®</sup> 45-13 tested and

remained within acceptable ranges for the survival of fathead minnow. Daily temperature monitoring of the test solutions and continuous temperature monitoring of the water in the surrounding waterbath established that the temperature in the test solutions ranged from 22 to 23 °C throughout the exposure period.

**3.2.2 Analytical Results** - The results of the analysis of the primary stock solution and the test solutions for NEODOL<sup>®</sup> 45-13 concentrations are summarized in Table 3. Results of the analysis of the primary stock solution (10 mg/mL) used to formulate the test solutions established that measured concentrations of NEODOL<sup>®</sup> 45-13 were consistent between stock solutions collected at 0, 24, 48 and 72 hours of exposure and averaged 99% of the nominal concentration. Analysis of the freshly prepared exposure solutions (0, 72-hours) resulted in measured concentrations which were generally consistent between sampling intervals. Measured concentrations for the aged (24-hours old) solutions at 24- and 96-hours established that the concentration of NEODOL<sup>®</sup> 45-13 generally decreased by an average of 36% between solution renewals. Mean measured concentrations based on the analysis of freshly prepared and aged solutions averaged 75% of nominal and defined the treatment levels as 0.32, 0.62, 1.1, 1.9 and 3.4 mg/L. These results established that consistent exposure conditions were maintained during the 96-hour study, which were sufficient to determine the acute toxicity of NEODOL<sup>®</sup> 45-13 to fathead minnow. Analytical results are presented in Battelle Ocean Sciences Study #SD-930119 (Appendix V).

**3.2.3 Biological Results** - The mean measured concentrations tested (based on the analysis of freshly prepared and aged solutions), the corresponding cumulative percent and number of mortalities and the observations made during the definitive exposure are presented in Table 4. All exposure solutions were observed to be clear and colorless and contained no visible signs of undissolved test material. Following 24 hours of exposure, 100% mortality was observed among fathead minnow exposed to the two highest concentrations tested (1.9 and 3.4 mg/L). At test termination (96 hours of exposure), 60% mortality was observed among fathead minnow exposed to the next lower exposure level (i.e., 1.1 mg/L). In addition, two of the surviving fathead minnow exposed to this treatment level exhibited a complete loss of equilibrium

and were observed at the surface of the solution. Throughout the 96-hour study, no mortality or sublethal effects were observed among fathead minnow exposed to the remaining test concentrations (0.32 and 0.62 mg/L) or the control. The 24-, 48-, 72- and 96-hour concentration-response (mortality) curves for this study are presented in Figures 1, 2, 3 and 4, respectively. Table 5 summarizes the 24-, 48-, 72- and 96-hour LC50 values and corresponding 95% confidence intervals, and presents the No-Observed-Effect Concentration (NOEC) through 96 hours. Based on mean measured concentrations of NEODOL<sup>®</sup> 45-13, the 96-hour LC50 value was estimated by nonlinear interpolation to be 1.0 mg/L with a corresponding 95% confidence interval calculated by binomial probability of 0.62 to 1.9 mg/L. The 96-hour NOEC established for this study was 0.62 mg/L.

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### PROTOCOL DEVIATIONS

1. The study protocol incorrectly states that approximately 500-mL water samples will be removed from each test vessel. During this study, water samples of 350-mL per replicate were pooled to yield the required 700-mL sample volume per concentration and control.
2. The protocol states that the test material will be heated to a temperature of 50 to 60°C until completely melted. On 12 April 1994, the test material was inadvertently heated to 75°C until completely melted. The sponsor confirmed that heating to 75°C would not affect the integrity of the sample.
3. The protocol states that biological observations will be made and recorded at 0, 24, 48, 72 and 96 hours of exposure. TSCA Guideline 797-1400 states that biological observations should be recorded at 24, 48, 72 and 96 hours after test initiation or within one hour of the designated time. During the study, biological observations were made and recorded at 0, 20, 44, 71 and 96 hours. The inadvertent documentation of biological observations prior to the prescribed intervals on test day 1 and 2 did not significantly impact the interpretation of the study results, since subsequent observations (e.g. 28 and 52 hours) were generally consistent with 20 and 24 hour observations.
4. The study protocol states that twenty organisms will be exposed to each test concentration or control, ten organisms per replicate vessel. During this study, following 72-hours of exposure, one of the organisms was unaccounted for in replicate vessel A of the 0.32 mg/L treatment level. Therefore, the population size exposed to this treatment level was 19 organisms. LC50 calculations at 72 and 96-hours were adjusted to account for the reduced population size at this treatment level. Adjustment of LC50 calculations to include the reduced population size results in an accurately defined LC50. The 0.32 mg/L treatment level was not considered in selection of the NOEC. Therefore, the reduction in population size in the 0.32 mg/L treatment level did not adversely impact the results of this study.

5. The study protocol states that dissolved oxygen concentrations will not be allowed to drop below 60% of saturation throughout the exposure period. During this study, dissolved oxygen concentrations in the 2.4 and 4.0 mg/L treatment levels (nominal) ranged from 45 - 53% of saturation in the aged solutions at the 24-hour interval, prior to solution renewal. The dissolved oxygen concentrations measured were sufficient for the survival and normal behavior of the exposed organisms, and mortalities observed at these treatment levels were not related to dissolved oxygen concentration. Therefore, this deviation did not adversely impact the results of this study.

It is our opinion that these deviations did not affect the results of this study.

SPRINGBORN LABORATORIES, INC.

Maura K. Collins      8/30/94

Maura K. Collins  
Study Director

Date

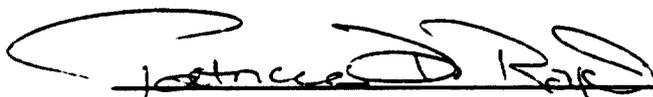
### QUALITY ASSURANCE UNIT STATEMENT

The raw data and report for "NEODOL® 45-13 - Acute Toxicity To Fathead minnow (*Pimephales promelas*) Under Static Renewal Conditions" were inspected by the Quality Assurance Unit (QAU) at Springborn Laboratories Inc., Environmental Sciences Division to determine adherence with the study protocol and laboratory standard operating procedures. In addition, inspection of certain phases of the in-life portion of the study was performed. Dates of study inspections, dates reported to the Study Director and to Management are listed below.

Based on these inspections, it was determined that this report accurately reflects the raw data collected during this study.

<u>Inspection Date</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
4/4/94	4/4/94	4/8/94
4/12/94	4/12/94	4/22/94
4/27 - 28/94	4/28/94	5/6/94
5/4/94	5/4/94	5/6/94
8/2 - 4/94	8/4/94	8/12/94
8/19 - 23/94	8/23/94	8/26/94
8/30/94	8/30/94	8/30/94

SPRINGBORN LABORATORIES, INC.

  
 Patricia D. Royal  
 Manager, Regulatory Affairs  
 and Quality Assurance Unit

8/30/94  
 Date

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Springborn Laboratories, Inc.

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**REFERENCES**

- APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, Washington, D.C., 2168 pp.
- ASTM Standard E729-80. 1980. Standard Practice for Conducting Acute Toxicity Tests with Fathead minnow, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.
- ASTM Standard E1022-84. 1985. Standard Practice for Conducting Bioconcentration Tests with Fathead minnow and Saltwater Bivalve Molluscs.
- Peltier, W.H. and C.I. Weber. 1985. *Methods for measuring the acute toxicity of effluents to freshwater and marine organisms*. 3rd ed. Environmental Monitoring and Support Laboratory, U.S. EPA, Cincinnati, Ohio. EPA-600/4-85/013.
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- U.S. EPA. 1985, 1987, 1992. Toxic Substances Control Test Act Guidelines. Federal Register 50(188): 39252-39516, September 27, 1985. Amended May 20, 1987, July 1992.
- U.S. EPA. 1989. Toxic Substances Control; Good Laboratory Practice Standards; Final Rule. (40 CFR, Part 792) Federal Register, Parts III, 48(230):53922-53944, August 17, 1989.

**TABLES**

**Table 1. The pH, dissolved oxygen concentration and temperature measurements recorded during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL® 45-13.**

Nominal Concentration (mg/L)	0-Hour		24-Hour <sup>a</sup>		48-Hour <sup>a</sup>		72-Hour <sup>a</sup>		96-Hour	
	A	B	A	B	A	B	A	B	A	B
<b>pH</b>										
Control	7.8	7.8	8.2/8.4	8.2/8.4	7.8/7.6	7.8/7.6	6.9/7.6	6.9/7.6	6.9	6.8
0.52	7.7	7.7	8.2/8.4	8.2/8.4	7.8/7.6	7.8/7.7	6.9/7.6	6.9/7.6	6.9	7.0
0.87	7.8	7.8	8.2/8.4	8.2/8.4	7.8/7.6	7.8/7.7	6.8/7.6	6.8/7.6	7.0	7.1
1.4	7.7	7.8	8.2/8.4	8.2/8.4	7.8/7.7	7.8/7.7	6.8/7.7	6.8/7.7	7.1	7.0
2.4	7.8	7.8	8.2/- <sup>b</sup>	8.2/-	-/-	-/-	-/-	-/-	-	-
4.0	7.7	7.8	8.2/-	8.2/-	-/-	-/-	-/-	-/-	-	-
<b>Dissolved Oxygen, mg/L (% saturation)</b>										
Control	9.1 (104)	9.1 (104)	6.1/8.2 (71/95)	6.9/8.3 (80/97)	7.0/8.2 (80/95)	7.0/8.3 (80/97)	5.9/8.1 (69/94)	6.2/8.2 (72/95)	6.9 (79)	7.0 (80)
0.52	9.2 (105)	9.1 (104)	6.3/8.2 (73/95)	5.6/8.3 (65/97)	6.9/8.2 (79/95)	6.9/8.3 (79/97)	5.9/8.1 (69/94)	6.0/8.1 (70/94)	6.9 (79)	6.8 (78)
0.87	9.1 (104)	9.1 (104)	5.3/8.2 (62/95)	6.7/8.3 (78/97)	6.5/8.3 (74/97)	6.5/8.3 (74/97)	5.6/8.1 (65/94)	6.3/8.3 (73/97)	6.8 (78)	6.9 (79)
1.4	9.1 (104)	9.2 (105)	6.6/8.3 (77/97)	6.0/8.3 (70/97)	7.1/8.4 (81/98)	6.9/8.4 (79/98)	6.5/8.4 (70/98)	5.9/8.4 (69/98)	6.9 (79)	7.0 (80)
2.4	9.1 (104)	9.1 (104)	4.6/- <sup>b</sup> (53/-)	4.2/- (49/-)	-/- (-/-)	-/- (-/-)	-/- (-/-)	-/- (-/-)	- (-)	- (-)
4.0	9.1 (104)	9.1 (104)	4.2/- (49/-)	3.9/- (45/-)	-/- (-/-)	-/- (-/-)	-/- (-/-)	-/- (-/-)	- (-)	- (-)
<b>Temperature (°C)<sup>c</sup></b>										
	22		23/23		22/23		23/23		22	

<sup>a</sup> Exposure solutions were renewed at this interval. Measurements are presented for aged/freshly prepared solutions.

<sup>b</sup> Due to mortality for 100% of the exposed organisms at the previous observation interval, no water quality measurements were taken at this interval.

<sup>c</sup> Values presented represent the daily temperatures measured (Fisher alcohol thermometer) in all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific Min-Max thermometer) of the surrounding water in the waterbath established a temperature range of 22 to 23 °C during the exposure period.

**Table 2. Total hardness, total alkalinity, specific conductance and acidity measured at 0-hour in the test solutions during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.**

Nominal Concentration (mg/L)		Total Hardness (mg/L as CaCO <sub>3</sub> )	Total Alkalinity (mg/L as CaCO <sub>3</sub> )	Specific Conductance (μmhos/cm)	Acidity (mg/L as CaCO <sub>3</sub> )
Control	A	72	48	250	4
	B	68	46	250	4
0.52	A	68	44	250	6
	B	68	46	250	4
0.87	A	72	46	250	4
	B	68	44	250	2
1.4	A	68	44	250	4
	B	68	46	250	4
2.4	A	68	46	250	4
	B	68	46	250	4
4.0	A	60	44	250	2
	B	68	44	250	2

**Table 3. Concentrations of NEODOL® 45-13 measured in the exposure solutions during the 96-hour toxicity test with fathead minnow (*Pimephales promelas*).**

Nominal Concentration (mg/L)	Measured Concentration (mg/L)				
	0-Hour <sup>a</sup>	24-Hour <sup>b</sup>	72-Hour <sup>a</sup>	96-Hour <sup>b</sup>	Mean <sup>c</sup>
Control	ND <sup>d</sup>	ND	ND	ND	NA <sup>e</sup>
0.52	0.49	0.18	0.39	0.24	0.32
0.87	0.81	0.47	0.70	0.48	0.62
1.4	1.3	0.83	1.2	0.93	1.1
2.4	2.2	1.7	NA	NA	1.9
4.0	3.5	3.2	NA	NA	3.4
Stock Solution <sup>f,g</sup> (10 mg/mL)	10	9.7	9.9	NA	NA

<sup>a</sup> Samples analyzed at this interval were removed from the freshly prepared exposure solutions.

<sup>b</sup> Samples analyzed at this interval were removed from the aged exposure solutions.

<sup>c</sup> Calculated values are based on actual analytical results and not on rounded values (two significant figures) presented in this table.

<sup>d</sup> ND = Not detected; less than the limit of detection (LOD)

<sup>e</sup> NA = Not Applicable

<sup>f</sup> Nominal concentration (as mg/mL) of stock solution is presented in parentheses.

<sup>g</sup> Stock solution analyzed at 48-hours of exposure resulted in a measured concentration of 9.8 mg/L.

**Table 4. Concentrations tested, corresponding cumulative percent and number of mortalities and observations made during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.**

Mean Measured Concentration (mg/L)	Cumulative Percent Mortality <sup>a</sup>											
	Day 0 (12:30)			Day 0 (19:15) <sup>b</sup>			Day 1 (8:45)			Day 1 (16:30) <sup>b</sup>		
	A	B	Mean	A	B	Mean	A	B	Mean	A	B	Mean
Control	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
0.32	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
0.62	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
1.1	0 (0)	0 (0)	0	0 <sup>c</sup> (0)	0 <sup>d</sup> (0)	0	10 <sup>c</sup> (1)	20 <sup>d</sup> (2)	15	10 <sup>c</sup> (1)	20 <sup>d</sup> (2)	15
1.9	0 (0)	0 (0)	0	40 <sup>e,f</sup> (4)	80 <sup>g</sup> (8)	60	100 (10)	100 (10)	100	100 (10)	100 (10)	100
3.4	0 (0)	0 (0)	0	100 (10)	100 (10)	100	100 (10)	100 (10)	100	100 (10)	100 (10)	100

<sup>a</sup> The actual number of dead fathead minnow is presented in parentheses.

<sup>b</sup> Supplemental biological observations were recorded during each interval.

<sup>c</sup> Three of the surviving fish exhibited partial loss of equilibrium.

<sup>d</sup> Two of the surviving fish exhibited partial loss of equilibrium.

<sup>e</sup> Four of the surviving fish exhibited complete loss of equilibrium and were observed at the bottom of the solution.

<sup>f</sup> Two of the surviving fish exhibited complete loss of equilibrium and were observed at the surface of the solution.

<sup>g</sup> Two of the surviving fish exhibited complete loss of equilibrium.

**Table 4. Continued. Concentrations tested, corresponding cumulative percent and number of mortalities and observations made during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL® 45-13.**

Mean Measured Concentration (mg/L)	Cumulative Percent Mortality <sup>a</sup>											
	Day 2 (8:30)			Day 2 (16:30) <sup>b</sup>			Day 3 (11:30)			Day 3 (16:30) <sup>b</sup>		
	A	B	Mean	A	B	Mean	A	B	Mean	A	B	Mean
Control	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
0.32	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 <sup>i</sup> (0)	0 (0)	0	0 <sup>i</sup> (0)	0 (0)	0
0.62	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
1.1	10 <sup>c,d</sup> (1)	20 <sup>c,d</sup> (2)	15	10 <sup>c,e</sup> (1)	30 <sup>f</sup> (3)	20	20 <sup>g</sup> (2)	50 <sup>c,e</sup> (5)	35	40 <sup>f</sup> (4)	50 <sup>f,h</sup> (5)	45
1.9	100 (10)	100 (10)	100	100 (10)	100 (10)	100	100 (10)	100 (10)	100	100 (10)	100 (10)	100
3.4	100 (10)	100 (10)	100	100 (10)	100 (10)	100	100 (10)	100 (10)	100	100 (10)	100 (10)	100

<sup>a</sup> The actual number of dead fathead minnow is presented in parentheses.  
<sup>b</sup> Supplemental biological observations were recorded during each interval.  
<sup>c</sup> One of the surviving fish exhibited partial loss of equilibrium.  
<sup>d</sup> One of the surviving fish exhibited complete loss of equilibrium.  
<sup>e</sup> Two of the surviving fish exhibited complete loss of equilibrium.  
<sup>f</sup> One of the surviving fish exhibited complete loss of equilibrium and was at the surface of the solution.  
<sup>g</sup> Three of the surviving fish exhibited complete loss of equilibrium.  
<sup>h</sup> Three of the surviving fish exhibited complete loss of equilibrium and were on the bottom of the solution.  
<sup>i</sup> One of the exposed organisms was unaccounted for following 72-hours of exposure, therefore nineteen fish were exposed to this treatment level.

**Table 4. Continued. Concentrations tested, corresponding cumulative percent and number of mortalities and observations made during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.**

Mean Measured Concentration (mg/L)	Cumulative Percent Mortality <sup>a</sup>		
	Day 4 (12:30) <sup>b</sup>		
	A	B	Mean
Control	0 (0)	0 (0)	0
0.32	0 <sup>c</sup> (0)	0 (0)	0
0.62	0 (0)	0 (0)	0
1.1	50 (5)	70 <sup>d</sup> (7)	60
1.9	100 (10)	100 (10)	100
3.4	100 (10)	100 (10)	100

<sup>a</sup> The actual number of dead fathead minnow is presented in parentheses.

<sup>b</sup> No supplemental biological observations were recorded because the definitive was terminated after this interval.

<sup>c</sup> One of the exposed organisms was unaccounted for following 72-hours of exposure, therefore nineteen fish were exposed to this treatment level.

<sup>d</sup> Two of the surviving fish exhibited complete loss of equilibrium and were on the surface of the solution.

**Table 5. The LC50 values, corresponding 95% confidence intervals and No Observed Effect Concentration (NOEC) established during the 96-hour static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.**

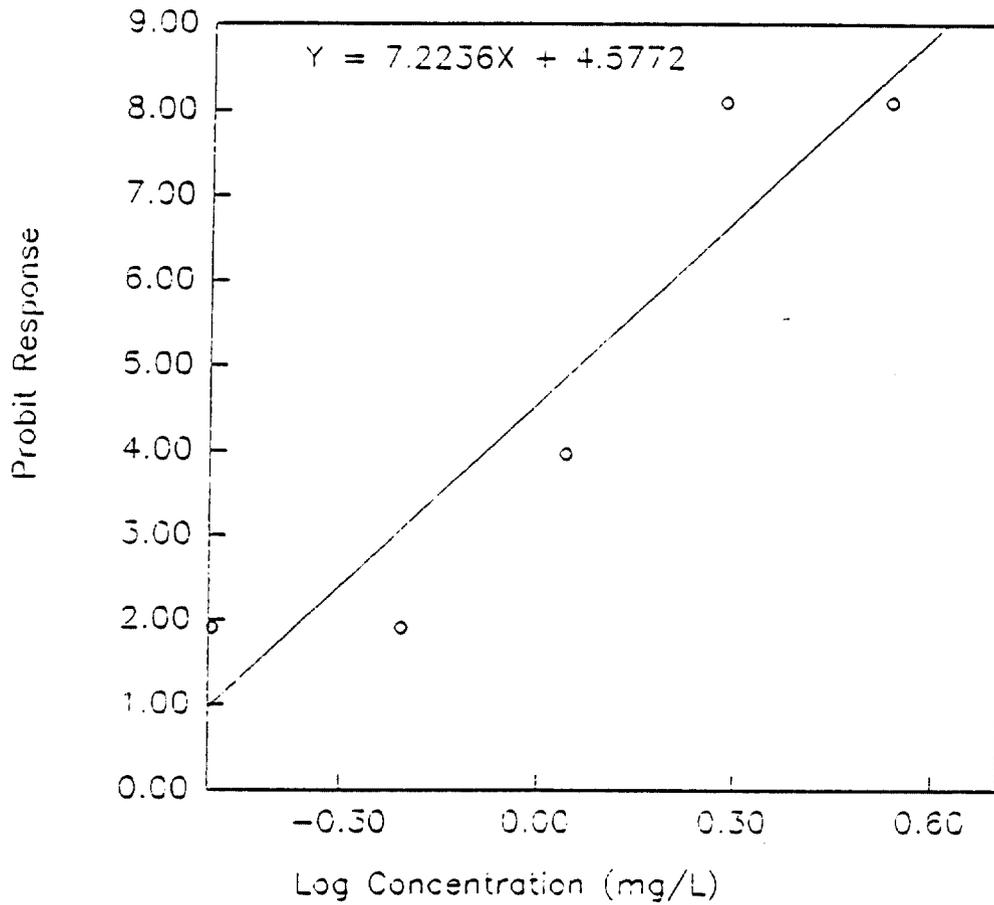
	LC50 (mg/L)	95% Confidence Interval	
		Lower (mg/L)	Upper (mg/L)
Day 1 <sup>a</sup>	1.3	1.1	1.9
Day 2 <sup>a</sup>	1.3	1.1	1.9
Day 3 <sup>a</sup>	1.1	0.62	1.9
Day 4 <sup>a</sup>	1.0	0.62	1.9

**NOEC through 96 hours = 0.62 mg/L**

<sup>a</sup> LC50 value estimated by nonlinear interpolation; 95% confidence interval calculated by binomial probability.

**FIGURES**

Figure 1. The 24-hour concentration-response (mortality) curve for the static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.



**Figure 2.** The 48-hour concentration-response (mortality) curve for the static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.

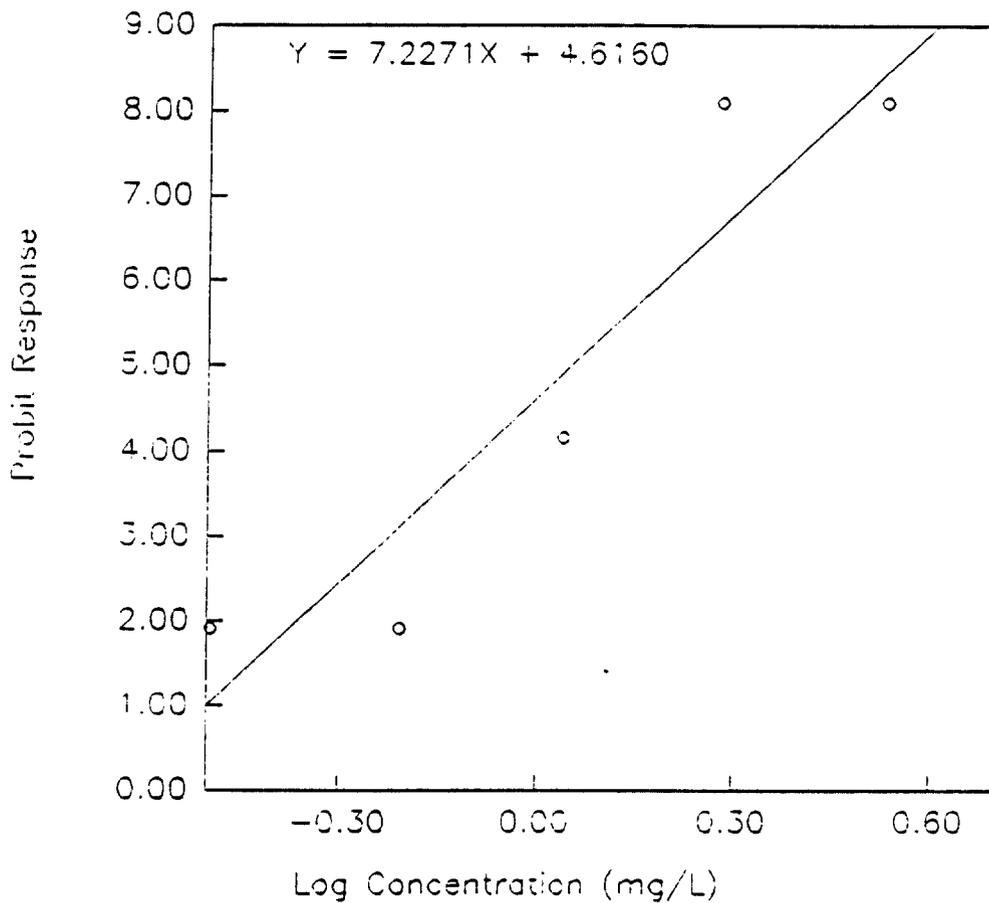


Figure 3. The 72-hour concentration-response (mortality) curve for the static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL<sup>®</sup> 45-13.

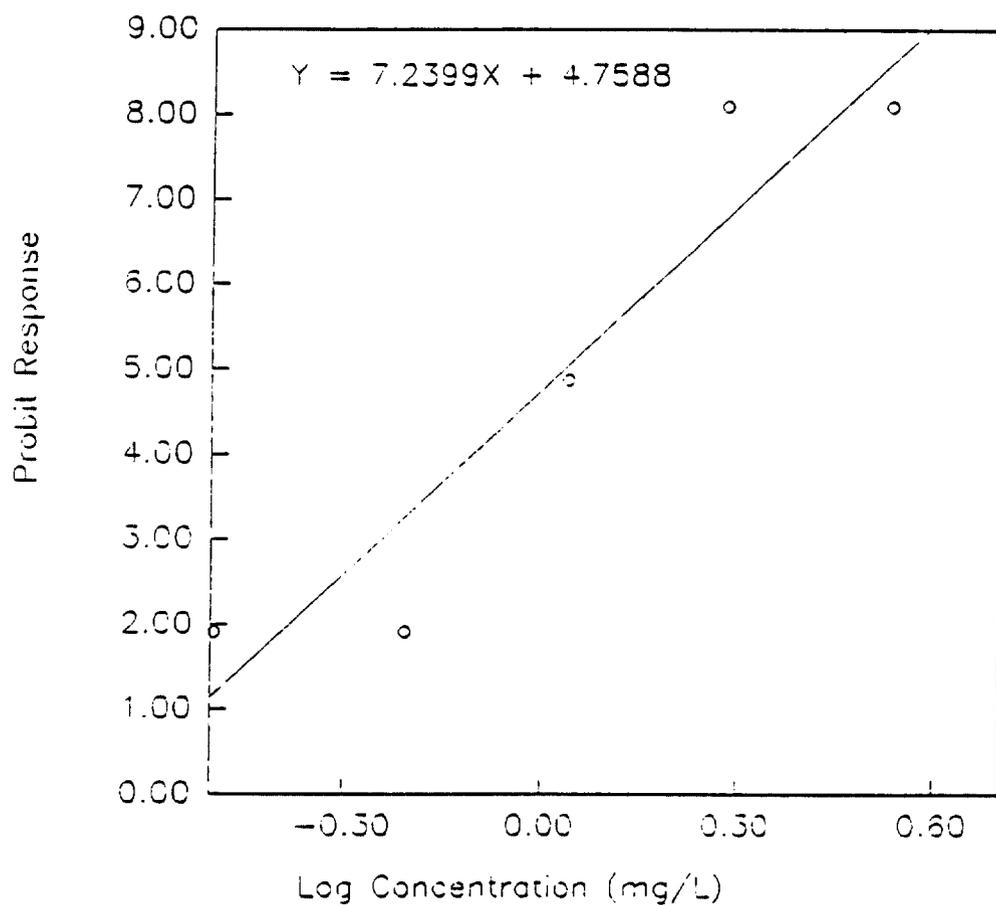
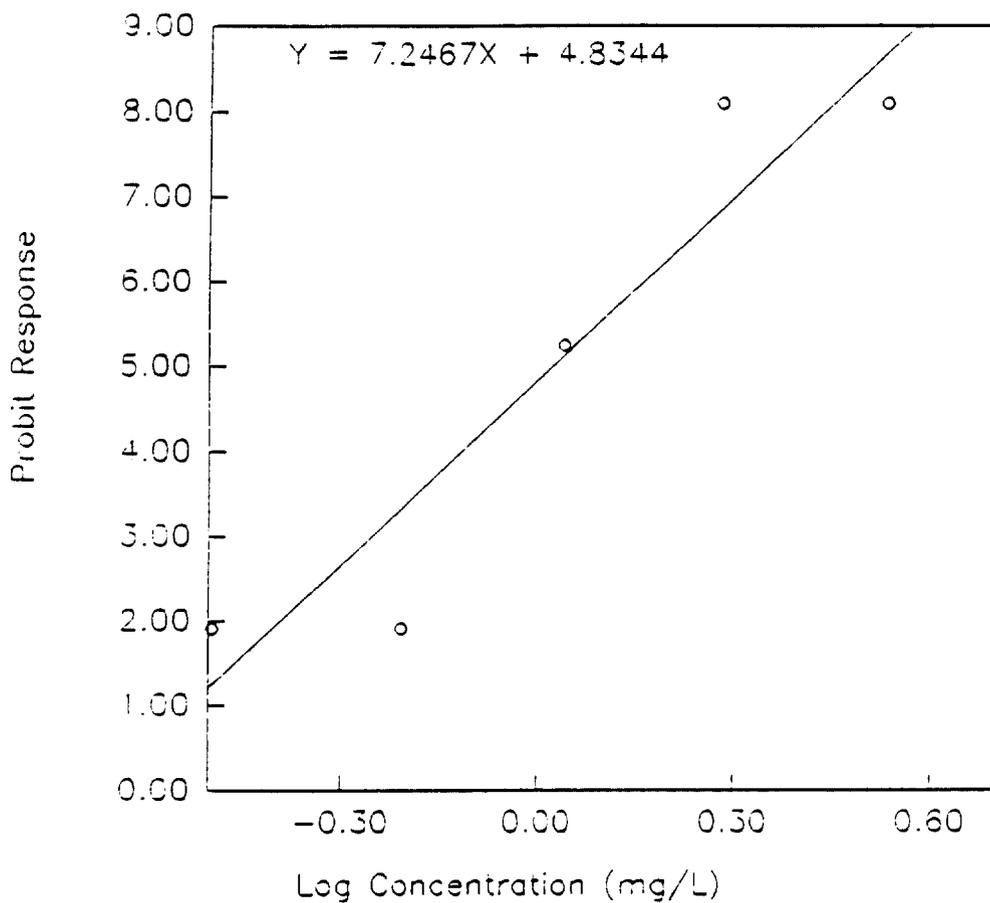


Figure 4. The 96-hour concentration-response (mortality) curve for the static renewal exposure of fathead minnow (*Pimephales promelas*) to NEODOL® 45-13.



**SIGNATURES AND APPROVAL**

**SUBMITTED BY:**

Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street  
Wareham, Massachusetts 02571-1075

**PREPARED BY:**

Maura K. Collins 8-30-94  
Maura K. Collins Date  
Study Director

Bette A. Trznadel 8-30-94  
Bette A. Trznadel Date  
Principal Investigator

Susan R. Shepherd 8-30-94  
Susan R. Shepherd for SRS Date  
Coordinator, Data Management  
and Reporting Unit

**APPROVED BY:**

Donald C. Surprenant 8/30/94  
Donald C. Surprenant Date  
Program Manager  
Environmental Toxicology

Patricia D. Royal 8/30/94  
Patricia D. Royal Date  
Manager, Regulatory Affairs  
and Quality Assurance Unit

This final report has been signed in accordance with SLI SOP No. 4.3.07(1).

**4.0 APPENDIX I - STUDY PROTOCOL**

Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street • Wareham, Massachusetts 02571 • (508) 295-2550 • Telex 4438041 • Facsimile (508) 295-8107

TEST PROTOCOL

PROTOCOL TITLE: Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with Fathead Minnows Following TSCA Test Guidelines § 797.1400

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: Shell Development Company  
Address: P.O. Box 1380  
Houston, Texas Phone: (713) ~~352-8040~~ <sup>261</sup> ~~2-24-94~~  
Sponsor Protocol/Project No.: WRC TOX No. 1209  
Test Substance: Neodol® 45-13  
Purity: 100% CAS# or LOT#: 68951-67-7  
Additional Comments and/or Modifications:

*Janie Johnson*, 2-24-94  
Sponsor Approval Date

TO BE COMPLETED BY SPRINGBORN LABORATORIES PRIOR TO TEST INITIATION:

Testing Facility: Springborn Laboratories, Inc., 790 Main St., Wareham, MA 02571  
Study Director: Maura K. Collins SLI Study No.: 777-0254-6107-101  
Test Concentrations: \*  
Solvent Used: \* CAS# or LOT#: \*  
Proposed Schedule: (Start) \* (Completion) \*  
Proposed Draft Report Date: \*

*Maura K. Collins*, 3 2 94  
Study Director Date

\* To be provided by amendment.

Springborn Laboratories Protocol #: 021494/TSCA/SHELL FM-SR



LETTERS AND REPORTS: Springborn Laboratories, Inc. accepts and agrees to issue only if the following conditions are met: 1) the client has provided the necessary information to conduct the study; 2) the client has provided the necessary resources to conduct the study; 3) the client has provided the necessary personnel to conduct the study; 4) the client has provided the necessary facilities to conduct the study; 5) the client has provided the necessary equipment to conduct the study; 6) the client has provided the necessary materials to conduct the study; 7) the client has provided the necessary services to conduct the study; 8) the client has provided the necessary support to conduct the study; 9) the client has provided the necessary assistance to conduct the study; 10) the client has provided the necessary cooperation to conduct the study; 11) the client has provided the necessary information to conduct the study; 12) the client has provided the necessary resources to conduct the study; 13) the client has provided the necessary personnel to conduct the study; 14) the client has provided the necessary facilities to conduct the study; 15) the client has provided the necessary equipment to conduct the study; 16) the client has provided the necessary materials to conduct the study; 17) the client has provided the necessary services to conduct the study; 18) the client has provided the necessary support to conduct the study; 19) the client has provided the necessary assistance to conduct the study; 20) the client has provided the necessary cooperation to conduct the study.

**Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with Fathead Minnows Following TSCA Test Guidelines § 797.1400.**

## 1.0 INTRODUCTION

The purpose of this test is to determine the acute lethal effects of an alcohol ethoxylate surfactant on fathead minnows under static renewal conditions. Test results will be reported as the 24-, 48-, 72- and 96-hour LC50 values (the median concentration which will kill 50% of the number of fish exposed) with 95% confidence limits. The No-Observed-Effect Concentration (NOEC) will also be reported. The test procedures performed during the biological portions of this study will meet or exceed the standard procedures described in the U.S. EPA Toxic Substance Control Act (TSCA) Test Guidelines § 797.1400 (U.S. EPA, 1985) and will meet the primary technical objectives of Shell Research Limited/Sittingbourne Research Centre guidelines (SOP No. 81, Edition No.8).

## 2.0 MATERIALS AND METHODS

### 2.1 TEST ORGANISMS.

- 2.1.1. **Justification for Test System.** Characteristics which make this test organism suitable for acute toxicity testing are their ease of culturing and handling, their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater fish species.
- 2.1.2. **Species.** Juvenile fathead minnow, *Pimephales promelas*, will be used to conduct the static acute toxicity test. The fish will be of approximately the same size and age, i.e., the length of the largest fish will not exceed the length of the smallest fish by more than two-fold. Fish will weigh less than 2.0 grams at the initiation of the study. Very young (not actively feeding), sexually mature, spawning and/or recently spent fish will not be used.
- 2.1.3. **Origin and Acclimation.** The fish will be obtained from in-house cultures. Fish will be gradually acclimated to the test conditions, and will be held for at least an additional 14 days in the dilution water prior to testing. They will be held a minimum of 48 hours at the required test temperature, during which time total mortality must not exceed three percent, or the fish will not be used.
- 2.1.4. **Feeding.** The fish will be fed a commercial pelleted food at least once daily prior to the test, but will not be fed during the final 48 hours before the test, nor during the 96-hour toxicity test. Periodic analyses of representative samples of the food will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the fish.

- 2.1.5. **Handling.** Fine-mesh dip nets will be used to transfer the fish from the culture vessel to the test chambers at test initiation, taking care to minimize possible stress due to handling. Fish that are damaged or dropped during transfer will be not used.
- 2.1.6. **Loading.** Fish biomass to solution ratio ("loading") will not exceed 0.5 grams per liter.
- 2.1.7. **Reference Tests.** In an effort to monitor the general health of the test organism culture, reference tests will be conducted, under static conditions, with *Pimephales promelas* using copper nitrate as the reference toxicant. The results of these tests will be evaluated based on nominal concentrations. The reference tests will be conducted using the same population of fish within 30 days of the definitive exposure (i.e., either 30 days prior to or 30 days following the definitive exposure).

## 2.2 PHYSICAL SYSTEM.

- 2.2.1. **Test Containers.** The test chambers used in the static acute bioassay will be 19-L clear glass vessels which will be chemically clean. Each test vessel will contain approximately 15 liters of test medium. This size will be adequate to meet the maximum allowable loading requirements (see above).
- 2.2.2. **Glassware Preparation.** All glassware used in testing will be thoroughly washed with detergent and rinsed with tap water. This will be followed by sequential rinsing with a 10% solution of nitric acid, acetone, distilled deionized water, isopropanol and finally distilled deionized water.
- 2.2.3. **Dilution Water.** Dilution water will consist of unadulterated water from a 100-meter bedrock well mixed in varying proportions with untreated town well water, and will be characterized as soft water with a typical total hardness of 50 - 70 mg/L as CaCO<sub>3</sub>, and alkalinity of 25 - 45 mg/L as CaCO<sub>3</sub>. The pH range will be 6.0 to 8.5 and the specific conductance will be 150 to 250 micromhos/cm. The well water will be fortified based on the formulation for soft water presented in "Methods for acute toxicity tests with fish, macroinvertebrates and amphibians" (US EPA, 1975). Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1985).

The dilution water will be prepared in 500-L batches. New batches of dilution water will be prepared when the previous batch is exhausted or when a water quality parameter (total hardness, alkalinity, etc.) has varied from the normal ranges. The dilution 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 dilution water. The total hardness, total alkalinity, acidity, pH, TOC, TSS and specific conductance of the dilution water will be monitored prior to use. Total organic carbon (TOC) will be monitored approximately once per month. Periodic analyses of representative samples of dilution water source will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs, unionized ammonia, residual chlorine and selected toxic metals, at concentrations which may be harmful to the fish.

**2.2.4. Replication and Control of Bias.** Two replicates will be included with each test concentration and control. Test aquaria will be positioned inside a water bath by stratified random design, and labeled by replicate and concentration (or control). Each replicate test vessel will contain ten individuals, i.e., 20 fish will be used per concentration or control(s). Fish will be added impartially to the test vessels by adding no more than two fish to each vessel until all vessels contain two fish. This procedure will be repeated until each test vessel contains ten fish. Test organisms will be added to the exposure solutions within 30 minutes of preparation.

### 2.3 CHEMICAL SYSTEM.

**2.3.1. Test Material.** Upon arrival at Springborn Laboratories, Inc., the external packaging of the test material will be inspected for damage. The packaging will be removed and the primary storage container will also be inspected for leakage or damage. The sample identity and percent active ingredient will be recorded and, unless different arrangements will be made with the study sponsor, the material will be stored in the dark at approximately 20°C until used.

**2.3.2. Toxicant Concentration Selection.** Toxicant concentrations for the acute toxicity test will be selected based on information provided by the Sponsor and obtained from a 96-hour preliminary range-finding study exposing fathead minnow to the test material. The preliminary test will consist of five widely spaced concentrations, usually of 15-L volume, each containing at least ten test fish. The range of concentrations selected for the definitive test is intended to include effect levels (> 50% mortality) and at least one no-effect level, but due to the nature of some test materials, one or both levels may not be observed. A geometric series of five concentrations and one control will be used for each definitive test after consultation with the Sponsor. A dilution factor of at least 60% will be used.

**2.3.3. Stock Preparation.** The surfactant will be heated in a glass container at a temperature of 50 - 60°C until completely melted. The melted test material will be stirred to ensure homogeneity. A glass pipet will be used to transfer the material for weighing. The test material will be weighed on an analytical balance for which a calibration log will be maintained. The stock solution will be mixed for several minutes. A Chemical Usage Log will also be maintained in which the amount, the date, the intended use and the user's initials are recorded each time test material is used. The primary stock will be prepared in distilled deionized water. The final concentration of the primary stock solution will be 1 - 2% active surfactant. A new primary stock solution will be prepared for each renewal of test solutions. Secondary stocks, if necessary, may be prepared in either distilled deionized water or in dilution water (if the volume displacement of dilution water during test solution preparation will be greater than 1%).

**2.3.4. Measurement of Exposure Solution and Stock Solution Concentration.** Samples from each test chamber of each concentration and control(s) will be removed at test initiation (new solutions) and test termination (old solutions). In addition, a set of samples will also be removed at 24 hours (old solutions) and at 72 hours (new solutions). Replicate

solutions at each test concentration will be pooled. All primary stock solutions prepared during the course of the test will also be sampled.

- 2.3.5. **Sampling.** Water samples (approximately 500-mL) will be taken from a point approximately midway between the surface, bottom and sides of each vessel. All samples will be collected in 700 mL borosilicate glass containers with Teflon<sup>®</sup>-lined caps which have been serially rinsed with deionized water, isopropanol and deionized water as specified in Section 2.2.2. Sampling containers will be completely filled to minimize head space. Samples will be preserved with 1% Formalin (i.e., 5 mL formalin/500 mL sample) and shipped within 24 hours to Battelle Ocean Sciences, Duxbury, Massachusetts.
- 2.3.6. **Analytical Chemistry.** Analyses of analytical samples will be conducted by Battelle Ocean Sciences, Duxbury, Massachusetts, using a Shell Development Company analytical method entitled "Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) and HPLC/ELSD (Evaporative Light Scattering Detection) in Dilute Aqueous Solutions".

#### 2.4 EXPERIMENTAL CONDITIONS.

- 2.4.1. **Measurement of Water Quality Variables.** At test initiation, total hardness, alkalinity, acidity and specific conductivity will be measured and recorded in each replicate vessel in each test concentration and control. Temperature, pH and dissolved oxygen concentration will be recorded daily at each replicate of each concentration and control. Measurements will be recorded for the aged and for the freshly prepared solutions on renewal days.
- 2.4.2. **Photoperiod.** A combination of fluorescent bulbs will be used to illuminate the aquaria, providing a wide spectrum that simulates natural sunlight. Light intensity at the water surface will be 30 to 100 footcandles. An 8-hour dark and 16-hour light photoperiod will be maintained during the test. There will be a transition period between light and dark.
- 2.4.3. **Dissolved Oxygen.** Total dissolved oxygen will exceed 90% of saturation (i.e., 7.9 mg/L at 22 °C) at the initiation of the test, and will not be allowed to drop below 60% of saturation (i.e., 5.3 mg/L at 22 °C) for the remainder of the test. Should the dissolved oxygen fall below 60% of saturation, appropriate action will be taken after consultation with the Study Sponsor.
- 2.4.4. **Temperature.** Water temperature of the test solutions will be maintained at 22 ± 2°C by maintaining the aquaria in a waterbath.
- 2.4.5. **pH.** The pH of the control solutions will be maintained in a range of 6.0 to 8.5.
- 2.4.6. **Biological Data.** At 0, 24, 48, 72, and 96 hours during exposure, observations of stress, abnormal behavioral activity and mortality will be made. Dead fish will be removed from exposure solutions twice daily. In addition, prior to test initiation and whenever test

organisms are observed, characteristics of the test solutions will also be observed and recorded, e.g., precipitated materials, cloudiness, etc.

- 2.4.7. **Renewal Scheme.** Test solutions will be prepared at 0, 24, 48, and 72 hours of exposure. Test organisms will be carefully transferred to the freshly prepared solutions.
- 2.4.8. **Initiation and Test Duration.** The study will be initiated when all test organisms have been impartially added to the exposure solutions. The study will be terminated following 96 hours of exposure at which time, mortality of the control organisms will not exceed 10% or the test will be considered unacceptable.

If 100% mortality occurs at any exposure level prior to test termination, water quality parameters, analytical samples and biological observations will only be recorded on the day which complete mortality was observed. Observations following the day on which complete mortality was observed will be discontinued.

### 3.0 DATA EVALUATION

Mortality data derived from the acute test will be used to statistically estimate a median lethal concentration (LC50) and its 95% confidence interval after each 24-hour interval of exposure. The LC50 is the estimated mean measured concentration of the test material in dilution water which produces 50% mortality in the test fish population at the stated times of exposure.

The computer program utilized estimates LC50 values using one of three statistical methods: probit analysis, moving average method, or binomial probability. The method selected will be determined by the data base (i.e., presence or absence of 100% response, number of partial responses, etc.). An LC50 value cannot be calculated if the mortality data derived is insufficient according to any of the three statistical methods. The probit method provides values of the slope, and includes 95% confidence intervals as well as appropriate statistical tests to evaluate goodness-of-fit.

Following 96 hours of exposure, data obtained on organism survival will be evaluated to establish the No-Observed-Effect Concentration (NOEC). This level is defined as the highest test concentration at and below which there were no toxicant-related mortalities or physical and behavioral abnormalities (e.g., lethargy).

The concentration-response data generated during this test will be provided to the Study Sponsor in Lotus® format.

### 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 at Springborn Laboratories as a result of the study.

## 5.0 REPORTING

The raw data generated at Springborn Laboratories and final draft of the report will be reviewed by the Quality Assurance Unit and Study Director. All measurements (e.g. water quality) will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. A single copy of the draft report will initially be submitted to the Study Sponsor for review. Upon acceptance by the Sponsor, three (3) copies of the final report will be submitted. All reports include, but are not limited to, the following information.

- \* Springborn Laboratories, Inc., report and project numbers.
- \* Laboratory and site, the dates of testing and personnel involved in the study, e.g., Quality Assurance Unit, Program Coordinator (if applicable), Study Director, Principal Investigator.
- \* All information pertaining to the test material which appears on the sample bottle, e.g., its source, percent active ingredient, physical properties, Sponsor's test material I.D., and sample number (if applicable).
- \* Characterization and origin of the dilution water.
- \* Scientific name of the test organisms, source, and culturing information.
- \* Range-finding study results.
- \* The 96-hour LC50 value with the 95% confidence limits and control performance of applicable copper nitrate reference test.
- \* Test container volume, dilution water volume, number of replicates used per concentration, and number of organisms tested per treatment.
- \* Definition of criteria used to determine the sublethal effects, and general observations on non-quantifiable effects.
- \* Description of exposure system and stock preparation.
- \* Test temperatures, dissolved oxygen concentration, and pH; photoperiod and light intensity; and specific conductance, total alkalinity and total hardness measured.
- \* Description of, or reference to, chemical and statistical procedures applied.
- \* Percentage of mortality observed in the controls and in each treatment level at each

observation period, in tabular form.

- \* The 24-, 48-, 72- and 96-hour LC50's with 95 percent confidence limits, and the No-Observed-Effect Concentration (NOEC), when possible. All calculations will be based on mean measured concentrations.
- \* Graph of the concentration response curve at each observation period for which an LC50 is calculated. Mean measured concentrations will be used to establish the concentration-response curve.
- \* Deviations from the protocol not addressed in protocol amendments together with a discussion of the impact on the study, signed by the Study Director.
- \* Good Laboratory Practice (GLP) compliance statement (for the biological portion of the study) signed by the Study Director.
- \* Dates of Quality Assurance reviews, signed by the QA 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 impact of the change on results of the study, if any. If necessary, amendments initially may be in the form of verbal authorization, followed by Springborn's written documentation of amendment. In such a case, the effective date of the amendment will be the date of verbal authorization.

#### 7.0 SPECIAL PROVISIONS

**GOOD LABORATORY PRACTICES (GLP):** All test procedures, documentation, records, and reports related to the biological portion of this study will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substance Control Act (FEDERAL REGISTER, Part III, 17 August, 1989)

**TEST MATERIAL DISPOSAL:** After 60 days of the issuance of the final test report, the test material will be returned to the Sponsor's project officer, at Sponsor expense, unless different arrangements are made.

**ARCHIVAL:** All raw data and the final report will be archived at Shell Development Company unless different arrangements are made.

*Springborn Laboratories Protocol #: 021494/TSCA/SHELL/IFM-SR*

*Page 3 of 9*

**8.0 REFERENCES**

- APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, Washington, DC. 2168 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. Environmental Protection Agency. 1989. *Toxic Substances Control: Good Laboratory Practice Standards: Final Rule*. (40 CFR, Part 792) Federal Register, Part III, 48(230):53922-53944. August 17, 1989.
- U.S. Environmental Protection Agency. 1985. *Toxic Substances Control Act Test Guidelines*. Federal Register 50(188):39252-39516, September 27, 1985. Amended May 20, 1987, July 1, 1991, and July 1, 1992.

**Springborn Laboratories, Inc.**

Environmental Sciences Division

790 Main Street • Wareham, Massachusetts 02571-1075 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

PROTOCOL AMENDMENT

AMENDMENT #: 1

DATE: 5 April 1994

PROTOCOL TITLE: "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with Fathead Minnows Following TSCA Test Guidelines 5797.1400."

SPECIES: *Pimephales promelas*

STUDY SPONSOR: Shell Development Company

TEST SUBSTANCE: Neodol® 45-13

SLI STUDY No.: 777.0294.6109.101

SPONSOR PROTOCOL/PROJECT NO.: WRC Tax No. 1209

AMENDMENT(S):

Amendment (Page 1):

Test Concentrations: 4.0, 2.4, 1.4, 0.87 and 0.52 mg A.I./L plus controls.

Solvent Used: NA CAS# or LOT#: NA

Proposed Schedule:

(Start) 4-4-94 (Completion) 4-8-94 (Draft Report) 5-9-94

Reason for Change:

This information is provided per instruction on page one of the Study Protocol.

Approval Signatures:	<u>Maura K Collins</u>	<u>4/5/94</u>
	Maura K. Collins	Date
	SLI Study Director	

	<u>Diane C. L. Wong</u>	<u>4/13/94</u>
	Diane C. L. Wong	Date
	Sponsor Study Monitor	

Springborn Laboratories Protocol #: 021494/TSCA/SHELL/FM-SR

Page 1 of 1  


LETTERS AND REPORTS: Springborn Laboratories, Inc. does not warrant the accuracy of the information contained in this report or the results of any tests conducted. The information contained in this report is for informational purposes only and does not constitute an offer of insurance or any other financial product. The information contained in this report is not intended to be used for any other purpose. The information contained in this report is not intended to be used for any other purpose. The information contained in this report is not intended to be used for any other purpose.

**5.0 APPENDIX II - CERTIFICATE OF ANALYSIS**

## Shell Development Company

A Division of Shell Oil Company

Westhollow Research Center  
P.O. Box 1380  
Houston, TX 77251-1380

February 24, 1994

Pamela M. Lincoln  
Springborn Laboratories, Inc.  
790 Main Street  
Wareham, MA 02571

Dear Ms. Lincoln:

The missing information, pertaining to test substance NEODOL 45-13, that you requested of me in your letter dated February 21, is as follows:

Lot Number:	TANK TM 996
% Active Ingredient:	100%
Net Amount Shipped:	1 1/4 qt.
Molecular Weight:	Avg. 777
Expiration Date:	February 1995

Analytical characterization acquired in support of test substance NEODOL 45-13 was performed at Westhollow Research Center (WRC). Methods and procedures used follow all applicable government regulations regarding Good Laboratory Practices as stated in 40 CFR 792. All records and raw data generated by these analyses will be retained in the WRC Analytical Special Collection of Files.

Analytical methods used to characterize the test substance were Hydroxyl Number (mg KOH/gm), % Water (%wt), Cloud Point, Ethylene Oxide Distribution, Polyethylene Glycol (%wt) and Carbon Number Distribution.

If you have any further questions, please feel free to contact me at (713) 544-8410.

Sincerely,

A handwritten signature in cursive script that reads "Harriet Smith".

Harriet Smith

**6.0 APPENDIX III - CULTURE FOOD ANALYSIS**

Zeigler Brothers, Inc. Salmon Starter #1*		
Date Submitted: 12/13/93 Date Reported: 1/26/94		
Test	Result	Limit of Quantitation
Pesticide Screen I;II;III	attached	
Arsenic	2.6 ppm	0.1
Cadmium	0.6 ppm	0.1
Lead	0.4 ppm	0.2
Mercury	<0.02 ppm	0.02
* Analyzed by Lancaster Laboratories		

Zeigler Brothers, Inc. Salmon Starter #1*		
Date Submitted: 12/13/93 Date Reported: 1/26/94		
Pesticide Screen I;II;III	Result	Limit of Quantitation
Alpha BHC	< 0.01 mg/kg	0.01
Beta BHC	< 0.01 mg/kg	0.01
Gamma BHC - Lindane	< 0.01 mg/kg	0.01
Delta BHC	< 0.01 mg/kg	0.01
Heptachlor	< 0.01 mg/kg	0.01
Aldrin	< 0.01 mg/kg	0.01
Heptachlor Epoxide	< 0.01 mg/kg	0.01
DDE	< 0.01 mg/kg	0.01
DDD	< 0.01 mg/kg	0.01
DDT	< 0.01 mg/kg	0.01
HCB	< 0.01 mg/kg	0.01
Mirex	< 0.01 mg/kg	0.01
Methoxychlor	< 0.05 mg/kg	0.05
Dieldrin	< 0.01 mg/kg	0.01
Endrin	< 0.01 mg/kg	0.01
Telodrin	< 0.01 mg/kg	0.01
Chlordane	< 0.05 mg/kg	0.05
Toxaphene	< 0.1 mg/kg	0.1
PCBs	< 0.2 mg/kg	0.2
Ronnel	< 0.01 mg/kg	0.01
Ethion	< 0.02 mg/kg	0.02
Trithion	< 0.05 mg/kg	0.05
Diazinon	< 0.1 mg/kg	0.1
Methyl Parathion	< 0.02 mg/kg	0.02
Ethyl Parathion	< 0.02 mg/kg	0.02
Malathion	< 0.05 mg/kg	0.05
Endosulfan I	< 0.01 mg/kg	0.01
Endosulfan II	< 0.01 mg/kg	0.01
Endosulfan Sulfate	< 0.03 mg/kg	0.03
Chlorpyrifos	< 0.01 mg/kg	0.01
* Analyzed by Lancaster Laboratories		

**7.0 APPENDIX IV - DILUTION WATER ANALYSIS**

Well <sup>1</sup> Water Sample*		
Date Collected: 7/29/93 Date Reported: 9/17/93		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 µg/l	0.01
Beta BHC	< 0.01 µg/l	0.01
Gamma BHC - Lindane	< 0.01 µg/l	0.01
Delta BHC	< 0.01 µg/l	0.01
Heptachlor	< 0.01 µg/l	0.01
Aldrin	< 0.01 µg/l	0.01
Heptachlor Epoxide	< 0.01 µg/l	0.01
DDE	< 0.01 µg/l	0.01
DDD	< 0.01 µg/l	0.01
DDT	< 0.01 µg/l	0.01
HCB	< 0.01 µg/l	0.01
Mirex	< 0.01 µg/l	0.01
Methoxychlor	< 0.05 µg/l	0.05
Dieldrin	< 0.01 µg/l	0.01
Endrin	< 0.01 µg/l	0.01
Telodrin	< 0.01 µg/l	0.01
Chlordane	< 0.3 µg/l	0.3
Toxaphene	< 4. µg/l	4.
PCBs	< 1. µg/l	1.
Ronnel	< 0.01 µg/l	0.01
Ethion	< 0.02 µg/l	0.02
Trithion	< 0.05 µg/l	0.05
Diazinon	< 0.1 µg/l	0.1
Methyl Parathion	< 0.02 µg/l	0.02
Ethyl Parathion	< 0.02 µg/l	0.02
Malathion	< 0.05 µg/l	0.05
Endosulfan I	< 0.01 µg/l	0.01
Endosulfan II	< 0.01 µg/l	0.01
Endosulfan Sulfate	< 0.03 µg/l	0.03
<sup>1</sup> Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		

Well <sup>1</sup> Water Sample*		
Date Collected: 8/9/93 Date Reported: 8/26/93		
Analysis	Result As Received	Limit of Quantitation
Mercury	< 0.00020 mg/l	0.00020
Arsenic	< 0.20 mg/l	0.20
Selenium	< 0.20 mg/l	0.2
Boron	< 0.040 mg/l	0.04
Thallium	< 0.30 mg/l	0.3
Aluminum	< 0.20 mg/l	0.2
Antimony	< 0.20 mg/l	0.2
Barium	< 0.10 mg/l	0.1
Beryllium	< 0.010 mg/l	0.01
Cadmium	< 0.010 mg/l	0.01
Calcium	7.71 mg/l	0.2
Chromium	< 0.050 mg/l	0.05
Cobalt	< 0.050 mg/l	0.05
Copper	< 0.020 mg/l	0.02
Iron	< 0.10 mg/l	0.1
Lead	< 0.10 mg/l	0.1
Magnesium	2.31 mg/l	0.1
Manganese	< 0.010 mg/l	0.01
Molybdenum	< 0.10 mg/l	0.1
Nickel	< 0.050 mg/l	0.05
Potassium	1.07 mg/l	0.5
Silver	< 0.020 mg/l	0.02
Sodium	14.0 mg/l	0.4
Titanium	< 0.010 mg/l	0.01
Vanadium	< 0.010 mg/l	0.01
Zinc	<0.040 mg/l	0.04
Total Organic Carbon ***	< 1. mg/L	1.

<sup>1</sup> Well water supplemented by Town of Wareham water

\* Analyzed by Lancaster Laboratories, Inc.

\*\*\* Represents "non-purgeable TOC"

## 8.0 APPENDIX V - ANALYTICAL METHODOLOGY

---

FINAL DATA REPORT

Study Title

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with Fathead Minnow

Data Requirements

United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA),  
Good Laboratory Practice Standards (40 CFR, Part 792)

Submitted To

Shell Development Company  
Westhollow Research Center  
Houston, TX 77251-1380

Performing Laboratory

Battelle Ocean Sciences  
397 Washington Street  
Duxbury, MA 02332

Author

Gregory S. Durell

Study Initiation Date

March 2, 1994

Study Completion Date

May 12, 1994

Battelle Study Number

SD-930119

SIGNATURE PAGE

*Gregory S. Durrell*

Gregory S. Durrell  
Analytical Chemistry Task Leader  
Battelle Ocean Sciences

*06/02/94*

Date

*Allen D. Uhler*

Allen D. Uhler  
Chemistry Department Manager  
Battelle Ocean Sciences

*6/06/94*

Date

*Harriet C. Smith*

Harriet C. Smith  
Project Monitor  
Shell Development Company

*June 6, 1994*

Date

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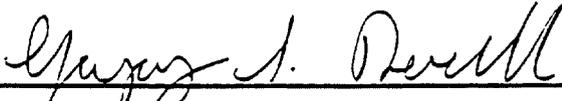
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COMPLIANCE STATEMENT

The work conducted at Battelle through Battelle Proposal/Agreement No. 882-H-0641 "Analysis of Non-ionic Surfactants in Water Samples by HPLC and ELSD" was performed in compliance with United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 CFR, Part 792), August 17, 1989.



\_\_\_\_\_  
Gregory S. Duffell  
Analytical Chemistry Task Leader  
Battelle Ocean Sciences



\_\_\_\_\_  
Date

QUALITY ASSURANCE STATEMENT

for

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with Fathead Minnow

In accordance with Good Laboratory Practice Standards (40 CFR, Part 792) dated August 17, 1989, this study has been monitored by Battelle Ocean Science's Quality Assurance Unit. Study audit dates and dates when the results were reported to the Study Director and management are listed in the following table.

To the best of my knowledge, the analyses reported here accurately represent the data generated during this study.

*Rosanna L. Buhl*

\_\_\_\_\_  
Rosanna L. Buhl  
Quality Assurance Coordinator  
Battelle Ocean Sciences

*6-6-94*

\_\_\_\_\_  
Date

## QUALITY ASSURANCE AUDITS

Conducted for SD-930119

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with Fathead Minnow

Audit Type	Audit Date	Date of Report to Analytical Task Leader	Date of Report to Study Director	Date of Report to Management
Initiation	02/28/94 03/28/94	none	none	none
Lab Inspection	05/09/94 05/10/94 05/12/94 05/23/94	05/24/94	06/06/94	06/06/94
Data Package	06/01-02/94	06/02/94	06/06/94	06/06/94
Report Review	06/01-2/94	06/02/94	06/06/94	06/06/94

STUDY PARTICIPANTS

SD-930119

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with Fathead Minnow

---

Mr. Gregory S. Durell	Analytical Chemistry Task Leader; HPLC Analysts
Mr. Richard Restucci	Laboratory Technician, HPLC Analyst (in training)
Mr. Lyle Roberts	Laboratory Technician (in training)

---

## 1.0 INTRODUCTION

The work reported in this document is a component of aquatic toxicological testing that has been requested and initiated by the Sponsor of this study. The toxicological testing was conducted by Springborn Laboratories. Battelle Ocean Sciences was responsible for providing chemical analytical support to the toxicological testing by determining concentrations of the Test Substance in samples received from Springborn Laboratories. The results of these chemical analyses are presented in this Final Data Report.

The objective of the work reported in this document was to perform chemical analysis of aqueous samples and primary stock solutions, for the determination of concentrations of alcohol ethoxylate surfactants using the analytical method titled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*. This analytical procedure was approved by the Analytical Chemistry Task Leader on February 25, 1994, and by the Sponsor's Project Monitor on March 7, 1994.

### 1.1 Test Substance Identification

The Test Substance analyzed in this study was an alcohol ethoxylate (AE) surfactant (Neodol 45-13<sup>®</sup>). The Test Substance was provided by the Sponsor (Shell Development Company). The Sponsor was also responsible for providing Battelle with the lot number, analysis, purity, stability, storage requirements, and all other relevant chemical and physical characterization data for the Test Substance. The Test System and Test Substance identity and characterization information, and other relevant test information for this study, as provided by the Sponsor, is summarized below.

Test System:	Fathead minnow
Test Substance:	Neodol <sup>®</sup> 45-13
Test Substance CAS#:	68951-67-7
Test Substance Lot#:	Tank TM996 (01/23/92) 1209 (WRC Tox Sample Number)
Test Substance Purity:	100%

<b>Test Substance Composition:</b>	A mixture of C <sub>4</sub> -C <sub>13</sub> alcohol ethoxylates with an average of 13 moles of ethylene oxide per mole of alcohol. Traces of free ethylene oxide (less than or equal to 6 ppm) may be present in the neat Test Substance.
<b>Test Substance Analysis:</b>	The percent purity data is based on process knowledge, and research and development prior to manufacture of the Test Substance used.
<b>Test Substance Solubility:</b>	Completely soluble in water. May form gel.
<b>Test Substance Stability:</b>	Stable. A stability and holding time of two years from receipt at Battelle was assigned to the Test Substance.
<b>Test Substance Storage Requirements:</b>	Cool, dry.

## 2.0 MATERIALS AND METHODS

### 2.1 Analytical Method Description

The analytical procedure used was developed for the determination of total alcohol ethoxylate surfactants in aqueous samples. The method involves an extraction step to isolate the surfactant from a water sample and a high performance liquid chromatography (HPLC) analytical procedure to quantify the nonionic surfactant concentration. The results are reported as total alcohol ethoxylate surfactant concentrations. Concentrations of the surfactant were also determined in primary stock solutions received from the toxicology testing laboratory.

In order to analyze alcohol ethoxylate (AE) surfactants in aqueous matrices at low levels the surfactant must first be isolated from the water, concentrated, and analyzed using an appropriate method of detection to quantitate the amount of surfactant originally present in the aqueous sample. The method used employs solid phase extraction (SPE) with a C<sub>8</sub> reverse phase cartridge for isolating the analyte and concentrating the sample. After elution of the surfactant from the SPE cartridge with methyl and isopropyl alcohol it is analyzed using an HPLC procedure (octyl HPLC-column separation and methanol/water mobile phase gradient) that employs an evaporative light scattering detector

(ELSD) for analyte detection and quantification. This method quantifies total AE surfactant without distinguishing between the various individual AEs or carbon chain distributions.

The primary stock solution surfactant concentration was determined by simply diluting the sample to the appropriate concentration using methanol and analyzing it by HPLC/ELSD. No extraction step was needed.

The analytical procedure is described in detail in the document entitled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*, which was developed specifically for these analyses. This Test Substance specific document was prepared by Battelle and approved by the Sponsor on March 7, 1994. The analytical procedure document, and associated study-specific analytical information, is included in Battelle's data-package for this study. This data-package will be provided to the Sponsor and a copy maintained by Battelle Ocean Sciences.

## 2.2 Laboratory Quality Control

The water samples were processed in analytical batches of no more than 20 test samples. Each batch of test samples included four laboratory quality control (QC) samples: one procedural blank (PB), one matrix spike (MS), one matrix spike duplicate (MSD), and one blank spike (BS). The procedural blank (which consists of Milli-Q laboratory water carried through all steps and treated as other samples) sample was used to ensure that there were no significant levels of laboratory contamination. The matrix spike (test sample spiked with the target analyte), matrix spike duplicate, and blank spike (Milli-Q water spiked with the target analyte) samples were used to demonstrate laboratory accuracy and precision; these QC samples were also carried through all sample processing procedures and treated as the rest of the samples.

A portion of a non-fortified (control) test sample was used to prepare the matrix spike samples because it contained no background analyte levels yet had a sample matrix that was representative of the test samples. The blank spike was processed to determine if the accuracy/recovery of the analyte was affected by the sample matrix.

Each sequence of samples analyzed by the HPLC/ELSD instrument was initiated with a six-point multilevel calibration. Test samples followed the initial calibration in the analysis sequence, and a calibration check standard was analyzed at least every 12 samples to verify the validity of the calibration.

Summarized below are the QC data quality objectives that applied for this study.

### Data Quality Objectives

QC Analysis	Criteria Objective
Blank spike analyte recovery	70%-120%
Matrix spike analyte recovery	70%-120%
Matrix spike/spike duplicate precision	≤ 30% RPD
Procedural blank	< 1 × limit of quantification (LOQ)
Instrument multilevel calibration	Correlation coefficient > 0.995
Instrument calibration check	15% RPD in determined and actual standard concentration

### 2.3 Calculations

#### Sample Concentration Calculations

An external standard method of calibration and quantification was used. Sample extract concentrations were determined by applying the multilevel quadratic calibration equation using a chromatography data system on which the analytical data were acquired during the instrumental analysis. A six-point calibration curve which bracketed the expected concentration range of exposure samples was generated at the initiation of the HPLC analysis. Calibration standard concentrations were approximately 22, 44, 83, 122, 164, and 204  $\mu\text{g}/\text{mL}$ . Original water sample concentrations were subsequently determined by applying the water extraction volume (WEV) and pre-injection volume (PIV) information. The PIV of the PB, BS, MS, and MSD samples, controls, and 520, 870 and 1,400 parts per billion (ppb) nominal concentration samples was 1.00 mL. For the 2,400 and 4,000 ppb nominal concentration samples the PIV was 4.00 mL. Analyte concentrations of the original water samples were determined in ppb. Analyte concentration of the primary stock solution samples were determined in parts per million (ppm).

$$\text{Determined Water Sample Concentration (ppb)} = \text{EC} \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$$\text{Primary Stock Solution Concentration (ppm)} = \text{EC} \times \text{DIL VOL}_1 \times (1/\text{DIL VOL}_2)$$

EC = Extract (HPLC sample) concentration ( $\mu\text{g}/\text{mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

DIL VOL<sub>1</sub> = Final volume of diluted Primary Stock subsample (mL)

DIL VOL<sub>2</sub> = Volume of Primary Stock subsample taken for the dilution (mL)

### Quality Control Sample Calculations

Two separate calculations were performed on the Quality Control (QC) sample data. Percent recoveries were determined for the blank spike and matrix spike samples, and the relative percent difference (%RPD) between the two percent recovery values was determined for the matrix spike/duplicate sample pair.

$$\% \text{ Recovery} = \text{WC}_0 \times (1/\text{WC}_s) \times 100\%$$

$$\% \text{RPD} = [ \% \text{REC}_{\text{MS}} - \% \text{REC}_{\text{MSD}} ] \times (2 / (\% \text{REC}_{\text{MS}} + \% \text{REC}_{\text{MSD}})) \times 100\%$$

WC<sub>0</sub> = Determined water sample concentration (ppb) — calculated as shown above

WC<sub>s</sub> = Spiked water sample concentration (ppb) — prepared concentration

%REC<sub>MS</sub> = Percent recovery of the matrix spike sample

%REC<sub>MSD</sub> = Percent recovery of the matrix spike duplicate sample

### Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) was determined for the analyses. The target analyte had to provide a detector response of a minimum 3:1 signal:noise ratio to be considered detected.

The water sample equivalent LOD was calculated by comparing the peak height equivalent to a signal:noise ratio of 3:1 in the sample to the peak height of the analyte in the low-level calibration standard, and applying the water extraction volume (WEV) and the pre-injection volume (PIV), as shown previously. The LOD was determined using two samples with the lowest nominal concentration and averaging the values obtained in the two determinations.

The LOQ was determined as the water sample concentration equivalent to a final extract concentration that is the same as the low calibration standard. The LOQ was determined using the PIVs and WEVs used for the samples with the lowest nominal concentrations.

$$\text{LOD (ppb)} = H_{3x} \times (C_{LS}/H_{LS}) \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$$\text{LOQ (ppb)} = C_{LS} \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$H_{3x}$  = Peak height equivalent to  $3 \times$  the noise in the sample

$H_{LS}$  = Peak height of analyte in the low-level calibration standard

$C_{LS}$  = Concentration of analyte in the low-level calibration standard ( $\mu\text{g/mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

### 3.0 RESULTS

#### 3.1 Analytical Results — Toxicological Test Samples

The water samples received for the first Fathead Minnow study (started on April 4, 1994) were not analyzed at the direction of the toxicology laboratory. This test was terminated and re-started by the toxicology laboratory on April 12, 1994. Only the second set of water samples received was extracted and analyzed.

The results of the chemical analyses of the samples received from the toxicological testing laboratory are presented in Table 1. The analyses of the test samples were performed in two analytical batches, the first batch containing the  $t=0$  hr and  $t=24$  hr (old) samples and the second batch containing the  $t=72$  hr (new) and  $t=96$  hr (old) samples. Table 1 also presents the data for the Primary Stock Solution analyses.

The measured analyte concentrations in the test samples that had been fortified with the Test Substance ranged from 176 ppb (for sample NC56, a sample with a nominal concentration of 520 ppb) to 3,542 ppb (for sample NC53, a sample with a nominal concentration of 4,000 ppb). The measured concentrations were between 34 percent (sample NC56) and 94 percent (samples NC49 and NC51) of the nominal concentration. On an average, the concentrations in the  $t=24$  (old) and  $t=96$  samples are lower than the  $t=0$  and  $t=72$  (new) samples, suggesting that there may be a some loss of the analyte with time. Additionally, for the  $t=24$  (old) and  $t=96$  samples the measured concentrations deviated more from the nominal concentrations for samples with lower concentrations than for samples with higher concentrations.

Table 1. Neodol® 45-13 Concentrations in Samples Received from the  
Toxicological Testing Laboratory

Barreille Sample ID	Test Sample Time/Type	Nominal Conc. (ppb)	Measured Conc. (ppb)
<b>Batch #1</b>			
NC48	t=0	0	ND
NC49	t=0	520	488
NC50	t=0	870	812
NC51	t=0	1,400	1,323
NC52	t=0	2,400	2,199
NC53	t=0	4,000	3,542
NC55	t=24, old	0	ND
NC56	t=24, old	520	176
NC57	t=24, old	870	472
NC58	t=24, old	1,400	826
NC59	t=24, old	2,400	1,660
NC60	t=24, old	4,000	3,198
<b>Batch #2</b>			
NC63	t=72, new	0	ND
NC64	t=72, new	520	390
NC65	t=72, new	870	696
NC66	t=72, new	1,400	1,179
NC68	t=96, old	0	ND
NC69	t=96, old	520	238
NC70	t=96, old	870	483
NC71	t=96, old	1,400	932
<b>Primary Stock Solution</b>		(ppm)	(ppm)
NH07	t=0, stock	10,000	10,420
NH08	t=24, stock	10,000	9,718
NH09	t=48, stock	10,000	9,783
NH10	t=72, stock	10,000	9,859

ND: Not detected; <LOD.

The concentrations measured for the Primary Stock Solutions were close to the expected concentrations, with measured concentrations ranging from 9.718 to 10.420 ppm for the four samples, all of which had nominal/expected concentrations of 10.000 ppm. The measured Primary Stock Solution concentrations were consistently within 4 percent of the expected concentration.

### 3.2 Analytical Results — Quality Control Samples

All quality control objectives were met for this work. The six-point multi-level instrument calibration used had a correlation coefficient of 0.999213 for the quadratic equation, and the continuing calibration check analyses ranged from 4.8 to 7.3 relative percent difference between the determined and actual standard concentration.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as described in Section 2.3, and were as follows.

#### Limit of Detection and Limit of Quantitation

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Limit of Detection (LOD)	26 ppb
Limit of Quantitation (LOQ)	221 ppb

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The concentrations for all samples with anticipated analyte concentrations (i.e., all samples except the laboratory procedural blanks and toxicological test control samples) had measurable levels of analyte and were determined to be above the LOD. One sample (NC56, the  $t=24$  hr 520 ppb nominal concentration sample) had a detectable (i.e., above the LOD) amount of target analyte which was determined to be below the LOQ — 176 ppb detected versus an LOQ of 221 ppb.

The results of the laboratory quality control (QC) sample analyses are presented in Table 2. The target analyte was not detected in either of the two procedural blank samples. The analyte recovery in the blank spike (BS) samples were 90% and 89% for analytical batches #1 and #2, respectively. The analyte recovery in the four matrix spike (MS/MSD) samples ranged from 90% to 92%, and these data suggest that there were no significant matrix effects on the analytical procedure. Acceptable precision was observed for both analytical batches. The relative percent differences in the

Table 2. Laboratory Quality Control Sample Analysis Results

Battelle Sample ID	QC Sample Type	Recovery (%)
Batch #1		
NH22PB	Procedural Blank	ND
NH23BS	Blank Spike	90.1
NH24MS	Matrix Spike	92.4
NH25MSD	Matrix Spike Duplicate	89.7
	MS/MSD %RPD:	3.0
Batch #2		
NH26PB	Procedural Blank	ND
NH27BS	Blank Spike	89.2
NH28MS	Matrix Spike	92.0
NH29MSD	Matrix Spike Duplicate	90.7
	MS/MSD %RPD:	1.4

ND: Not detected; &lt;LOD.

measured analyte recoveries for the MS/MSD duplicate analyses were 3% and 1% for analytical batches #1 and #2, respectively.

The QC data indicate that the laboratory analysis was in control for this work. There were no identified circumstances or occurrences during the conduct of this work that may have affected the quality or integrity of the data.

#### 4.0 ARCHIVING OF DATA

Study records that will be maintained by Battelle include, but are not limited to:

- Verified copies of all raw data and documentation records
- Verified copy of the signed and approved Analytical Chemistry Method, and associated amendments and deviations
- All correspondence, memos, or notes pertaining to the study
- Verified copy of the signed Final Data Report
- Test Substance records, including receipt and inventory, and physical and chemical characterization data, as supplied by the Sponsor

All project files, including verified copies of the raw data and the Final Data Report, will be archived by Battelle after the submission of this Final Data Report. The Battelle Quality Assurance Unit manages the limited-access data archival. Additionally, a small amount of Test Substance will be archived by Battelle.

**APPENDIX A**

**Deviations to Analytical Method**

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BATTELLE OCEAN SCIENCES  
Miscellaneous Documentation Form

Page 1 of 1

Project Title: Gap-Filling Project

Study Number: SD-930119

Battelle Project Number: N8328-0002

This is a Deviation to Analytical Method:

Yes X No       

Entered by: Gregory S. Durell

Date: May 26, 1994

---

Subject: Miscellaneous Deviations to Analytical Method

- The temperature recorded for Refrigerator #2, where standards and samples were stored, ranged from 1 to 10°C in March and April. Unextracted water samples were to be stored at approximately 4°C, as indicated in the Analytical Procedure Document. Although this temperature range is larger than what may be considered covered by "approximately 4°C", it is not expected to have impacted the integrity of the samples or results, because of the stability of the test substance.
- The nebulizer gas flow (nitrogen) for the HPLC detector (ELSD) was maintained at 9 L/min and not 11 L/min as specified in the Analytical Procedure Document. This analytical modification was requested by the Sponsor, based on method verification work performed at the Sponsor's laboratory, in order to achieve the necessary sensitivity. This method modification was requested by the Sponsor by telephone on 04/06/94 and confirmed by telephone on 04/08/94, as indicated in the Analytical Task Leader's telephone log.

Approved: Gregory S. DurellDate: 05/26/94

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**BATTELLE OCEAN SCIENCES**  
Miscellaneous Documentation Form

Page 1 of 1

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**Project Title:** Gap-Filling Project

**Study Number:** SD-930119

**Battelle Project Number:** N8328-0002

**This is a Deviation to Analytical Method:**

Yes X No \_\_\_\_\_

**Entered by:** Gregory S. Durell  
Richard P. Restucci

**Date:** May 26, 1994  
June 3, 1994

---

**Subject:** Miscellaneous Deviations

- The monthly calibration with a 70 g weight, as specified in SOP 3-073, was not performed on the Mettler AC100 balance that was used to prepare standards for this study. However, the balance was always calibrated on the day it was used with a weight of suitable for representing the weight actually being measured for this study.
- According to the Project-specific Analytical Information memo, seven calibration levels with an approximate concentration range of 20 - 380 ug/mL were to be analyzed prior to sample analysis. However, six levels at a range of 20 - 204 ug/mL were analyzed with the Fathead Minnow study.

---

**Approved:** AD Uhl

**Date:** 6/06/94

**NEODOL® 45-13 - ACUTE TOXICITY TO  
DAPHNIDS (*Daphnia magna*) UNDER STATIC  
RENEWAL CONDITIONS**

**TSCA TEST GUIDELINES § 797.1300**

**Submitted to:**

**Shell Development Company  
Westhollow Technology Center  
P.O. Box 1380  
Houston, Texas 77251**

**SLI Report #94-6-5317**

**Sponsor Protocol/Project No.: WRC TOX No. 1209**

**SLI Study #777.0294.6110.110**

**Study Director: Maura K. Collins**

**Contains No CBI**

**Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street  
Wareham, Massachusetts 02571-1075**

**Analytical Support:  
Battelle Ocean Sciences  
397 Washington Street  
Duxbury, Massachusetts 02332**

**12 August 1994**

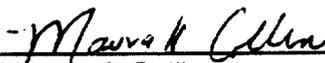
**FINAL REPORT**

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**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The data and report presented for "Neodol<sup>®</sup> 45-13 - Acute Toxicity to Daphnids (*Daphnia magna*) Under Static Renewal Conditions" were produced and compiled in accordance with all pertinent TSCA Good Laboratory Practice regulations (40 CFR, Part 792) with the following exception: routine water and food contaminant screening analyses for pesticides, PCBs and metals were conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, Pennsylvania. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc.). Stability, characterization, and verification of the test material identity and maintenance of records on the test material are the responsibility of the Study Sponsor. At the termination of the testing program, all remaining test material will be sent to the Study Sponsor. Archival of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES

 8.12.04  
Maura K. Collins Date  
Study Director

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**SUMMARY****NEODOL<sup>®</sup> 45-13 - Acute Toxicity to Daphnids  
(*Daphnia magna*) Under Static Renewal Conditions**

**SPONSOR:** Shell Development Company  
P.O. Box 1380  
Houston, Texas 77251

**PROTOCOL TITLE:** "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with *Daphnia magna*, Following TSCA Test Guidelines § 797.1300," Springborn Protocol #021494/TSCA/SHELL/DM-SR, and Protocol Amendment #1 dated 5 April 1994.

**REPORT NUMBER:** 94-6-5317

**STUDY NUMBER:** 777.0294.6110.110

**TEST MATERIAL:** NEODOL<sup>®</sup> 45-13, CAS #68951-67-7, Lot LR #20944-178, (TANK<sup>TM</sup> 996), a clear liquid reported by the Study Sponsor to contain 100% active ingredient, received 17 February 1994.

**TEST DATES:** 5 to 7 April 1994

**TEST ORGANISM:** *Daphnia magna*, inoculum - ≤ 24 hrs in age, source - Springborn culture

**DILUTION WATER:** Fortified well water  
pH: 8.3  
Specific conductivity: 500  $\mu$ mhos/cm  
Total hardness as CaCO<sub>3</sub>: 180 mg/L  
Total alkalinity as CaCO<sub>3</sub>: 120 mg/L

**TEST CONDITIONS:** 48-hour duration, 20 °C, illumination at 60 footcandles (646 lux), photoperiod of 16 hours light and 8 hours dark

**NOMINAL TEST CONCENTRATIONS:** 0.50, 0.85, 1.4, 2.4 and 4.0 mg/L

**MEAN MEASURED CONCENTRATIONS:** 0.32, 0.65, 1.2, 1.9 and 3.6 mg/L

---

**EFFECT CRITERION:** Immobilization as defined by lack of movement by the test organisms except for minor activity of the appendages.

**RESULTS:** Based on mean measured concentrations, the 48-hour EC50 value was calculated by nonlinear interpolation to be 1.2 mg/L (95% confidence interval calculated by binomial probability of 0.65 to 1.9 mg/L). The 48-hour No-Observed-Effect Concentration (NOEC) was determined to be 0.65 mg/L.

## 1.0 INTRODUCTION

The purpose of this study was to estimate the acute toxicity (EC50) of Neodol<sup>®</sup> 45-13 to daphnids (*Daphnia magna*) under static renewal conditions. The EC50 is defined as the concentration of test material in dilution water which causes immobilization of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of potential acute hazards resulting from release of the test material into aquatic environments. The study was initiated on 2 March 1994, 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 48-hour definitive test was conducted from 5 to 7 April 1994 at the Environmental Sciences Division of Springborn Laboratories, Inc. (SLI), in Wareham, Massachusetts. All original raw data and the final report produced during this study are stored at Shell Development Company.

## 2.0 MATERIALS AND METHODS

### 2.1 Protocol

Procedures used in this acute toxicity study followed those described in the Springborn protocol entitled "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with *Daphnia magna*, Following TSCA Test Guidelines § 797.1300," Springborn Protocol #021494/TSCA/SHELL/DM-SR, and Protocol Amendment #1 dated 5 April 1994 (Appendix I). The methods described in this protocol meet or exceed the standard procedures described in the U.S. EPA Toxic Substances Control Act (TSCA) Test Guidelines § 797.1300 (U.S. EPA, 1992) and meet the primary technical objectives of the Shell Research Limited/Sittingbourne Research Centre guidelines (SBT SOP No. 167, Edition No. 9).

### 2.2 Test Material

The test material, Neodol<sup>®</sup> 45-13, was received from Shell Development Company, Houston, Texas on 17 February 1994. Upon receipt at Springborn, the test material was stored at room temperature (approximately 20 °C) in a dark ventilated cabinet. Test concentrations were adjusted for purity of the test material and are reported as milligrams of active ingredient per liter (mg/L). The following information describes the test material:

---

Empirical Formula:	not available
Chemical Name:	not available
Physical Appearance:	clear liquid
Lot No.:	20944-178 (TANK TM 996)
CAS Registry No.:	68951-67-7
Purity:	100% (Appendix II)
Molecular Weight:	777 g/mole (average)
Water Solubility:	complete, may form gel
Vapor Pressure:	< 0.1 mm Hg at 75 °F

### 2.3 Test Organisms

The *Daphnia magna* used in this toxicity test were obtained from laboratory cultures maintained at Springborn. The culture water was prepared by fortifying well water based on the formula for hard water (ASTM, 1980) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. This water had total hardness and alkalinity ranges as calcium carbonate (CaCO<sub>3</sub>) of 160 to 180 mg/L and 110 to 130 mg/L, respectively, a pH range of 7.9 to 8.3, a temperature of 20 ± 2 °C, a dissolved oxygen concentration of greater than 60% of saturation and a specific conductivity range of 400 to 600 micromhos per centimeter (μmhos/cm) (SLI Hard Recon Water Log Book, Vol. 15).

The daphnid culture area received a daily regulated photoperiod of 16 hours of light and 8 hours of darkness. Light at an intensity of 30 to 100 footcandles (320 to 1100 lux) at the surface of the culture solutions was provided by Durotest Vitalite<sup>®</sup> fluorescent bulbs. The ambient air temperature in the culture area was controlled in order to maintain the culture solution temperature at 20 ± 2 °C. Daphnids were fed a combination of a trout food (Ziegler Brothers<sup>®</sup> Salmon Starter #1) suspension and a unicellular green algae (*Ankistrodesmus falcatus*) once daily. The food solution contained 5.0 mg/mL trout food and approximately 4 x 10<sup>7</sup> cells/mL of algae. Routine analyses were conducted on representative samples of the food sources for the presence of metals, pesticides and PCBs (Appendix III). Food sources were considered to be of acceptable quality since the total concentration of pesticides measured was less than 0.3 mg/kg (ASTM, 1985) (SLI Invertebrate culture log for *Daphnia magna*, Vol. 9).

## 2.4 Reference Test

A copper nitrate reference test was conducted with the test organism population from 13 to 15 April 1994. The resulting 48-hour EC50 was calculated by Trim Spearman-Karber analysis to be 45  $\mu\text{g/L}$  (95% confidence interval of 40 to 50  $\mu\text{g/L}$ ) (SLI *Daphnia magna* Copper Nitrate Reference Log, Vol. II). In addition to the above data, culture records document the ability of this population of *Daphnia magna* to actively feed, grow and reproduce over a period of several generations. Based on the results of the reference test and the successful culture of *Daphnia magna*, it was established that this population was suitable for testing.

## 2.5 Test Dilution Water

The dilution water used during this study was from the same source as the culture water described above and had a total hardness and total alkalinity (as  $\text{CaCO}_3$ ) of 180 mg/L and 120 mg/L, respectively, a pH of 8.3 and a specific conductivity of 500  $\mu\text{mhos/cm}$  (SLI IWQ Log Book, Vol. 15). Representative samples of the dilution water source were analyzed for the presence of metals, pesticides and PCBs (Appendix IV). None of these compounds were detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM Standard Practice (ASTM, 1980). In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. Based on these analyses, the TOC concentration of the dilution water source ranged from 0.427 to 0.793 mg/L for the months of March and April 1994 (SLI TOC Master Log). In addition, TOC concentration and total suspended solids (TSS) analyses were conducted at Springborn on the batch of dilution water used during this study. The TOC concentration of the batch of hard reconstituted water, Batch #1764, ranged from 0.851 to 2.29 mg/L for the month of April 1994. The TSS for this batch was less than 2.0 mg/L for the month of April 1994. Several species of daphnids are maintained in water from the same source as the dilution water utilized in this study and have successfully survived and reproduced over several generations. The success of the cultured daphnids, in combination with the previously mentioned analyses, confirms the acceptability of this dilution water for bioassays.

## 2.6 Test Conditions

Test vessels were positioned in stratified random order in a waterbath designed to maintain test solution temperatures at  $20 \pm 2$  °C. Test solutions were not aerated. The test area was illuminated with Dura-Tert Vita Lite and General Electric Coolwhite fluorescent bulbs at an intensity of 646 lux. Light intensity was measured using a General Electric type 214 light meter. The photoperiod during testing was the same as that provided in the daphnid culture area. The culture area received a regulated photoperiod of 16 hours of light and 8 hours of darkness. Sudden transitions from light to dark and vice versa were avoided.

## 2.7 Test Concentrations

Selection of nominal Neodol<sup>®</sup> 45-13 concentrations for the 48-hour definitive toxicity test with *Daphnia magna* was based on toxicity information developed at Springborn through preliminary testing. The nominal concentrations selected were 0.50, 0.85, 1.4, 2.4 and 4.0 mg/L.

## 2.8 Test Procedures

The static renewal toxicity test was conducted in 250-mL glass beakers which contained 200 mL of test solution. The exposure solution in each test vessel had a depth of 6.2 cm and a surface area of 33 cm<sup>2</sup>. Four replicates were maintained for each test concentration and control. A 10 mg/mL stock solution was prepared by heating the glass bottle containing the test material to a temperature ranging from 50 to 60 °C until the test material was completely melted. One gram of test material was then removed using a glass pipet, and was diluted to 100 mL with distilled deionized water in a 100-mL volumetric flask. This stock solution was stirred for several minutes to ensure that the test material completely dissolved in the distilled deionized water. The resultant stock solution was clear and colorless with no sign of undissolved test material.

Test solutions with nominal Neodol<sup>®</sup> 45-13 concentrations of 0.50, 0.85, 1.4, 2.4 and 4.0 mg/L were prepared by diluting the appropriate amount of the stock solution (10 mg/mL) with 2000 mL of dilution water. Two thousand milliliters (2000 mL) of solution were prepared for each concentration in order to accommodate analytical sampling requirements and to provide sufficient volume for water quality analyses. The exposure solutions were stirred for 30 seconds with a

magnetic laboratory stir plate and a Teflon<sup>®</sup>-coated stir bar. The test solutions were observed to be clear and colorless with no sign of undissolved test material. Two hundred milliliters (200 mL) of the appropriate test solution was placed in each of four replicate beakers. Following this procedure, all exposure solutions were observed to be clear and colorless and contained no visible signs of undissolved test material. One set of control vessels was also established which contained the same dilution water and was maintained under the same conditions as the treatment vessels, but contained no NEODOL<sup>®</sup> 45-13. Test solutions were renewed at 24 hours of exposure following the procedure mentioned above. A duplicate set of exposure vessels was established at 24 hours to prepare renewal solutions.

The definitive study was initiated when daphnids were added to each test vessel (5 daphnids per replicate, 20 daphnids per treatment level and control). Daphnids were added to the test vessels within thirty minutes of exposure solution preparation one at a time until all test vessels contained one daphnid. This procedure was repeated until all replicate test vessels contained 5 daphnids. At the renewal period, the daphnids were carefully transferred, one at a time, from the old test solutions into their respective newly prepared test solutions using a wide bore pipet. Daphnids that were observed to be immobilized at the time of renewal were not transferred into the new test solutions. Daphnids were not fed during the study.

## 2.9 Test Monitoring

The number of immobilized daphnids in each replicate test vessel was recorded at 24 and 48 hours of exposure. Biological observations and observations of the physical characteristics of each replicate test solution were also made and recorded at 0, 24 and 48 hours. The pH, dissolved oxygen concentration and temperature were measured in each replicate vessel of all test concentrations and the control at 0, 24 and 48 hours of exposure. Water quality measurements performed at 24 hours were made in both the old and new test solutions when applicable (i.e., less than 100% immobilization at the previous interval). Total hardness, total alkalinity, acidity and specific conductivity were measured at 0-hour in each replicate vessel of the control and treatment solutions. In addition, the temperature of the surrounding water in the

waterbath used to control the exposure solution temperature was continuously monitored throughout the exposure period using a minimum/maximum thermometer.

Total hardness concentration presented in this report was measured by the EDTA titrimetric method and total alkalinity concentration was determined by potentiometric titration to an endpoint of pH 4.5 (APHA *et al.*, 1985). Acidity was determined by potentiometric titration to an endpoint of pH 8.3. Specific conductivity was measured with a Yellow Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe. A Jenco Model 601A pH meter and combination electrode was used to measure pH. Dissolved oxygen concentration was measured with a YSI Model #57 dissolved oxygen meter and probe. Daily temperature was measured with a Fisher Scientific thermometer. Continuous temperature monitoring was performed using a Fisher Scientific Min/Max thermometer.

## **2.10 Analytical Measurements**

During the definitive exposure period, water samples were removed from the four replicate solutions of each treatment level and the control at 0, 24 and 48 hours for the analysis of Neodol® 45-13 concentration. Samples analyzed at 0 hour were removed from the excess test solution remaining in the volumetric flasks prior to division into the replicate test vessels. At the 24-hour interval, both old and new test solutions were analyzed for Neodol® 45-13 concentration. Samples removed from the old solutions were composited (replicates A, B, C and D) for each treatment level and the control. New solution samples were removed from excess test solution remaining in the volumetric flasks prior to division into the replicate test vessels. Samples analyzed at 48 hours were removed from the composited test solution (replicates A, B, C and D) for each treatment level and the control after biological observations and water quality measurements were taken. Each exposure solution sample was collected from the approximate midpoint of the test vessel with a volumetric pipet. Samples were collected in 700-mL borosilicate glass containers with Teflon®-lined caps. Containers were completely filled to minimize head space and were preserved with 1.0% formalin. Within 24 hours of preparation, the samples were shipped to Battelle Ocean Sciences, Duxbury, Massachusetts, for analysis using the Shell Development Company analytical method entitled "Analysis of Alcohol Ethoxylate Surfactants

Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions". This method is described in Battelle Ocean Sciences Study #SD-930118 (Appendix V). All of the glassware used in testing and sample collection was thoroughly washed with detergent and rinsed with tap water, and then washed with sequential rinses of a 10% solution of nitric acid, acetone, distilled-deionized water, isopropanol and distilled-deionized water.

### **2.11 Determination of EC50 and NOEC Values**

The mean measured concentrations tested and the corresponding biological-response data (immobilization) derived from the toxicity test were used to estimate 24- and 48-hour median effect concentrations (EC50) and 95% confidence intervals. The EC50 is defined as the concentration of the test material in dilution water which caused immobilization of 50% of the test organism population at the stated time interval. If at least one test concentration caused immobilization of greater than or equal to 50% of the test population, then a computer program, modified from the program of C. Stephan (Peltier *et al*, 1985), was used to calculate the EC50 values and 95% confidence intervals.

Three statistical methods were available in the computer program: moving average angle analysis, probit analysis, and nonlinear interpolation with 95% confidence intervals calculated by binomial probability. Moving average angle and probit analyses yield statistically sound results only if at least two concentrations produce immobilization of between 0 and 100% in the test population. The selection of reported EC50 values and 95% confidence intervals was based upon an examination of the database and the results of the computer analysis. Selection criteria included the establishment of a concentration-effect relationship, the number of concentrations causing partial responses, and the span of responses bracketing the EC50 value. If two or more statistical methods produced acceptable results, then the method which yielded the smallest 95% confidence interval was selected. The No-Observed-Effect Concentration (NOEC) during the 48-hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there were no toxicant-related immobilization or physical and behavioral abnormalities (e.g., lethargy, flared carapace), with respect to the control.

### 3.0 RESULTS

#### 3.1 Preliminary Test

Prior to initiating the definitive study, a preliminary range-finding test was conducted at Springborn from 2 to 4 March 1994 at nominal concentrations of 0.10, 0.40, 1.0 and 4.0 mg/L. At 24 hours of exposure, 80% immobilization was observed among organisms exposed to the highest test concentration (4.0 mg/L). In addition, two organisms at this exposure level exhibited erratic swimming behavior on bottom of vessel. After 48 hours exposure, all organisms at the 4.0 mg/L treatment were immobilized. At test termination, no immobilization was observed in the remaining lower test concentrations (i.e.,  $\leq 1.0$  mg/L). Based on the results of this range-finding test, nominal concentrations of 0.50, 0.85, 1.4, 2.4 and 4.0 mg/L were selected for the definitive study with Neodol<sup>®</sup> 45-13.

#### 3.2 Definitive Test

**3.2.1 Evaluation of Test Conditions** - The measurements of the water quality parameters (i.e., pH, dissolved oxygen concentration, temperature) recorded during the definitive study are presented in Table 1. Total hardness, total alkalinity, total acidity and specific conductivity measured at 0-hour of the definitive study are presented in Table 2. Throughout the exposure period, the pH and dissolved oxygen saturation for the control and test solutions ranged from 8.0 to 8.3 and 8.1 to 8.9 mg/L, respectively. Analysis of the control and test solutions at test initiation established a total hardness (as CaCO<sub>3</sub>) ranging from 172 to 180 mg/L, a total alkalinity (as CaCO<sub>3</sub>) range of 116 - 122 mg/L, a specific conductivity of 500  $\mu$ mhos/cm and an acidity (as CaCO<sub>3</sub>) of 2 mg/L. These results demonstrate that the water quality parameters measured were unaffected by the concentrations of Neodol<sup>®</sup> 45-13 tested and remained within acceptable ranges for the survival of daphnids. Daily temperature monitoring of the test solutions and continuous temperature monitoring of the water in the surrounding waterbath established that the temperature in the test solutions remained at 20 °C throughout the exposure period.

**3.2.2 Analytical Results** - The results of the analyses of the primary stock solutions and the exposure solutions for Neodol<sup>®</sup> 45-13 concentration during the exposure period are

presented in Table 3. Results of the analyses of the primary stock solutions (10 mg/mL) used to formulate the test solutions established an average concentration of Neodol® 45-13 of 99% of the nominal concentration. Measured concentrations for all treatment levels were generally consistent between sampling intervals, and established that the expected concentration gradient was maintained in both the old and newly prepared solutions. These results demonstrate that the test system maintained consistent exposure conditions throughout the 48-hour study. Mean measured concentrations for the lowest treatment level (0.32 mg/L) averaged 65% of nominal while the remaining treatment levels tested ranged from 77 - 90% of nominal. The test concentrations were defined as 0.32, 0.65, 1.2, 1.9 and 3.6 mg/L. Analytical results are presented in Battelle Ocean Sciences Study #SD-930118 (Appendix V).

**3.2.3 Biological Results** - The mean measured concentrations tested, (based on 0, 24 old and new solutions and 48 hour analysis) the corresponding cumulative percent and number of immobilizations and the observations made during the definitive exposure are presented in Table 4. Throughout the 48-hour test, all exposure solutions were observed to be clear and colorless and contained no visible signs of undissolved test material. Following 24 hours of exposure, 90% immobilization was observed among daphnids exposed to the highest concentration tested (3.6 mg/L). In addition, several of the mobile daphnids exposed to this treatment level were observed to be lethargic or erratically swimming at the bottom of the test solution. Several daphnids exposed to 1.9 mg/L of Neodol® 45-13 were observed to be lethargic. At test termination (48 hours of exposure), immobilization of 100% was observed among daphnids exposed to 1.9 and 3.6 mg/L Neodol® 45-13. Immobilization of 40% was observed among daphnids exposed to the 1.2 mg/L test concentration. In addition, several daphnids exposed to 1.2 mg/L Neodol® 45-13 were observed to be lethargic. No immobilization or sublethal effects were observed among daphnids exposed to the remaining lower test concentrations (i.e., 0.65 and 0.32 mg/L). The 24-hour and 48-hour concentration-response (immobilization) curves for this study are presented in Figures 1 and 2, respectively.

Table 5 summarizes the 24- and 48-hour EC50 values and corresponding 95% confidence intervals, and presents the No-Observed-Effect Concentration (NOEC) through 48 hours. Based

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on mean measured concentrations of Neodol® 45-13, the 48-hour EC50 value was calculated by nonlinear interpolation to be 1.2 mg/L with a corresponding 95% confidence interval calculated by binomial probability of 0.65 to 1.9 mg/L. The NOEC established for this study was 0.65 mg/L.

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**PROTOCOL DEVIATION**

The study protocol states that the water samples will be collected in 500-mL glass containers which are completely filled to minimize headspace. During this study, water samples (approximately 700 mL volume) were collected in 700-mL glass containers which were completely filled to minimize headspace. The increase in sample volume was necessary to avoid headspace within the sampling container.

It is our opinion that this deviation did not affect the results of this study.

SPRINGBORN LABORATORIES, INC.

Maura K. Collins      8/29/94

Maura K. Collins  
Study Director

Date

### QUALITY ASSURANCE UNIT STATEMENT

The raw data and report for "NEODOL® 45-13 - Acute Toxicity To Daphnids (*Daphnia magna*) Under Static Renewal Conditions" were inspected by the Quality Assurance Unit (QAU) at Springborn Laboratories Inc., Environmental Sciences Division to determine adherence with the study protocol and laboratory standard operating procedures. In addition, inspection of certain phases of the in-life portion of the study was performed. Dates of study inspections, dates reported to the Study Director and to Management are listed below.

Based on these inspections, it was determined that this report accurately reflects the raw data collected during this study.

<u>Inspection Date</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
4/8/94	4/8/94	4/8/94
4/19/94	4/19/94	4/22/94
4/27/94	4/27/94	5/6/94
7/22 - 26/94	7/26/94	7/29/94
8/2/94	8/2/94	8/12/94
8/12/94	8/12/94	8/12/94

SPRINGBORN LABORATORIES, INC.

  
Doreen S. Newhouse      12 Aug 94  
 Doreen S. Newhouse      Date  
 Supervisor, Quality Assurance Unit

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**TABLES**

**Table 1. The pH, dissolved oxygen concentration and temperature measurements recorded during the 48-hour static exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.**

Nominal Concentration (mg/L)	0-Hour				24-Hour <sup>a</sup>				48-Hour			
	A	B	C	D	A	B	C	D	A	B	C	D
	pH											
Control	8.1	8.1	8.1	8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.2	8.3	8.3	8.3
0.50	8.1	8.1	8.1	8.1	8.0/8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.2	8.2	8.2	8.2
0.85	8.1	8.1	8.1	8.1	8.1/8.1	8.1/8.1	8.1/8.0	8.1/8.1	8.2	8.2	8.2	8.2
1.4	8.1	8.1	8.1	8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.0/8.1	8.3	8.0	8.3	8.3
2.4	8.1	8.1	8.1	8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.1/8.1	8.3	8.3	8.3	8.2
4.0	8.1	8.1	8.1	8.1	8.1/8.1	8.0/8.0	8.0/8.0	8.1/8.1	8.3	8.3	8.3	8.3
	Dissolved Oxygen, mg/L (% saturation)											
Control	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.1/8.6 (89/94)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.3 (91)	8.3 (91)	8.4 (92)	8.3 (91)
0.50	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.2/8.7 (90/95)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.4 (92)	8.4 (92)	8.4 (92)	8.3 (91)
0.85	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.2/8.6 (90/94)	8.2/8.7 (90/95)	8.3/8.6 (91/94)	8.2/8.6 (90/94)	8.3 (91)	8.3 (91)	8.4 (92)	8.4 (92)
1.4	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.1/8.6 (89/94)	8.2/8.6 (90/94)	8.3 (91)	8.3 (91)	8.3 (91)	8.3 (91)
2.4	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.3/8.6 (91/94)	8.2/8.7 (90/95)	8.2/8.6 (90/94)	8.2/8.7 (90/95)	8.4 (92)	8.3 (91)	8.3 (91)	8.4 (92)
4.0	8.9 (98)	8.9 (98)	8.9 (98)	8.9 (98)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.2/8.6 (90/94)	8.3 (91)	8.3 (91)	8.4 (92)	8.3 (91)
	Temperature (°C) <sup>b</sup>											
	20				20/20				20			

<sup>a</sup> Exposure solutions were renewed at the 24-hour interval. Measurements are presented as Old/New.  
<sup>b</sup> Values presented represent the daily temperatures measured (Fisher Scientific thermometer) in all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific Min-Max thermometer) of the surrounding water in the waterbath established a temperature of 20 °C throughout the exposure period.

**Table 2. Total hardness, total alkalinity, specific conductivity and acidity measured at 0-hour in the test solutions during the 48-hour static exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.**

Nominal Concentration (mg/L)		Total Hardness (mg/L as CaCO <sub>3</sub> )	Total Alkalinity (mg/L as CaCO <sub>3</sub> )	Specific Conductivity (μmhos/cm)	Acidity (mg/L as CaCO <sub>3</sub> )
Control	A	176	116	500	2
	B	176	120	500	2
	C	172	118	500	2
	D	176	120	500	2
0.50	A	180	118	500	2
	B	180	120	500	2
	C	180	118	500	2
	D	176	118	500	2
0.85	A	176	124	500	2
	B	180	122	500	2
	C	180	124	500	2
	D	180	124	500	2
1.4	A	176	122	500	2
	B	176	122	500	2
	C	180	120	500	2
	D	180	122	500	2
2.4	A	176	120	500	2
	B	176	122	500	2
	C	176	120	500	2
	D	176	120	500	2
4.0	A	176	118	500	2
	B	180	120	500	2
	C	176	120	500	2
	D	180	120	500	2

**Table 3. Concentrations of Neodol<sup>®</sup> 45-13 measured in the replicate test solutions during the 48-hour static renewal exposure of daphnids (*Daphnia magna*).**

Nominal Concentration (mg/L)	0-Hour Measured Concentration (mg/L)	24-Hour Measured Concentration (mg/L) <sup>a</sup>	48-Hour Measured Concentration (mg/L)	Mean Measured Concentration (mg/L) <sup>bc</sup>	% Nominal <sup>f</sup>
Control	ND <sup>d</sup>	ND/ND	ND	NA <sup>e</sup>	NA
0.50	0.34	0.27/0.37	0.32	0.32 (0.041)	65%
0.85	0.67	0.54/0.76	0.64	0.65 (0.088)	77%
1.4	1.2	1.1/1.2	1.2	1.2 (0.080)	84%
2.4	1.9	1.8/1.8	2.0	1.9 (0.098)	78%
4.0	3.8	3.3/3.6	3.7	3.6 (0.22)	90%
stock (10,000)	9927	9936	NA	NA	NA

Mean % Nominal : 79%

<sup>a</sup> Exposure solutions were renewed at the 24-hour interval. Measurements are presented as Old/New solutions.

<sup>b</sup> Mean measured concentrations were calculated using the actual unrounded analytical results (0, 24 and new solutions, 48-hour analysis) and not the rounded (two significant figures) values presented in this table.

<sup>c</sup> Standard deviation is presented in parentheses.

<sup>d</sup> ND = Not detectable; below the limit of detection.

<sup>e</sup> NA = Not Applicable

<sup>f</sup> Calculations were performed using the actual unrounded analytical data and not the rounded values presented in this column.

**Table 4. Mean measured concentrations tested, corresponding cumulative percent and number of immobilized organisms and observations made during the 48-hour static acute exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.**

Mean Measured Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>									
	24-Hour					48-Hour				
	A	B	C	D	Mean	A	B	C	D	Mean
Control	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.32	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.65	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
1.2	0 <sup>b</sup> (0)	0 (0)	0 <sup>c</sup> (0)	0 <sup>b</sup> (0)	0	60 <sup>c</sup> (3)	0 <sup>c</sup> (0)	60 <sup>c</sup> (3)	40 <sup>d</sup> (2)	40
1.9	40 <sup>d</sup> (2)	60 <sup>c</sup> (3)	40 <sup>d</sup> (2)	20 <sup>d</sup> (1)	40	100 (5)	100 (5)	100 (5)	100 (5)	100
3.6	100 (5)	100 (5)	80 <sup>e</sup> (4)	80 <sup>b</sup> (4)	90	100 (5)	100 (5)	100 (5)	100 (5)	100

<sup>a</sup> The actual number of immobilized daphnids is presented in parentheses.

<sup>b</sup> One of the mobile daphnids was lethargic.

<sup>c</sup> Two of the mobile daphnids were lethargic.

<sup>d</sup> Several of the mobile daphnids were lethargic.

<sup>e</sup> One of the mobile daphnids exhibited erratic swimming behavior and was on the bottom of the test vessel.

**Table 5. The EC50 values, corresponding 95% confidence intervals and No Observed Effect Concentration (NOEC) established during the 48-hour static renewal exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.**

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	EC50 (mg/L)	95% Confidence Interval	
		Lower (mg/L)	Upper (mg/L)
24-Hour <sup>a</sup>	2.2	1.9	2.6
48-Hour <sup>b</sup>	1.2	0.65	1.9

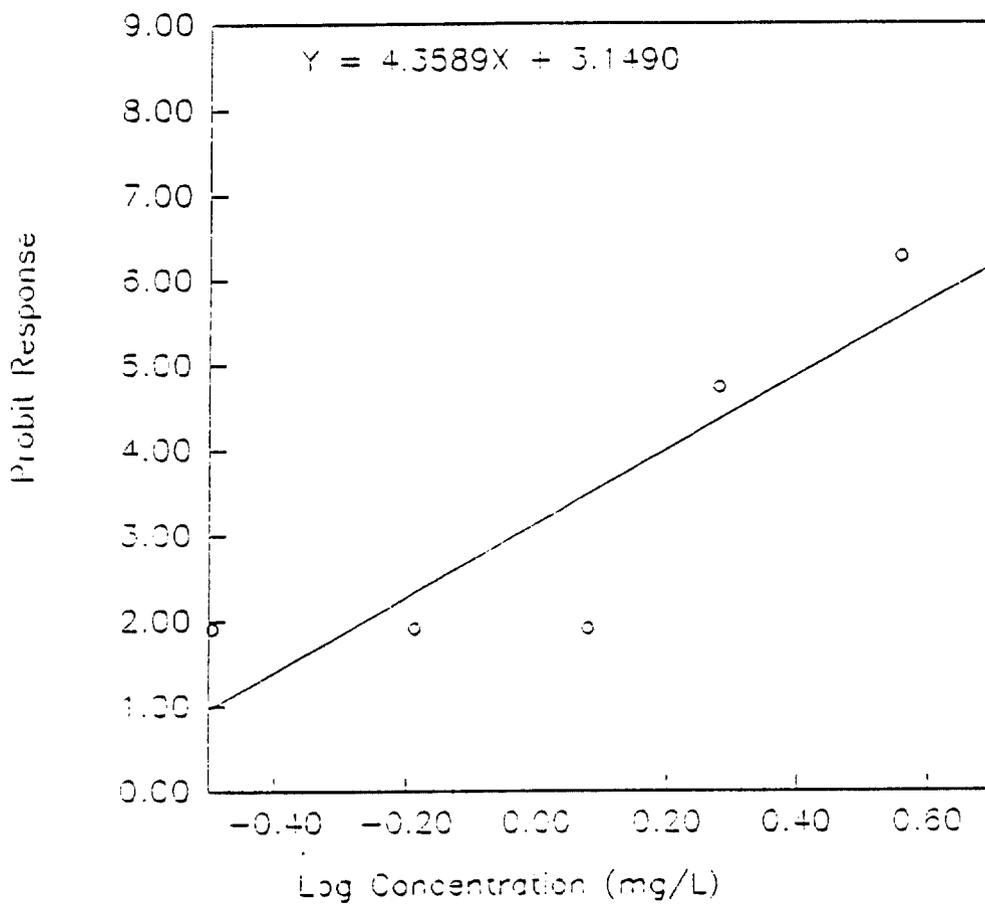
---

**NOEC through 48 hours = 0.65 mg/L**

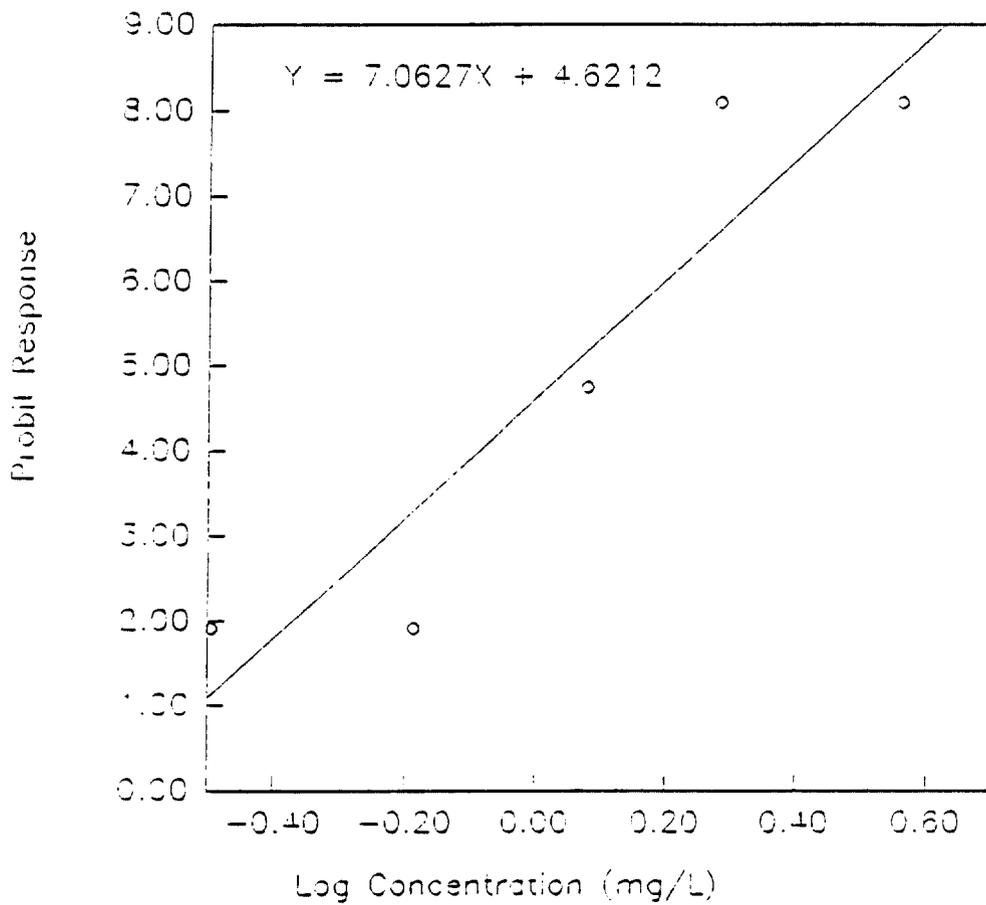
- 
- <sup>a</sup> EC50 value and the corresponding 95% confidence interval were calculated by probit analysis.  
<sup>b</sup> EC50 value calculated by nonlinear interpolation; 95% confidence interval calculated by binomial probability.

**FIGURES**

**Figure 1.** The 24-hour concentration-response (immobilization) curve for the static renewal exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.



**Figure 2.** The 48-hour concentration-response (immobilization) curve for the static renewal exposure of daphnids (*Daphnia magna*) to Neodol<sup>®</sup> 45-13.



**SIGNATURES AND APPROVAL**

**SUBMITTED BY:**

Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street  
Wareham, Massachusetts 02571-1075

**PREPARED BY:**

Maura K. Collins 8/12/94  
Maura K. Collins Date  
Study Director

James J. O'Brien 8/12/94  
James J. O'Brien Date  
Principal Investigator

Susan P. Shepherd 12 Aug 94  
Susan P. Shepherd Date  
Coordinator, Data Management  
and Reporting Unit

**APPROVED BY:**

Donald C. Surprenant 8/12/94  
Donald C. Surprenant Date  
Program Manager  
Environmental Toxicology

Doreen S. Newhouse 17 Aug 94  
Doreen S. Newhouse Date  
Supervisor, Quality Assurance Unit

This final report has been signed in accordance with SLI SOP No. 4.3.07(1).

#### 4.0 APPENDIX I - STUDY PROTOCOL



Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with *Daphnia magna*, Following TSCA Test Guidelines § 797.1300

## 1.0 INTRODUCTION

The purpose of this test is to determine the acute effects of an alcohol ethoxylate surfactant on the water flea, *Daphnia magna*, under static renewal conditions. Test results will be reported as 24- and 48-h EC50 values, (the median concentration that will immobilize 50% of the number of daphnids exposed) with 95% confidence limits. The No-Observed-Effect Concentration (NOEC) will also be reported. The test procedures performed during the biological portions of this study will meet or exceed the standard procedures described in the U.S. EPA Toxic Substances Control Act (TSCA) Test Guidelines § 797.1300 (U.S. EPA, 1985) and will meet the primary technical objectives of the Shell Research Limited/Sittingbourne Research Centre guidelines (S&T SOP No. 167, Edition No. 3).

## 2.0 MATERIALS AND METHODS

### 2.1 TEST ORGANISMS.

- 2.1.1. Justification for Test System. Characteristics which make this test organism suitable for acute toxicity testing are their ease of culturing and handling, their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater invertebrate species.
- 2.1.2. Species. The daphnid crustacean (water flea), *Daphnia magna*, will be the species used in this test. Test organisms will be  $\leq$  24 hours old at the initiation of the test. Daphnids will be obtained by removing all immature daphnids from the culture vessel, thus isolating sexually mature daphnids 24 hours prior to initiating the test. All organisms will originate from the same culture population. Young produced by these organisms will be subsequently pipetted into the test beakers. Young for testing will not be taken from cultures where adults contain eponidia.
- 2.1.3. Origin and Acclimation. *D. magna* will be obtained from cultures maintained at Springborn Laboratories, Inc. Daphnids will be cultured in 2-L glass vessels containing 1 L of water. Water used to culture the daphnids will be prepared in the same manner and will have the same characteristics as described for dilution water. Culture water will be maintained at the required test temperature ( $20 \pm 2^\circ\text{C}$ ). Each culture vessel will be cleaned once weekly. Young will not be used if more than 20% of the culture stock die within the 48 hours preceding the test.
- 2.1.4. Feeding. While being maintained in culture prior to the test, organisms will be fed daily a combination of a trout food (Ziegler Brothers<sup>®</sup> Salmon Starter #1) suspension and a unicellular green algae, *Ankistrodesmus falcatus*. The food solution will be prepared to

contain 5 mg/mL trout food and approximately  $4 \times 10^7$  cells/mL of algae. An aliquot of 0.5 mL of the trout food suspension and 2 mL of algae will be manually introduced to each culture vessel once daily. Daphnids will not be fed during the 48-hour exposure period. Periodic analyses of representative samples of the food will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the daphnids.

- 2.1.5. **Handling.** Wide-orifice pipets will be used to transfer the daphnids, taking care to minimize possible stress due to handling. Daphnids that are damaged or dropped during transfer will not be used.
- 2.1.6. **Reference Tests.** In an effort to monitor the general health of the test organism culture, reference tests will be conducted, under static conditions, with *Daphnia magna* using copper nitrate as the reference toxicant. The results of these tests will be evaluated based on nominal concentrations. The reference tests will be conducted using young from the same culture population within 30 days of the definitive exposure (i.e., either 30 days prior to or 30 days following the definitive exposure).

## 2.2 PHYSICAL SYSTEM.

- 2.2.1. **Test Containers.** The test chambers used in the static acute bioassay will be 250-mL glass beakers which are chemically clean. Each beaker will contain 200 mL of the test solution or the control dilution water. The test vessels will be loosely covered.
- 2.2.2. **Glassware Preparation.** All glassware used in testing will be thoroughly washed with detergent and rinsed with tap water. This will be followed by sequential rinsing with a 10% solution of nitric acid, acetone, distilled deionized water, isopropanol and finally distilled deionized water.
- 2.2.3. **Dilution Water.** Dilution water will consist of hard fortified well water with a total hardness of 160 to 180 mg/L as  $\text{CaCO}_3$ . Hard water will be used since *D. magna* are generally found in hard water habitats in the natural environment. The well water (total hardness about 30 mg/L as  $\text{CaCO}_3$ ) 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 amcorite XAC-7 resin column (30 cm long and 1.5 cm wide) and an activated carbon bed. This filtration will effectively remove any potential organic contaminants from the water.

Quality of the dilution water used to conduct daphnid acute tests will be judged by the ability of the daphnid cultures to survive and reproduce in the water free of stress. The dilution water will be prepared in 1,900-L batches. New batches of dilution water will be prepared when the previous batch is exhausted, when a water quality parameter (total hardness, alkalinity, etc.) has varied from the normal range, or after two weeks of holding. The dilution 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 dilution water. The total hardness, total alkalinity, acidity, pH, TOC, TSS and specific conductance of the dilution water will be monitored prior to use. Total organic carbon (TOC) will be monitored approximately once per month. Periodic analyses of representative samples of dilution water source will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs, unionized ammonia, residual chlorine and selected toxic metals, at concentrations which may be harmful to the daphnids.

- 2.2.4. **Replication and Control of Bias.** Four replicates will be included with each test concentration and control. Test beakers will be labeled by replicate letter and concentration (or control), and will be positioned in stratified random order. The daphnids will be impartially added to the test vessels by adding one daphnid to the first beaker, one to the next beaker and so forth until each beaker contains one test organism. This procedure will be repeated until each beaker contains five daphnids. Test organisms will be added to the exposure solutions within 30 minutes of preparation.

### 2.3 CHEMICAL SYSTEM.

- 2.3.1. **Test Material.** Upon arrival at Springborn Laboratories, Inc., the external packaging of the test material will be inspected for damage. The packaging will be removed and the primary storage container will also be inspected for leakage or damage. The sample identity will be recorded and the material will be stored in the dark at approximately 20°C until used, unless specified differently by the test Sponsor.
- 2.3.2. **Toxicant Concentration Selection.** Toxicant concentrations for the acute toxicity test will be selected based on information provided by the Sponsor and obtained from a 48-hour preliminary range-finding study exposing *D. magna* to the test material. The range-finding study will consist of five widely-spaced concentrations, usually of 1.0-L volume, each containing ten daphnids. The range of concentrations selected for the definitive test is intended to include both 100% effect and no-effect levels, but due to the nature of some compounds, one or both levels may not be observed. Five concentrations and one control will be used for each definitive test, each concentration consisting of twenty test daphnids after consultation with the Sponsor. A dilution ratio of 1.5 to 2.0 will be used.
- 2.3.3. **Stock Preparation.** The surfactant will be heated in a glass container at a temperature of 50 - 60°C until completely melted. The melted test material will be stirred to ensure homogeneity. A glass pipet will be used to transfer the material for weighing. The test material will be weighed on an analytical balance for which a calibration log is maintained. The stock solution will be mixed for several minutes. A Chemical Usage Log will also be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test material is used. The primary stock will be prepared in distilled deionized water. The final concentration of the primary stock solution will be 1 - 2% active surfactant. A new primary stock solution will be prepared for each renewal of test solutions. Secondary stocks, if necessary, may be prepared in either distilled deionized water or in dilution water (if the volume displacement of dilution water during preparation of the test solutions will be greater than 1%).

- 2.3.4. **Measurement of Exposure Solution and Stock Solution Concentration.** Samples from each test chamber of each concentration and control(s) will be removed at test initiation (new solutions), midterm (old and new solutions) and test termination (old solutions). Samples at test initiation (new) will be taken by removing the appropriate aliquot from each test solution prior to splitting the solution into replicate chambers. Samples of the aged replicate solutions at each test concentration will be pooled. All primary stock solutions prepared during the test will also be sampled.
- 2.3.5. **Sampling.** Water samples (approximately 500-mL) will be taken from a point approximately midway between the surface, bottom and sides of each vessel. All samples will be collected in 700 mL borosilicate glass containers with Teflon<sup>®</sup>-lined caps which have been serially rinsed with deionized water, isopropanol and deionized water as specified in Section 2.2.2. Samples will be preserved with 1% formalin (i.e., 5 mL formalin/500 mL sample) and shipped within 24 hours to Battelle Ocean Sciences, Duxbury, Massachusetts.
- 2.3.6. **Analytical Chemistry.** Analyses of analytical samples will be conducted by Battelle Ocean Sciences, Duxbury, Massachusetts, using a Shell Development Company analytical method entitled "Analysis of Alconol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) and HPLC/ELSD (Evaporative Light Scattering Detection) in Dilute Aqueous Solutions".

#### 2.4 EXPERIMENTAL CONDITIONS.

- 2.4.1. **Measurement of Water Quality Variables.** At test initiation, total hardness, alkalinity, acidity and specific conductance will be measured in each replicate vessel of each test concentration and control. Temperature, pH and dissolved oxygen will be recorded daily in each replicate of each concentration and control. Measurements will be recorded for the aged and for the freshly prepared solutions on renewal days.
- 2.4.2. **Photoperiod.** The tests will be illuminated at a light intensity of 30 - 100 footcandles using a combination of fluorescent bulbs. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer. There will be a transition period between light and dark.
- 2.4.3. **Dissolved Oxygen.** Total dissolved oxygen will not be allowed to drop below 60% or exceed 105% of saturation for the duration of the test. Should the dissolved oxygen fall below 60% of saturation, appropriate action will be taken after consultation with the Study Sponsor.
- 2.4.4. **Temperature.** Water temperature of the test solutions will be maintained at 20 ± 2°C by conducting the test in a waterbath.
- 2.4.5. **pH.** The pH of the control solutions will be maintained in a range of 6.0 to 8.5.

- 2.4.6. **Biological Data.** The number of immobilized daphnids in each test vessel will be recorded after 24 and 48 hours of test initiation. Immobilization is defined as the lack of movement by the test organisms except for minor activity of the appendages. In addition, prior to test initiation and whenever test organisms are observed, characteristics of the test solutions will also be observed and recorded, e.g., precipitated materials, cloudiness, etc.
- 2.4.7. **Renewal Scheme.** Test solutions will be prepared at 0 and 24 hours of exposure. Daphnids will be carefully transferred to the freshly prepared solutions using a wide-bore pipet.
- 2.4.8. **Initiation and Test Duration.** The study will be initiated when all test organisms have been impartially added to the exposure solutions. The study will be terminated following 48 hours of exposure at which time mortality of the control organisms will not exceed 10% or the test will be considered unacceptable.

If 100% immobilization occurs at any exposure level prior to test termination, water quality parameters, analytical samples and biological observations will only be recorded on the day which complete immobilization was observed. Observations following the day on which complete immobilization was observed. Observations following the day on which complete immobilization was observed will be discontinued.

### 3.0 DATA EVALUATION

Test results derived from the acute test will be used to statistically estimate a median effective concentration (EC50) and its 95% confidence interval after 24 and 48 hours of exposure. The EC50 is the estimated mean measured concentration of the test material in dilution water which produces 50% immobility in the test populations of daphnids at the stated times of exposure.

The computer program utilized estimates EC50 values using three statistical methods: probit analysis, moving average method, and binomial probability. The method selected and reported will be determined by the data base (i.e., presence or absence of 100% response, number of partial responses, etc.). An EC50 value cannot be calculated if the data derived is insufficient according to any of the three statistical methods. The probit method provides values of the slope, including 95% confidence intervals, as well as appropriate statistical tests to evaluate goodness-of-fit.

Following 48 hours of exposure, data obtained on organism survival will be evaluated to establish the No-Observed-Effect Concentration (NOEC). This level is defined as the highest test concentration at and below which there were no toxicant-related mortalities or physical and behavioral abnormalities (e.g., lethargy).

The concentration-response data generated during this test will be provided to the Study Sponsor in Lotus<sup>®</sup> format.

#### 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 at Springborn Laboratories as a result of the study.

#### 5.0 REPORTING

The raw data generated at Springborn Laboratories and final draft of the report will be reviewed by the Quality Assurance Unit and Study Director. All measurements (e.g. water quality) will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. A single copy of the draft report will initially be submitted to the study Sponsor for review. Upon acceptance by the Sponsor, three (3) copies of the final report will be submitted. All reports include, but will not be limited to, the following information:

- Springborn Laboratories, Inc., report and project numbers.
- Laboratory and site, the dates of testing and personnel involved in the study, e.g., Quality Assurance Unit, Program Coordinator (if applicable), Study Director, Principal Investigator.
- All information pertaining to the test material which appears on the sample bottle, e.g., its source, percent active ingredient, physical properties, Sponsor's test material I.D., and sample number (if applicable).
- Characterization and origin of the dilution water.
- Scientific name of the test organisms, source, and culturing information.
- Range-finding study results.
- The 48-hour EC50 value with the 95% confidence limits and control performance of applicable copper nitrate reference test.
- Test container volume, dilution water volume, number of replicates used per concentration, and number of daphnids used per treatment.
- Definition of criteria used to determine the sublethal effects, and general observations on non-quantifiable effects.
- Description of exposure system and stock preparation.
- Test temperatures, dissolved oxygen concentration, and pH; photoperiod and light intensity; and specific conductance, total alkalinity and total hardness measured.

- Description of, or reference to, chemical and statistical procedures applied.
- Percentage of daphnids that were immobilized in the controls and in each treatment at each observation period, in tabular form.
- The 24- and 48-hour EC50's with 95 percent confidence limits, and the No-Observed-Effect Concentration (NCEC), when possible. All calculations will be based on mean measured concentrations.
- Graph of the concentration-response curve at each observation period for which an EC50 is calculated. Mean measured concentrations will be used to establish the concentration-response curve.
- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study, signed by the Study Director.
- Good Laboratory Practice (GLP) compliance statement (for the biological portion of this study) signed by the Study Director.
- Dates of Quality Assurance reviews, signed by the QA 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 impact of the change on results of the study, if any. If necessary, amendments initially may be in the form of verbal authorization, followed by Springborn's written documentation of the amendment. In such a case, the effective date of the amendment will be the date of verbal authorization.

#### 7.0 SPECIAL PROVISIONS

**GOOD LABORATORY PRACTICES (GLP):** All test procedures, documentation, records, and reports related to the biological portion of this study will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substance Control Act: (FEDERAL REGISTER, Part IV, 17 August, 1989)

**TEST MATERIAL DISPOSAL:** After 60 days of the issuance of the final test report, the test material will be returned to the Sponsor's project officer, at Sponsor expense, unless different arrangements are made.

**ARCHIVAL:** All raw data and the final report will be archived at Shell Development Company unless different arrangements are made.

### 8.0 REFERENCES

- APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, Washington, DC. 2168 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. Environmental Protection Agency. 1989. *Toxic Substances Control; Good Laboratory Practice Standards; Final Rule*. (40 CFR, Part 792) Federal Register, Part III, 48(230):53922-53944, August 17, 1989.
- U.S. Environmental Protection Agency. 1985. *Toxic Substances Control Act Test Guidelines*. Federal Register 50(188):39252-39516, September 27, 1985. Amended May 20, 1987, July 1, 1991 and July 1, 1992.

**Springborn Laboratories, Inc.**

Environmental Sciences Division

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**PROTOCOL AMENDMENT**

AMENDMENT #: 1

DATE: 5 April 1994

PROTOCOL TITLE: "Alcohol Ethoxylate Surfactants: Protocol for Conducting an Acute Toxicity Test, Under Static Renewal Conditions, with *Daphnia magna*, Following TSCA Test Guidelines §797.1300."

SPECIES: *Daphnia magna*

STUDY SPONSOR: Shell Development Company

TEST SUBSTANCE: Neodol® 45-13

SLI STUDY No.: 777.0294.6110.110

SPONSOR PROTOCOL PROJECT NO.: WRC Tox No. 1209

AMENDMENT(S):

Amendment (Page 1):

Test Concentrations: 4.0, 2.4, 1.4, 0.35 and 0.50 mg A.L./L plus controls.

Solvent Used: NA

CAS# or LOT#: NA

Proposed Schedule:

(Start) 4-5-94

(Completion) 4-7-94

(Draft Report) 5-3-94

Reason for Change:

This information is provided per instruction on page one of the Study Protocol.

Amendment (Section 2.4.1):

The study protocol states that total hardness, alkalinity, acidity and specific conductance will be measured in each replicate vessel of each test concentration and control at test initiation.

Springborn Laboratories Protocol #: 021-494-TSCA/SHELL DM-SR

Page 1 of 2  
 Springborn

Springborn Laboratories, Inc. warrants that the information contained herein was prepared by its employees or agents and that the information is true and correct to the best of its knowledge and belief. It is understood that the information is provided for informational purposes only and is not intended to constitute an offer of insurance or any other financial product. The information is not to be used for any other purpose without the express written consent of Springborn Laboratories, Inc.

Amended. total hardness, alkalinity, acidity and specific conductance will be measured in an extra set of replicate solutions (identified as A., B., C., D.) of each test concentration and control at test initiation. The extra set of replicate exposure solutions will be established with the same exposure solution prepared for the biological exposure.

**Reason for Change:**

The additional set of exposure solutions will allow for the measurement of total hardness, alkalinity, acidity and specific conductance in replicate solutions while maintaining consistent volumes of test solution in vessels containing test organisms.

Approval Signatures:

Maura K Collins 4-5-94  
Maura K. Collins Date  
SLL Study Director

Diane C. L. Wong 4/13/94  
Diane C. L. Wong Date  
Sponsor Study Monitor

**5.0 APPENDIX II - CERTIFICATE OF ANALYSIS**

## Shell Development Company

A Division of Shell Oil Company

Westhollow Research Center  
P.O. Box 1080  
Houston, TX 77251-1080

February 24, 1994

Pameia M. Lincoln  
Springborn Laboratories, Inc.  
790 Main Street  
Wareham, MA 02571

Dear Ms. Lincoln:

The missing information, pertaining to test substance NEODOL 45-13, that you requested of me in your letter dated February 21, is as follows:

Lot Number:	TANK TM 996
% Active Ingredient:	100%
Net Amount Shipped:	1 1/4 qt.
Molecular Weight:	Avg. 777
Expiration Date:	February 1995

Analytical characterization acquired in support of test substance NEODOL 45-13 was performed at Westhollow Research Center (WRC). Methods and procedures used follow all applicable government regulations regarding Good Laboratory Practices as stated in 40 CFR 792. All records and raw data generated by these analyses will be retained in the WRC Analytical Special Collection of Files.

Analytical methods used to characterize the test substance were Hydroxyl Number (mg KOH/gm), % Water (%wt), Cloud Point, Ethylene Oxide Distribution, Polyethylene Glycol (%wt) and Carbon Number Distribution.

If you have any further questions, please feel free to contact me at (713) 544-3410

Sincerely,

A handwritten signature in cursive script that reads "Harriet Smith".

Harriet Smith

**6.0 APPENDIX III - CULTURE FOOD ANALYSIS**

Zeigler Brothers, Inc. Salmon Starter #1*		
Date Submitted: 12/13/93 Date Reported: 1/26/94		
Pesticide Screen I;II;III	Result	Limit of Quantitation
Alpha BHC	< 0.01 mg/kg	0.01
Beta BHC	< 0.01 mg/kg	0.01
Gamma BHC - Lindane	< 0.01 mg/kg	0.01
Delta BHC	< 0.01 mg/kg	0.01
Heptachlor	< 0.01 mg/kg	0.01
Aldrin	< 0.01 mg/kg	0.01
Heptachlor Epoxide	< 0.01 mg/kg	0.01
DDE	< 0.01 mg/kg	0.01
DDD	< 0.01 mg/kg	0.01
DDT	< 0.01 mg/kg	0.01
HCB	< 0.01 mg/kg	0.01
Mirex	< 0.01 mg/kg	0.01
Methoxychlor	< 0.05 mg/kg	0.05
Dieldrin	< 0.01 mg/kg	0.01
Endrin	< 0.01 mg/kg	0.01
Telodrin	< 0.01 mg/kg	0.01
Chlordane	< 0.05 mg/kg	0.05
Toxaphene	< 0.1 mg/kg	0.1
PCBs	< 0.2 mg/kg	0.2
Ronnel	< 0.01 mg/kg	0.01
Ethion	< 0.02 mg/kg	0.02
Trithion	< 0.05 mg/kg	0.05
Diazinon	< 0.1 mg/kg	0.1
Methyl Parathion	< 0.02 mg/kg	0.02
Ethyl Parathion	< 0.02 mg/kg	0.02
Malathion	< 0.05 mg/kg	0.05
Endosulfan I	< 0.01 mg/kg	0.01
Endosulfan II	< 0.01 mg/kg	0.01
Endosulfan Sulfate	< 0.03 mg/kg	0.03
Chlorpyrifos	< 0.01 mg/kg	0.01
* Analyzed by Lancaster Laboratories		

Zeigler Brothers, Inc. Salmon Starter #1*		
Date Submitted: 12/13/93 Date Reported: 1/26/94		
Test	Result	Limit of Quantitation
Pesticide Screen I;II;III	attached	
Arsenic	2.6 ppm	0.1
Cadmium	0.6 ppm	0.1
Lead	0.4 ppm	0.2
Mercury	<0.02 ppm	0.02
* Analyzed by Lancaster Laboratories		

Ankistrodesmus Grab Sample*		
Date Collected: 7/28/93 Date Reported: 9/6/93		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 mg/kg	0.01
Beta BHC	< 0.01 mg/kg	0.01
Gamma BHC - Lindane	< 0.01 mg/kg	0.01
Delta BHC	< 0.01 mg/kg	0.01
Heptachlor	< 0.01 mg/kg	0.01
Aldrin	< 0.01 mg/kg	0.01
Heptachlor Epoxide	< 0.01 mg/kg	0.01
DDE	< 0.01 mg/kg	0.01
DDD	< 0.01 mg/kg	0.01
DDT	< 0.01 mg/kg	0.01
HCB	< 0.01 mg/kg	0.01
Mirex	< 0.01 mg/kg	0.01
Methoxychlor	< 0.05 mg/kg	0.05
Dieldrin	< 0.01 mg/kg	0.01
Endrin	< 0.01 mg/kg	0.01
Teodrin	< 0.01 mg/kg	0.01
Chlordane	< 0.05 mg/kg	0.05
Toxaphene	< 0.1 mg/kg	0.1
PCBs	< 0.2 mg/kg	0.2
Ronnel	< 0.01 mg/kg	0.01
Ethion	< 0.02 mg/kg	0.02
Trithion	< 0.05 mg/kg	0.05
Diazinon	< 0.1 mg/kg	0.1
Methyl Parathion	< 0.02 mg/kg	0.02
Ethyl Parathion	< 0.02 mg/kg	0.02
Malathion	< 0.05 mg/kg	0.05
Endosulfan I	< 0.01 mg/kg	0.01
Endosulfan II	< 0.01 mg/kg	0.01
Endosulfan Sulfate	< 0.03 mg/kg	0.03
Chlorpyrifos	< 0.01 mg/kg	0.01
* Analyzed by Lancaster Laboratories, Inc.		

Ankistrodesmus Grab Sample*		
Date Collected: 7/28/93 Date Reported: 9/6/93		
Analysis	Result As Received	Limit of Quantitation
Pesticide Screen I,II,III	attached	
Sodium	26. mg/l	10.

\* Analyzed by Lancaster Laboratories, Inc.

**7.0 APPENDIX IV - DILUTION WATER ANALYSIS**

Well Water Sample*		
Date Collected: 7/29/93 Date Reported: 9/17/93		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 µg/l	0.01
Beta BHC	< 0.01 µg/l	0.01
Gamma BHC - Lindane	< 0.01 µg/l	0.01
Delta BHC	< 0.01 µg/l	0.01
Heptachlor	< 0.01 µg/l	0.01
Aldrin	< 0.01 µg/l	0.01
Heptachlor Epoxide	< 0.01 µg/l	0.01
DDE	< 0.01 µg/l	0.01
DDD	< 0.01 µg/l	0.01
DDT	< 0.01 µg/l	0.01
HCB	< 0.01 µg/l	0.01
Mirex	< 0.01 µg/l	0.01
Methoxychlor	< 0.05 µg/l	0.05
Dieldrin	< 0.01 µg/l	0.01
Endrin	< 0.01 µg/l	0.01
Teodrin	< 0.01 µg/l	0.01
Chlordane	< 0.3 µg/l	0.3
Toxaphene	< 4. µg/l	4.
PCBs	< 1. µg/l	1.
Ronnel	< 0.01 µg/l	0.01
Ethion	< 0.02 µg/l	0.02
Trithion	< 0.05 µg/l	0.05
Diazinon	< 0.1 µg/l	0.1
Methyl Parathion	< 0.02 µg/l	0.02
Ethyl Parathion	< 0.02 µg/l	0.02
Malathion	< 0.05 µg/l	0.05
Endosulfan I	< 0.01 µg/l	0.01
Endosulfan II	< 0.01 µg/l	0.01
Endosulfan Sulfate	< 0.03 µg/l	0.03
Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		

Well Water Sample <sup>1</sup>		
Date Collected: 8/9/93 Date Reported: 8/26/93		
Analysis	Result As Received	Limit of Quantitation
Mercury	< 0.00020 mg/l	0.00020
Arsenic	< 0.20 mg/l	0.20
Selenium	< 0.20 mg/l	0.2
Boron	< 0.040 mg/l	0.04
Thallium	< 0.30 mg/l	0.3
Aluminum	< 0.20 mg/l	0.2
Antimony	< 0.20 mg/l	0.2
Barium	< 0.10 mg/l	0.1
Beryllium	< 0.010 mg/l	0.01
Cadmium	< 0.010 mg/l	0.01
Calcium	7.71 mg/l	0.2
Chromium	< 0.050 mg/l	0.05
Cobalt	< 0.050 mg/l	0.05
Copper	< 0.020 mg/l	0.02
Iron	< 0.10 mg/l	0.1
Lead	< 0.10 mg/l	0.1
Magnesium	2.21 mg/l	0.1
Manganese	< 0.010 mg/l	0.01
Molybdenum	< 0.10 mg/l	0.1
Nickel	< 0.050 mg/l	0.05
Potassium	1.07 mg/l	0.5
Silver	< 0.020 mg/l	0.02
Sodium	14.0 mg/l	0.4
Titanium	< 0.010 mg/l	0.01
Vanadium	< 0.010 mg/l	0.01
Zinc	< 0.040 mg/l	0.04
Total Organic Carbon ***	< 1. mg/L	1.
<sup>1</sup> Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		
*** Represents non-purgeable TOC		

**8.0 APPENDIX V - ANALYTICAL METHODOLOGY**

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FINAL DATA REPORT

Study Title

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Daphnia magna*

Data Requirements

United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA),  
Good Laboratory Practice Standards (40 CFR, Part 792)

Submitted To

Shell Development Company  
Westhollow Research Center  
Houston, TX 77251-1380

Performing Laboratory

Battelle Ocean Sciences  
397 Washington Street  
Duxbury, MA 02332

Author

Gregory S. Durell

Study Initiation Date

March 2, 1994

Study Completion Date

May 11, 1994

Battelle Study Number

SD 930113

SIGNATURE PAGE

*Gregory S. Dorell*

Gregory S. Dorell  
Analytical Chemistry Task Leader  
Barteile Ocean Sciences

*06/02/94*

Date

*Allen D. Uhler*

Allen D. Uhler  
Chemistry Department Manager  
Barteile Ocean Sciences

*6/02/94*

Date

*Harriet C. Smith*

Harriet C. Smith  
Project Monitor  
Shell Development Company

*June 14, 1994*

Date

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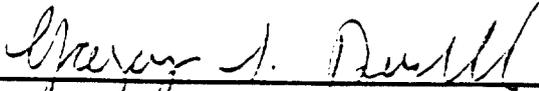
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COMPLIANCE STATEMENT

The work conducted at Battelle through Battelle Proposal/Agreement No. 382-H-0641 "Analysis of Non-ionic Surfactants in Water Samples by HPLC and ELSD" was performed in compliance with United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 CFR, Part 792), August 17, 1989.



\_\_\_\_\_  
Gregory S. Durell  
Analytical Chemistry Task Leader  
Battelle Ocean Sciences

06/02/94

\_\_\_\_\_  
Date

QUALITY ASSURANCE STATEMENT

for

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Daphnia magna*

In accordance with Good Laboratory Practice Standards (40 CFR, Part 792) dated August 17, 1989, this study has been monitored by Battelle Ocean Science's Quality Assurance Unit. Study audit dates and dates when the results were reported to the Study Director and management are listed in the following table.

To the best of my knowledge, the analyses reported here accurately represent the data generated during this study.

Rosanna L. Buhl

Rosanna L. Buhl  
Quality Assurance Coordinator  
Battelle Ocean Sciences

6-6-90

Date

## QUALITY ASSURANCE AUDITS

Conducted for SD-930118

Measurement of Neodol<sup>®</sup> 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Daphnia magna*

Audit Type	Audit Date	Date of Report to Analytical Task Leader	Date of Report to Study Director	Date of Report to Management
Initiation	02/23/94 03/23/94	none	none	none
Lab Inspection	05/09/94 05/10/94 05/12/94 05/23/94	05/24/94	06/06/94	06/06/94
Data Package	06/01-02/94	06/02/94	06/06/94	06/06/94
Report Review	06/01-02/94	06/02/94	06/06/94	06/06/94

STUDY PARTICIPANTS

SD-930113

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Daphnia magna*

---

Mr. Gregory S. Durell	Analytical Chemistry Task Leader: HPLC Analysts
Mr. Richard Restucci	Laboratory Technician, HPLC Analyst (in training)
Mr. Lyle Roberts	Laboratory Technician (in training)

---

## 1.0 INTRODUCTION

The work reported in this document is a component of aquatic toxicological testing that has been requested and initiated by the Sponsor of this study. The toxicological testing was conducted by Springborn Laboratories. Bartelle Ocean Sciences was responsible for providing chemical analytical support to the toxicological testing by determining concentrations of the Test Substance in samples received from Springborn Laboratories. The results of these chemical analyses are presented in this Final Data Report.

The objective of the work reported in this document was to perform chemical analysis of aqueous samples and primary stock solutions, for the determination of concentrations of alcohol ethoxylate surfactants using the analytical method titled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*. This analytical procedure was approved by the Analytical Chemistry Task Leader on February 25, 1994, and by the Sponsor's Project Monitor on March 7, 1994.

### 1.1 Test Substance Identification

The Test Substance analyzed in this study was an alcohol ethoxylate (AE) surfactant (Neodol 45-13<sup>®</sup>). The Test Substance was provided by the Sponsor (Shell Development Company). The Sponsor was also responsible for providing Bartelle with the lot number, analysis, purity, stability, storage requirements, and all other relevant chemical and physical characterization data for the Test Substance. The Test System and Test Substance identity and characterization information, and other relevant test information for this study, as provided by the Sponsor, is summarized below.

Test System:	<i>Daphnia magna</i>
Test Substance:	Neodol <sup>®</sup> 45-13
Test Substance CAS#:	68951-67-7
Test Substance Lot#:	Tank TM996 (01/23/92) 1209 (WRC Tox Sample Number)
Test Substance Purity:	100%

<b>Test Substance Composition:</b>	A mixture of C <sub>12</sub> -C <sub>15</sub> alcohol ethoxylates with an average of 13 moles of ethylene oxide per mole of alcohol. Traces of free ethylene oxide (less than or equal to 5 ppm) may be present in the neat Test Substance.
<b>Test Substance Analysis:</b>	The percent purity data is based on process knowledge, and research and development prior to manufacture of the Test Substance used.
<b>Test Substance Solubility:</b>	Completely soluble in water. May form gel.
<b>Test Substance Stability:</b>	Stable. A stability and holding time of two years from receipt at Barteile was assigned to the Test Substance.
<b>Test Substance Storage Requirements:</b>	Cool, dry.

## 2.0 MATERIALS AND METHODS

### 2.1 Analytical Method Description

The analytical procedure used was developed for the determination of total alcohol ethoxylate surfactants in aqueous samples. The method involves an extraction step to isolate the surfactant from a water sample and a high performance liquid chromatography (HPLC) analytical procedure to quantify the nonionic surfactant concentration. The results are reported as total alcohol ethoxylate surfactant concentrations. Concentrations of the surfactant were also determined in primary stock solutions received from the toxicology testing laboratory.

In order to analyze alcohol ethoxylate (AE) surfactants in aqueous matrices at low levels the surfactant must first be isolated from the water, concentrated, and analyzed using an appropriate method of detection to quantitate the amount of surfactant originally present in the aqueous sample. The method used employs solid phase extraction (SPE) with a C<sub>18</sub> reverse phase cartridge for isolating the analyte and concentrating the sample. After elution of the surfactant from the SPE cartridge with methyl and isopropyl alcohol it is analyzed using an HPLC procedure (octyl HPLC-column separation and methanol/water mobile phase gradient) that employs an evaporative light scattering detector

(ELSD) for analyte detection and quantification. This method quantifies total AE surfactant without distinguishing between the various individual AEs or carbon chain distributions.

The primary stock solution surfactant concentration was determined by simply diluting the sample to the appropriate concentration using methanol and analyzing it by HPLC/ELSD. No extraction step was needed.

The analytical procedure is described in detail in the document entitled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*, which was developed specifically for these analyses. This Test Substance specific document was prepared by Battelle and approved by the Sponsor on March 7, 1994. The analytical procedure document, and associated study-specific analytical information, is included in Battelle's data-package for this study. This data-package will be provided to the Sponsor and a copy maintained by Battelle Ocean Sciences.

## 2.2 Laboratory Quality Control

The water samples were processed in analytical batches of no more than 20 test samples. Each batch of test samples included four laboratory quality control (QC) samples: one procedural blank (PB), one matrix spike (MS), one matrix spike duplicate (MSD), and one blank spike (BS). The procedural blank (which consists of Milli-Q laboratory water carried through all steps and treated as other samples) sample was used to ensure that there were no significant levels of laboratory contamination. The matrix spike (test sample spiked with the target analyte), matrix spike duplicate, and blank spike (Milli-Q water spiked with the target analyte) samples were used to demonstrate laboratory accuracy and precision; these QC samples were also carried through all sample processing procedures and treated as the rest of the samples.

A portion of a non-fortified (control) test sample was used to prepare the matrix spike samples because it contained no background analyte levels yet had a sample matrix that was representative of the test samples. The blank spike was processed to determine if the accuracy/recovery of the analyte was affected by the sample matrix.

Each sequence of samples analyzed by the HPLC/ELSD instrument was initiated with a six-point multilevel calibration. Test samples followed the initial calibration in the analysis sequence, and a calibration check standard was analyzed at least every 12 samples to verify the validity of the calibration.

Summarized below are the QC data quality objectives that applied for this study.

### Data Quality Objectives

QC Analysis	Criteria Objective
Blank spike analyte recovery	70%-120%
Matrix spike analyte recovery	70%-120%
Matrix spike/spike duplicate precision	≤30% RPD
Procedural blank	< 1 × limit of quantification (LOQ)
Instrument multilevel calibration	Correlation coefficient > 0.995
Instrument calibration check	15% RPD in determined and actual standard concentration

### 2.3 Calculations

#### Sample Concentration Calculations

An external standard method of calibration and quantification was used. Sample extract concentrations were determined by applying the multilevel quadratic calibration equation using a chromatography data system on which the analytical data were acquired during the instrumental analysis. A six-point calibration curve which bracketed the expected concentration range of exposure samples was generated at the initiation of the HPLC analysis. Calibration standard concentrations were approximately 22, 44, 83, 122, 164, and 204  $\mu\text{g}/\text{mL}$ . Original water sample concentrations were subsequently determined by applying the water extraction volume (WEV) and pre-injection volume (PIV) information. The PIV of the PB, BS, MS, and MSD samples, controls, and 500, 850 and 1,400 parts per billion (ppb) nominal concentration samples was 1.00 mL. For the 2,400 and 4,000 ppb nominal concentration samples the PIV was 4.00 mL. Analyte concentrations of the original water samples were determined in ppb. Analyte concentration of the primary stock solution samples were determined in parts per million (ppm).

$$\text{Determined Water Sample Concentration (ppb)} = \text{EC} \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$$\text{Primary Stock Solution Concentration (ppm)} = \text{EC} \times \text{DIL VOL} \times (1/\text{DIL VOL})$$

EC = Extract (HPLC sample) concentration ( $\mu\text{g}/\text{mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

DIL VOL<sub>1</sub> = Final volume of diluted Primary Stock subsample (mL)

DIL VOL<sub>2</sub> = Volume of Primary Stock subsample taken for the dilution (mL)

### Quality Control Sample Calculations

Two separate calculations were performed on the Quality Control (QC) sample data. Percent recoveries were determined for the blank spike and matrix spike samples, and the relative percent difference (%RPD) between the two percent recovery values was determined for the matrix spike/duplicate sample pair.

$$\% \text{ Recovery} = \text{WC}_2 \times (1/\text{WC}_1) \times 100\%$$

$$\% \text{RPD} = [ \% \text{REC}_{\text{MS}} - \% \text{REC}_{\text{MSD}} ] \times (2 / (\% \text{REC}_{\text{MS}} + \% \text{REC}_{\text{MSD}})) \times 100\%$$

WC<sub>2</sub> = Determined water sample concentration (ppb) — calculated as shown above

WC<sub>1</sub> = Spiked water sample concentration (ppb) — prepared concentration

%REC<sub>MS</sub> = Percent recovery of the matrix spike sample

%REC<sub>MSD</sub> = Percent recovery of the matrix spike duplicate sample

### Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) was determined for the analyses. The target analyte had to provide a detector response of a minimum 3:1 signal:noise ratio to be considered detected.

The water sample equivalent LOD was calculated by comparing the peak height equivalent to a signal:noise ratio of 3:1 in the sample to the peak height of the analyte in the low-level calibration standard, and applying the water extraction volume (WEV) and the pre-injection volume (PIV), as shown previously. The LOD was determined using two samples with the lowest nominal concentration and averaging the values obtained in the two determinations.

The LOQ was determined as the water sample concentration equivalent to a final extract concentration that is the same as the low calibration standard. The LOQ was determined using the PIVs and WEVs used for the samples with the lowest nominal concentrations.

$$\text{LOD (ppb)} = H_{3x} \times (C_{LS}/H_{LS}) \times PIV \times (1/\text{WEV}) \times 1000$$

$$\text{LOQ (ppb)} = C_{LS} \times PIV \times (1/\text{WEV}) \times 1000$$

$H_{3x}$  = Peak height equivalent to  $3 \times$  the noise in the sample

$H_{LS}$  = Peak height of analyte in the low-level calibration standard

$C_{LS}$  = Concentration of analyte in the low-level calibration standard ( $\mu\text{g/mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

### 3.0 RESULTS

#### 3.1 Analytical Results — Toxicological Test Samples

The results of the chemical analyses of the samples received from the toxicological testing laboratory are presented in Table 1. The analyses of the test samples were performed in two analytical batches, the first batch containing the  $t=0$  hr and  $t=24$  hr (new) samples and the second batch containing the  $t=24$  hr (old) and  $t=48$  hr (old) samples. Table 1 also presents the data for the Primary Stock Solution analyses.

The measured analyte concentrations in the test samples that had been fortified with the Test Substance ranged from 269 ppb (for sample ND16, a sample with a nominal concentration of 500 ppb) to 3,825 ppb (for sample ND06, a sample with a nominal concentration of 4,000 ppb). The measured concentrations were between 54 percent (sample ND16) and 96 percent (sample ND06) of the nominal concentration, with the majority of the samples having a measured concentration ranging from approximately 70 to approximately 90 percent of the nominal concentration. In general, on a relative basis, the measured concentrations deviated more from the nominal concentrations for samples with lower concentrations than for samples with higher concentrations.

The concentrations measured for the Primary Stock Solutions were slightly lower than the expected concentrations, with measured concentrations of 9,927 and 9,936 ppm for the two samples, both of which had nominal/expected concentrations of 10,000 ppm. The measured Primary Stock Solution concentrations were 1 percent lower than the expected concentration.

Table 1. Neodol® 45-13 Concentrations in Samples Received from the  
Toxicological Testing Laboratory:

Bartelle Sample ID	Test Sample Time/Type	Nominal Conc. (ppb)	Measured Conc. (ppb)
<b>Batch #1</b>			
ND01	☐	0	ND
ND02	☐	500	337
ND03	☐	850	673
ND04	☐	1,400	1,156
ND05	☐	2,400	1,936
ND06	☐	4,000	3,825
ND08	☐24, new	0	ND
ND09	☐24, new	500	367
ND10	☐24, new	350	756
ND11	☐24, new	1,400	1,249
ND12	☐24, new	2,400	1,787
ND13	☐24, new	4,000	3,564
<b>Batch #2</b>			
ND15	☐24, old	0	ND
ND16	☐24, old	500	269
ND17	☐24, old	350	542
ND18	☐24, old	1,400	1,067
ND19	☐24, old	2,400	1,773
ND20	☐24, old	4,000	3,290
ND21	☐48, old	0	ND
ND22	☐48, old	500	317
ND23	☐48, old	850	641
ND24	☐48, old	1,400	1,217
ND25	☐48, old	2,400	1,966
ND26	☐48, old	4,000	3,564
<b>Primary Stock Solution</b>		<b>(ppm)</b>	<b>(ppm)</b>
NH11	☐, stock	10,000	9,927
NH12	☐24, stock	10,000	9,936

ND: Not detected; <LOD.

### 3.2 Analytical Results — Quality Control Samples

All quality control objectives were met for this work. The six-point multi-level instrument calibration used had a correlation coefficient of 0.999445 for the quadratic equation, and the continuing calibration check analyses ranged from 7.2 to 8.3 relative percent difference between the determined and actual standard concentration.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as described in Section 2.3, and were as follows.

#### Limit of Detection and Limit of Quantitation

Limit of Detection (LOD)	34 ppb
Limit of Quantitation (LOQ)	221 ppb

The concentrations for all samples with anticipated analyte concentrations (i.e., all samples except the laboratory procedural blanks and toxicological test control samples) had measurable levels of analyte and were determined to be above the LOD and the LOQ.

The results of the laboratory quality control (QC) sample analyses are presented in Table 2. The target analyte was not detected in either of the two procedural blank samples. The analyte recovery in the blank spike (BS) samples were 86% and 85% for analytical batches #1 and #2, respectively. The analyte recovery in the four matrix spike (MS/MSD) samples ranged from 37% to 92%, and these data suggest that there were no significant matrix effects on the analytical procedure. Acceptable precision was observed for both analytical batches. The relative percent differences in the measured analyte recoveries for the MS/MSD duplicate analyses were 2% and 4% for analytical batches #1 and #2, respectively.

Table 2. Laboratory Quality Control Sample Analysis Results

Barrelle Sample ID	QC Sample Type	Recovery (%)
Batch #1		
NH14PB	Procedural Blank	ND
NH15BS	Blank Spike	35.3
NH16MS	Matrix Spike	38.3
NH17MSD	Matrix Spike Duplicate	36.5
	MS/MSD %RPD:	2.1
Batch #2		
NH18PB	Procedural Blank	ND
NH19BS	Blank Spike	85.1
NH20MS	Matrix Spike	87.5
NH21MSD	Matrix Spike Duplicate	91.5
	MS/MSD %RPD:	4.4

ND: Not detected; &lt;LOD.

The QC data indicate that the laboratory analysis was in control for this work. There were no identified circumstances or occurrences during the conduct of this work that may have affected the quality or integrity of the data.

#### 4.0 ARCHIVING OF DATA

Study records that will be maintained by Battelle include, but are not limited to:

- Verified copies of all raw data and documentation records
- Verified copy of the signed and approved Analytical Chemistry Method, and associated amendments and deviations
- All correspondence, memos, or notes pertaining to the study
- Verified copy of the signed Final Data Report
- Test Substance records, including receipt and inventory, and physical and chemical characterization data, as supplied by the Sponsor

All project files, including verified copies of the raw data and the Final Data Report, will be archived by Battelle after the submission of this Final Data Report. The Battelle Quality Assurance Unit manages the limited-access data archival. Additionally, a small amount of Test Substance will be archived by Battelle.

**APPENDIX A**

**Deviations to Analytical Method**

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BATTELLE OCEAN SCIENCES  
Miscellaneous Documentation Form

Page 1 of 1Project Title: Gap-Filling ProjectStudy Number: SD-930118Battelle Project Number: N8323-0002This is a Deviation to Analytical Method: Yes X No       Entered by: Gregory S. DurrellDate: May 26, 1994

---

Subject: Miscellaneous Deviations to Analytical Method

- The temperature recorded for Refrigerator #2, where standards and samples were stored, ranged from 1 to 10°C in March and April. Unextracted water samples were to be stored at approximately 4°C, as indicated in the Analytical Procedure Document. Although this temperature range is larger than what may be considered covered by "approximately 4°C", it is not expected to have impacted the integrity of the samples or results, because of the stability of the test substance.
- The nebulizer gas flow (nitrogen) for the HPLC detector (ELSD) was maintained at 9 L/min and not 11 L/min as specified in the Analytical Procedure Document. This analytical modification was requested by the Sponsor, based on method verification work performed at the Sponsor's laboratory, in order to achieve the necessary sensitivity. This method modification was requested by the Sponsor by telephone on 04/06/94 and confirmed by telephone on 04/08/94, as indicated in the Analytical Task Leader's telephone log.

Approved: Gregory S. DurrellDate: 06/10/94

BATTELLE OCEAN SCIENCES  
Miscellaneous Documentation Form

Page 1 of 1

Project Title: Gap-Filling Project

Study Number: SD-930118

Battelle Project Number: N8328-0002

This is a Deviation to Analytical Method:

Yes X No       

Entered by: Gregory S. Dureil  
Richard P. Restucci

Date: May 26, 1994  
June 3, 1994

Subject: Miscellaneous Deviations

- The monthly calibration with a 70 g weight, as specified in SOP 3-073, was not performed on the Mettler AC100 balance that was used to prepare standards for this study. However, the balance was always calibrated on the day it was used with a weight of suitable for representing the weight actually being measured for this study.
- According to the Project-specific Analytical Information memo, seven calibration levels with an approximate concentration range of 20 - 380 ug/mL were to be analyzed prior to sample analysis. However, the actual concentration range analyzed with the Daphnia study was 20 - 290 ug/mL.

Approved: ASJUR

Date: 6/06/94

**NEODOL® 45-13 - TOXICITY TO THE  
FRESHWATER GREEN ALGA, *Selenastrum  
capricornutum***

**TSCA Test Guideline § 797.1050**

**Submitted to:**

**Shell Development Company  
Westhollow Technology Center  
P.O. Box 1380  
Houston, Texas 77251-1380**

**SLI Report #94-4-5252**

**Sponsor Protocol/Project No.: WRC TOX No. 1209**

**SLI Study #777.0294.6111.430**

**Study Director: James R. Hoberg**

**Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street  
Wareham, Massachusetts 02571-1075**

**Analytical Support:  
Battelle Ocean Services  
397 Washington Street  
Duxbury, Massachusetts 02332**

**Contains No CBI**

**23 August 1994**

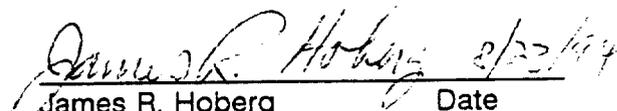
**FINAL REPORT**

---

**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The data and report presented for "**NEODOL® 45-13 - Toxicity to the Freshwater Green Alga, *Selenastrum capricornutum***" were produced and compiled in accordance with all pertinent TSCA Good Laboratory Practice Regulations (40 CFR, Part 792) with the following exception: routine water contaminant screening analyses for pesticides, PCBs and metals were conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, Pennsylvania. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc.). Stability, characterization, and verification of the test material identity and maintenance of records on the test material are the responsibility of the Study Sponsor. At the termination of the testing program, all remaining test material will be sent to the Study Sponsor. Archival of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.

  
James R. Hoberg      Date  
Study Director

---

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**SUMMARY****NEODOL® 45-13 - Toxicity to the Freshwater Green Alga, *Selenastrum capricornutum***

**SPONSOR:** Shell Development Company

**PROTOCOL TITLE:** "Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with the Freshwater Green Alga, *Selenastrum capricornutum*, Following TSCA Guideline § 797.1050," Springborn Laboratories Protocol #072993/TSCA/SHELL/SEL and Protocol Amendments #1 and #2 dated 17 March and 6 April 1994.

**REPORT NUMBER:** 94-4-5252

**STUDY NUMBER:** 777.0294.6111.430

**TEST MATERIAL:** NEODOL® 45-13, CAS Registry No. 68951-67-7, Lot No. Tank TM 996, WRC TOX. Sample No. 1209, a clear liquid reported by the Study Sponsor to contain 100% active ingredient, received 17 February 1994.

**TEST DATES:** 4 to 8 April 1994

**TEST ORGANISM:** *Selenastrum capricornutum*, inoculum - 3 days since previous transfer, source - Springborn culture

**DILUTION WATER:** Algal Assay Procedure (AAP) medium

**TEST CONDITIONS:** 96 hour duration, 24 to 25 °C, continuous illumination at 3800 to 5400 lux (350 to 500 footcandles), shaking at 100 rpm

**NOMINAL TEST CONCENTRATIONS:** 0.013, 0.025, 0.050, 0.10 and 0.20 mg/L

**INITIAL MEASURED CONCENTRATIONS:** 0.0089, 0.023, 0.038, 0.085 and 0.20 mg/L

**EFFECT CRITERION:** Inhibition of cell density relative to the control

**RESULTS:**

Based on initial measured concentrations, the 96-hour EC50 value was calculated to be 0.083 mg/L (95% confidence limits of 0.038 to 0.19 mg/L).

The 96-hour No-Observed-Effect Concentration (NOEC) was determined to be 0.023 mg/L.

## 1.0 INTRODUCTION

The objective of this study was to determine the effect of NEODOL<sup>®</sup> 45-13 on the growth of the freshwater green alga *Selenastrum capricornutum*. The results are based on initial measured concentrations and are reported as the 96-hour No-Observed-Effect Concentration (NOEC) and EC10, EC50 and EC90 values (i.e., the concentrations of test material that reduce culture density by 10, 50 and 90%, respectively, as compared with the control). The study was initiated on 2 March 1994, 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 96-hour definitive test was conducted from 4 to 8 April 1994 at the Environmental Sciences Division of Springborn Laboratories, Inc. (SLI), in Wareham, Massachusetts. All original raw data and the final report produced during this study are stored at Shell Development Company.

## 2.0 MATERIALS AND METHODS

### 2.1 Protocol

Procedures used in this acute toxicity study followed those described in the Springborn Laboratories protocol entitled "Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with the Freshwater Green Alga, *Selenastrum capricornutum*, Following TSCA Guideline § 797.1050," Springborn Laboratories Protocol #072993/TSCA/SHELL/SEL and Protocol Amendments #1 and #2 dated 17 March and 6 April 1994, respectively (Appendix I). The methods described in this protocol meet or exceed the standard procedures described in the U.S. EPA Toxic Substance Control Act (TSCA) Test Guidelines § 797.1050 (U.S. EPA, 1985) as amended in the Federal Register on 20 May 1987 (U.S. EPA, 1987) and meet the primary technical objectives of The Shell Research Limited/Sittingbourne Research Center guidelines (SBT SOP No. 169, Edition No. 8).

### 2.2 Test Material

The test material, NEODOL<sup>®</sup> 45-13, was received from Shell Development Company, Houston, Texas on 17 February 1994. Upon receipt at Springborn, the test material was stored at room temperature (approximately 20 °C) in a dark ventilated cabinet. Test concentrations are

reported as milligrams per liter of test solution (mg/L). The following information describes the test material received:

Empirical Formula:	not available
Chemical Name:	not available
Physical Appearance:	clear liquid
Lot No.:	20944-178 (TANK TM 996)
CAS Registry No.:	68951-67-7
Purity:	100% (Appendix II)
Molecular Weight:	777 g/mole (average)
Water Solubility:	complete, may form gel
Vapor Pressure:	< 0.1 mm Hg at 75 °F

### 2.3 Test Organism

The alga used in this toxicity test was the freshwater green alga *Selenastrum capricornutum*, strain 1648, Class Chlorophyceae. The alga was originally obtained from the Carolina Biological Supply Company, Burlington, North Carolina, and was maintained in stock culture at Springborn.

The culture medium used was Algal Assay Procedure (AAP) medium prepared with sterile, deionized water. The components used to formulate AAP medium are presented in Table 1. Representative samples of the dilution water source used in the preparation of the culture medium were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix III). None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed in agreement with U.S. EPA guidelines. In addition, a representative sample of AAP medium is analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration was 0.89 mg/L for the month of April 1994 (Springborn TOC and TSS Master Log, 1994).

The pH of the culture medium was adjusted to pH  $7.5 \pm 0.1$  with either 0.10 N hydrochloric acid or 0.10 N sodium hydroxide. Stock cultures were grown in 125-mL glass flasks containing 50 mL of medium. The flasks were covered with stainless steel caps which permitted gas exchange.

The stock cultures were maintained within the following conditions: a shaking rate of  $100 \pm 10$  rpm, a temperature of  $24 \pm 2$  °C and continuous illumination at the surface of the medium at an approximate light intensity of 3200 to 5400 lux (300 to 500 footcandles) for a minimum of three days prior to test initiation (SLI Algae Conditions Daily Log, 1994). Temperature was controlled using an environmental chamber. Lighting was supplied by Duro-Test, Inc. Vita-Lite® fluorescent lights. Culture flasks were agitated continuously on a Lab-Line orbital shaker.

Stock cultures were transferred to fresh medium approximately twice weekly. The inoculum used to initiate the toxicity test with NEODOL® 45-13 was taken from a stock culture that had been transferred to fresh medium three days before testing.

#### **2.4 Reference Test**

A copper nitrate reference test was conducted with the test organism culture from 11 to 15 April 1994. The resulting 96-hour EC50 based on measured test concentrations as copper, was calculated to be 0.057 mg/L (95% confidence interval of 0.045 to 0.069 mg/L). Based on the results of the reference test and the successful culture of *Selenastrum capricornutum*, it was established that this culture was suitable for testing.

#### **2.5 Test Dilution Water**

The AAP medium used to prepare the exposure solutions was formulated in the same manner as the culture medium. Several liters of AAP medium were prepared using deionized water, autoclaved and equilibrated to test temperature. The pH of this medium was 7.5 and required no pH adjustment prior to use.

#### **2.6 Test Concentrations**

Based on the results of a preliminary test conducted from 10 to 14 March 1994, nominal test concentrations of 0.013, 0.025, 0.050, 0.10 and 0.20 mg/L were selected for the definitive exposure.

## 2.7 Preparation of Test Solution

Prior to preparation of the stock solution, the test material was heated to a temperature range of 50 to 60 °C and stirred with a glass rod to ensure homogeneity. Forty grams (40.000 g) of test material was then removed using a glass pipet, and was diluted in 2000 mL of distilled, deionized water to create a 20,000 mg/L primary stock solution. This primary stock solution was stirred for 15 minutes to ensure that the test material completely dissolved, at which time the solution was observed to be clear and colorless. The secondary stock 1000 mg/L, was then prepared by diluting 50 mL of the 20,000 mg/L primary stock solution to a volume of 1000 mL with AAP medium. Test solutions with nominal concentrations of 0.013, 0.025, 0.050, 0.10 and 0.20 mg/L were prepared by diluting the appropriate volume of the 1000 mg/L secondary stock solution with AAP medium to a volume of 2000 or 3000 mL. Additional untreated AAP medium was prepared and designated the control. All of the test solutions were observed to be clear and colorless with no sign of undissolved test material present.

Replicate sterile 500-mL Erlenmeyer flasks, three per treatment level for the three highest concentrations, 0.050, 0.10 and 0.20 mg/L and four replicates for the two lowest treatments, 0.025 and 0.013 mg/L and the control were prepared. The additional replicate in the lowest treatments and control was required for analytical sample volumes. Each vessel was conditioned by rinsing with the appropriate test solution. Two hundred and fifty mL of the appropriate test solution was then added to each replicate flask. Four control flasks which contained AAP medium were maintained under the same conditions as the treatment level flasks but contained no NEODOL® 45-13. All test vessels were fitted with stainless steel caps which permitted gas exchange.

## 2.8 Test Initiation

Approximately thirty minutes after the test solutions were prepared and added to the test flasks, 1.375 mL of an inoculum of *Selenastrum capricornutum* cells at an approximate density of  $182 \times 10^4$  cells/mL was aseptically introduced into each flask (including additional vessels established for analytical samples). This inoculum provided the required cell density of approximately  $1.0 \times 10^4$  cells/mL.

## 2.9 Test Monitoring

**2.9.1 Algal Growth.** At each 24-hour interval, cell counts were conducted on each replicate vessel (A, B and C) using a hemacytometer (Neubauer Improved) and an Olympus compound microscope. One sample was taken from each flask for counting with the exception of the additional replicate established for 0.013, 0.025 mg/L treatment levels and the control. One or more hemacytometer fields, each 0.10 x 0.10 cm in surface area and 0.010 cm deep and containing 0.00010 mL of culture, were examined for each sample until at least 400 algal cells or four fields were counted. Observations of the health of the cells were made and recorded at each 24-hour interval.

**2.9.2 Recovery.** At test termination, since no test concentration completely inhibited algal growth, a 3.25 mL sample was removed from the composite of the three replicates of the highest test concentration (0.20 mg/L) which most severely inhibited algal growth. This sample was then diluted with fresh AAP medium to prepare a subculture with a nominal test concentration equal to the highest test concentration (0.013 mg/L) in which no growth inhibition was observed. This subculture was used to determine if the effects of the test material on the algae were algistatic (in which case cells would resume growth in the subculture) or algicidal (in which case no growth would occur in the subculture). The subculture was incubated under test conditions for four days (i.e., until growth was observed, as determined by cell counts taken every other day).

**2.9.3 Test Conditions.** The test was conducted in an environmental chamber designed to maintain the following test conditions: a temperature of  $24 \pm 2$  °C, continuous lighting with a light intensity within the range of 3800 to 5400 lux (350 to 500 footcandles) and a shaking table rate of  $100 \pm 10$  rpm.

Temperature was measured continuously with a Taylor Thermometer Company, Inc. minimum/maximum thermometer located in a flask of water adjacent to the test flasks in the environmental chamber. The shaking rate of the orbital shakers was recorded daily. The light intensity of the test area was measured with a General Electric Type 214 light meter at 0 hour and each 24-hour interval of the exposure period. Light intensity was measured in footcandles

and converted to lux based on the equivalency of 1 footcandle = 10.76 lux. Test flasks were randomly placed on the shaking table at test initiation based upon computer-generated random numbers. Following each observation interval, the test vessels were returned to the initial random positions established at test initiation.

Water quality parameters (pH and conductivity) were measured prior to test initiation and at the termination of the 96-hour exposure period. Measurements at 0 hour were conducted on the test solution remaining in the 2000 or 3000-mL volumetric flasks after the individual test flasks had been filled. At test termination, the three replicates (A, B and C) for each test concentration and the control were then individually composited and a portion of each composite solution was transferred to a 100-mL beaker for conductivity and pH measurements. Test solution pH was measured with a LaMotte Model HA pH meter, and conductivity was measured with a Yellow Springs Instrument Model #33 salinity-conductivity-temperature meter.

**2.9.4 Chemical Analysis.** At test initiation (0 hour) and test termination (96 hours), a single sample from each test solution and the control was analyzed for NEODOL<sup>®</sup> 45-13 concentration. Sample containers were approximately 700 mL borosilicate glass bottles with teflon<sup>®</sup> lined screw caps. Samples (approximately 700 mL) collected at 0 hour were removed from the excess test solution remaining in the 2000 or 3000-mL volumetric flasks after the test vessels were filled. Additionally, a sample of the primary stock solution used to formulate the test solutions was collected for analysis at test initiation. Samples (500 to 700 mL) collected at 96 hours were removed from the composited test solution (replicates A, B, C and D where applicable) for each treatment level and the control after cell counts and water quality measurements were taken. At test termination, the test solutions were centrifuged at 1500 rpm for 15 minutes to remove algal cells from the test solutions prior to analysis. Following centrifuging, 500 to 700 mL of the supernatant of each composited solution was poured into the sample containers. On the day of collection, all samples were preserved with 1% formalin and delivered to Battelle Ocean Sciences, Duxbury, Massachusetts for analysis. Samples were analyzed in accordance with methods described in Battelle Ocean Sciences Study #SD-930120 (Appendix IV). All of the glassware used in testing and sample collection was thoroughly washed

with sequential rinses of a 10% solution of nitric acid, acetone, distilled-deionized water, isopropanol and distilled-deionized water.

## 2.10 Determination of EC50 and NOEC Values

The cell density of each culture at each 24-hour interval was calculated by dividing the number of cells counted by the total number of fields examined. A mean and standard deviation was calculated for the cell density of each treatment level and the control.

The highest test concentration that caused no statistical adverse effect on cell density, the No-Observed-Effect Concentration (NOEC), was determined using Williams' Test (Williams, 1971, 1972). The data were first checked for normality using the Shapiro-Wilks' Test (Weber *et al*, 1989) and for homogeneity of variance using Bartlett's Test (Horning and Weber, 1985). All statistical determinations were made at the 95% level of certainty, except in the case of Bartlett's and Shapiro-Wilks' Tests, where a level of 99% certainty was applied.

EC10, EC50 and EC90 values (the concentration of test material which reduced cell densities by 10, 50 and 90%, respectively) were calculated based on cell density after 24, 48, 72, and 96 hours of exposure. The EC10, EC50 and EC90 values and their 95% confidence limits were determined by linear regression of response (percent reduction of cell density as compared with the control) vs. initial measured exposure concentration over the range of test concentrations where a clear exposure-response relationship was observed. Four linear regressions were estimated based on (a) untransformed data, (b) untransformed response vs. logarithm-transformed concentration, (c) probit-transformed response vs. untransformed concentration, and (d) probit-transformed response vs. logarithm-transformed concentration. The regression that best fit the data was selected based on the highest coefficient of determination ( $r^2$ ). This regression equation was then applied to estimate the EC values and their 95% confidence limits, using the method of inverse prediction (Sokal and Rohlf, 1981).

### 3.0 RESULTS AND DISCUSSION

#### 3.1 Preliminary Testing

A preliminary range-finding exposure was conducted at Springborn from 10 to 14 March 1994 at nominal concentrations of 0.010, 0.10, 1.0, 10, 100 and 1000 mg/L. Duplicate exposure vessels were established for each concentration and control. Following 96 hours of exposure, cell densities in the treatment levels (0.010 to 1000 mg/L) averaged 122, 4.0, 4.0, 0.38, 0.63 and  $0 \times 10^4$  cells/mL, respectively. The control solution averaged  $120 \times 10^4$  cells/mL. Based on these results, nominal concentrations of 0.013, 0.025, 0.050, 0.10 and 0.20 mg/L were selected for the definitive study.

#### 3.2 Definitive Testing

**3.2.1 Evaluation of Test Conditions** - Conductivity, pH, temperature and light intensity measurements recorded during the test are presented in Table 2. Conductivity of the exposure solutions ranged from 80 to 110  $\mu$ mhos/cm throughout the exposure period. The pH of the exposure solutions ranged from 7.3 to 7.5 at test initiation, increasing to 10.1 to 10.2 at test termination. This pH change is common in static algal cultures due to photosynthesis by the algae. Continuous temperature monitoring established that the temperature ranged from 24 to 25 °C throughout the study period. The shaking rate was maintained throughout the exposure at a constant rate of 100 rpm. Light intensity of the test area ranged from 3800 to 5400 lux (350 to 500 footcandles).

**3.2.2 Analytical Results** - The results of the analysis of the primary stock solution and the test solutions for NEODOL<sup>®</sup> 45-13 concentrations are summarized in Table 3. Results of the analysis of the primary stock solution (20,000 mg/L) used to formulate the test solutions indicate the concentration of NEODOL<sup>®</sup> 45-13 was measured at 19,000 mg/L. Measured concentrations at 0-hour for the treatment levels were 0.0089, 0.023, 0.038, 0.085 and 0.20 mg/L. Concentrations measured at the 96-hour interval were measured as 0.0056, 0.0064, 0.014, 0.016 and 0.045 mg/L. Since these data establish that there was a considerable loss of NEODOL<sup>®</sup> 45-13 over the 96 hour exposure period, the results of this test are based on the measured concentrations of

NEODOL<sup>®</sup> 45-13 established at test initiation (i.e., 0.0089, 0.023, 0.038, 0.085 and 0.20 mg/L). Analytical results are presented in Battelle Ocean Sciences Study #SD-930120 (Appendix IV).

**3.2.3 Biological Results** - Cell densities determined at each observation interval are presented in Table 4. At test termination, cell fragments and bloated cells were observed in the four highest treatment levels (0.023, 0.038, 0.085 and 0.20 mg/L). Cells exposed to the remaining treatment level (0.0089 mg/L) and the control were observed to be normal throughout the exposure. At test termination, cell densities followed the established concentration gradient, decreasing with increasing test concentration. Cell density in the 0.038, 0.085 and 0.20 mg/L treatment levels averaged 77, 50 and 23 x 10<sup>4</sup> cells/mL, respectively, which was significantly reduced compared to the cell density of the control (104 x 10<sup>4</sup> cells/mL). Cell density at the 0.0089 and 0.023 mg/L treatment levels was 107 and 102 x 10<sup>4</sup> cells/mL, respectively, which was comparable to the control. Based on these results, the 96-hour No-Observed-Effect Concentration (NOEC) for cell density was determined to be 0.023 mg/L.

Since no test concentration completely inhibited algal growth, a subsample of (3.25 mL) was removed from a composite of the highest test concentration (0.20 mg/L) which most severely inhibited algal growth to determine algistatic/algicidal effects. This subsample was diluted to 50 mL with fresh AAP medium to yield a nominal concentration of 0.013 mg/L and resulted in an estimated cell density of 1.5 x 10<sup>4</sup> cells/mL. After two days, 31 x 10<sup>4</sup> cells/mL were observed and after four days, 123 x 10<sup>4</sup> cells/mL were observed. The growth of *S. capricornutum* after transfer to fresh medium indicates that NEODOL<sup>®</sup> 45-13 had an algistatic, rather than an algicidal, effect at the 0.20 mg/L (nominal) test concentration.

Table 5 presents the EC10, EC50 and EC90 values and their corresponding 95% confidence limits. The 96-hour EC50 value for NEODOL<sup>®</sup> 45-13, based on cell density, was calculated to be 0.083 mg/L (95% confidence limits of 0.038 and 0.19 mg/L).



**QUALITY ASSURANCE UNIT STATEMENT**

The raw data and report for "NEODOL® 45-13 - Toxicity to the Freshwater Green Alga, *Selenastrum capricornutum*" were inspected by the Quality Assurance Unit (QAU) at Springborn Laboratories Inc., Environmental Sciences Division to determine adherence with the study protocol and laboratory standard operating procedures. In addition, inspection of certain phases of the in-life portion of the study was performed. Dates of study inspections, dates reported to the Study Director and to Management are listed below.

Based on these inspections, it was determined that this report accurately reflects the raw data collected during this study.

<u>Inspection Date</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
4/8/94	4/8/94	4/8/94
4/11/94	4/11/94	4/22/94
4/20/94	4/21/94	4/22/94
4/27/94	4/27/94	5/6/94
7/11/94, 7/12/94	7/12/94	7/15/94
8/23/94	8/23/94	8/23/94

SPRINGBORN LABORATORIES, INC.

*Doreen S Newhouse 23 Aug 94*  
 \_\_\_\_\_  
 Doreen S. Newhouse Date  
 Supervisor,  
 Quality Assurance Unit

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**TABLES**

**Table 1. Composition of algal growth medium (AAP medium) used in this study.**

Compound	Concentration
NaNO <sub>3</sub>	25.5 mg/L
MgCl <sub>2</sub> · 6H <sub>2</sub> O	12.16 mg/L
CaCl <sub>2</sub> · 2H <sub>2</sub> O	4.41 mg/L
MgSO <sub>4</sub> · 7H <sub>2</sub> O	14.7 mg/L
K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O	1.368 mg/L
NaHCO <sub>3</sub>	15.0 mg/L
H <sub>3</sub> BO <sub>3</sub>	185.5 µg/L
Na <sub>2</sub> SeO <sub>4</sub> <sup>a</sup>	1.88 µg/L
MnCl <sub>2</sub> · 4H <sub>2</sub> O	415.4 µg/L
ZnCl <sub>2</sub>	3.270 µg/L
CoCl <sub>2</sub> · 6H <sub>2</sub> O	1.43 µg/L
CuCl <sub>2</sub> · 2H <sub>2</sub> O	0.012 µg/L
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	7.26 µg/L
FeCl <sub>3</sub> · 6H <sub>2</sub> O	159.8 µg/L
Na <sub>2</sub> EDTA · 2H <sub>2</sub> O	300.0 µg/L

pH was adjusted to  $7.5 \pm 0.1$  with 0.1 N NaOH or 0.1 N HCl

<sup>a</sup> Additional nutrient required, personal communication. Dr. R.R.L. Guillard, June 1991.

Source: Miller, W.E., J.C. Greene and T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. EPA 600/9-78-018. U.S. Environmental Protection Agency, Washington, D.C.

**Table 2. Conductivity, pH, temperature and light intensity measured during the 96-hour exposure of *Selenastrum capricornutum* to NEODOL® 45-13.**

Nominal Concentration (mg/L)	pH		Conductivity (µmhos/cm)	
	0-Hour	96-Hour	0-Hour	96-Hour
Control	7.5	10.1	90	100
0.013	7.5	10.2	90	100
0.025	7.5	10.2	90	110
0.050	7.3	10.2	90	110
0.10	7.4	10.1	80	110
0.20	7.4	10.2	90	110

**Minimum/Maximum Temperature (°C)**

0 - 24-hour	24 - 48-hour	48 - 72-hour	72 - 96-hour
24/25	24/25	24/25	24/25

**Light Intensity**

	0-hour	24-hour	48-hour	72-hour	96-hour
footcandles:	350-500	350-500	350-500	350-500	350-500
lux:	3800-5400	3800-5400	3800-5400	3800-5400	3800-5400

**Table 3. Concentrations of NEODOL<sup>®</sup> 45-13 measured in the exposure solutions during the 96-hour toxicity test with *Selenastrum capricornutum*.**

Nominal Concentration (mg/L)	Measured Concentration (mg/L)		
	0-Hour	% nominal at 0-Hour	96-Hour
Control	ND <sup>a</sup>	NA <sup>c</sup>	0.0040
0.013	0.0089	68	0.0056
0.025	0.023	90	0.0064
0.050	0.038	76	0.014
0.10	0.085	85	0.016
0.20	0.20	98	0.045
Stock Solution <sup>b</sup> (20,000)	19,000	97	NA

<sup>a</sup> ND = Not detectable.

<sup>b</sup> Nominal concentration of stock solution is presented in parentheses.

<sup>c</sup> NA = Not applicable.

**Table 4. Cell density ( $\times 10^4$  cells/mL) of *Selenastrum capricornutum* after 24, 48, 72 and 96 hours of exposure to NEODOL<sup>®</sup> 45-13.**

Initial Measured Concentration (mg/L)	Cell Density ( $\times 10^4$ cells/mL)				96-Hour % Inhibition
	Observation Interval (Hours)				
	24	48	72	96	
Control A	4	16	48	102	
B	2	16	47	107	
C	3	15	48	103	
Mean(SD) <sup>a</sup>	3(1)	15(<1)	48(1)	104(3)	NA
0.0089 A	5	11	38	111	
B	7	15	43	107	
C	6	15	51	103	
Mean(SD) <sup>a</sup>	6(1)	14(2)	44(7)	107(4)	-2.7
0.023 A	3	12	30	87	
B	4	17	22	103	
C	3	14	24	115	
Mean(SD) <sup>a</sup>	3(1)	14(3)	25(4) <sup>bc</sup>	102(14) <sup>bc</sup>	2.2
0.038 A	4	14	20	77	
B	4	17	17	72	
C	3	11	19	81	
Mean(SD) <sup>a</sup>	4(1)	14(3)	19(1) <sup>bc</sup>	77(5) <sup>bcd</sup>	26
0.085 A	3	8	11	47	
B	4	10	14	51	
C	4	9	17	52	
Mean(SD) <sup>a</sup>	4(1)	9(1)	14(3) <sup>bc</sup>	50(2) <sup>bcd</sup>	52
0.20 A	1	7	8	22	
B	1	5	12	22	
C	2	8	12	26	
Mean(SD) <sup>a</sup>	1(1) <sup>b</sup>	7(2) <sup>bc</sup>	11(2) <sup>bc</sup>	23(2) <sup>bcd</sup>	78

- <sup>a</sup> Mean and standard deviation (SD) are calculated from original raw data, not from the rounded values (two significant figures) presented in this table.
- <sup>b</sup> Cell fragments were observed.
- <sup>c</sup> Bloated cells were observed.
- <sup>d</sup> Significantly different when compared to control data, according to William's Test.

**Table 5. EC10, EC50 and EC90 values for NEODOL® 45-13 calculated from results (cell density) of the 96-hour toxicity test with *Selenastrum capricornutum*.**

X = Initial measured concentration (mg/L).  
Y = Percent Inhibition (in cell density, compared with control)

24-Hour Results	EC10	EC50	EC90
EC value (mg/L):	0.13	0.21	0.29
95% Confidence Limits:	0.033 - 0.25	0.11 - 0.35	0.18 - 0.46
Regression Equation:	Probit (Y) = 1.5 + 17 X		
r <sup>2</sup> :	0.74		
N:	15		
Concentration Range <sup>a</sup> :	0.0089 - 0.20 mg/L		
48-Hour Results	EC10	EC50	EC90
EC value (mg/L):	0.029	0.14	0.68
95% Confidence Limits:	0.0064 - 0.096	0.042 - 0.63	0.19 - 6.1
Regression Equation:	Y = 100 + 58 Log(X)		
r <sup>2</sup> :	0.75		
N:	12		
Concentration Range <sup>a</sup> :	0.023 - 0.20 mg/L		
72-Hour Results	EC10	EC50	EC90
EC value (mg/L):	0.0056	0.037	0.24
95% Confidence Limits:	0.0011 - 0.020	0.0099 - 0.13	0.069 - 1.1
Regression Equation:	Y = 120 + 49 Log(X)		
r <sup>2</sup> :	0.81		
N:	15		
Concentration Range <sup>a</sup> :	0.0089 - 0.20 mg/L		

<sup>a</sup> Exposure-response relationship was judged to be linear over this concentration range; values for this concentration range were included in the linear regression.

**Table 5. Continued. EC10, EC50 and EC90 values for NEODOL® 45-13 calculated from results (cell density) of the 96-hour toxicity test with *Selenastrum capricornutum*.**

X = Initial measured concentration (mg/L).  
Y = Percent Inhibition (in cell density, compared with control)

96-Hour Results	EC10	EC50	EC90
EC value (mg/L):	0.020	0.083	0.35
95% Confidence Limits:	0.0085 - 0.043	0.038 - 0.19	0.16 - 0.92
Regression Equation:	Y = 120 + 64 Log(X)		
r <sup>2</sup> :	0.92		
N:	15		
Concentration Range <sup>a</sup> :	0.0089 - 0.20 mg/L		

<sup>a</sup> Exposure-response relationship was judged to be linear over this concentration range; values for this concentration range were included in the linear regression.

**SIGNATURES AND APPROVAL**

**SUBMITTED BY:**

Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street  
Wareham, Massachusetts 02571-1075

**PREPARED BY:**

James R. Hoberg  
James R. Hoberg  
Study Director

8/23/94  
Date

Carlene Thomas  
Carlene Thomas  
Principal Investigator

8-23-94  
Date

Susan P. Shepherd  
Susan P. Shepherd  
Coordinator, Data Management  
and Reporting Unit

23 Aug 94  
Date

**APPROVED BY:**

Donald C. Surprenant  
Donald C. Surprenant  
Program Manager  
Environmental Toxicology

8/23/94  
Date

Doreen S. Newhouse  
Doreen S. Newhouse  
Supervisor,  
Quality Assurance Unit

23 Aug 94  
Date

This final report has been signed in accordance with SLI SOP No. 4.3.07(1).

#### **4.0 APPENDIX I - STUDY PROTOCOL**

Springborn Laboratories, Inc.  
Environmental Sciences Division  
790 Main Street • Wareham, Massachusetts 02571 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-3107

TEST PROTOCOL

PROTOCOL TITLE: Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with the Freshwater Green Alga, *Selenastrum capricornutum*, Following TSCA Guideline § 797.1050

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: Shell Development Company

Address: P.O. Box 1380

Houston, Texas Phone: (713) <sup>541</sup> ~~498~~-8040 <sup>DCU</sup> ~~2-24-94~~

Sponsor Study No.: WRC Tox No. 1209

Test Substance: Neodol® 45-13

Purity: 100% CAS# or LOT#: 68151-67-7

Additional Comments and/or Modifications: \_\_\_\_\_

Frank Young 2-24-94  
Sponsor Approval Date

TO BE COMPLETED BY SLI PRIOR TO TEST INITIATION:

Testing Facility: Springborn Laboratories, Inc., 790 Main St., Wareham MA 02571

Study Director: James R. Hoberg SLI Study No.: 777.0294.0111.430

Test Concentrations: \*

Solvent Used: \* CAS# or LOT#: \*

Proposed Schedule: (Start) \* (Completion) \*

Proposed Draft Report Date: \*

James R. Hoberg 3/2/94  
Study Director Date

\* To be provided by amendment.

Springborn Laboratories Protocol #: 072993/TSCA/SELL/SEL



LETTERS AND REPORTS: Springborn Laboratories, Inc. letters and reports are issued for the exclusive use of the clients to whom they are addressed. No duplication from reports of use of the Springborn Laboratories, Inc. name is permitted except as expressly authorized in writing. Letters and reports apply only to the specific material, products or processes tested, examined or analyzed and are not necessarily indicative of the business of Springborn Laboratories or Springborn Laboratories, Inc. The liability of Springborn Laboratories, Inc. with respect to services rendered shall be limited to the amount of the consideration paid for such services and not include any consequential damages.

Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with  
the Freshwater Green Alga, *Selenastrum capricornutum*, Following TSCA  
Guideline § 797.1050

## 1.0 INTRODUCTION

The purpose of this test is to determine the effects of an alcohol ethoxylate surfactant on the growth of *Selenastrum capricornutum* under static conditions. The results of this study will be reported as the 96-hour EC10, EC50 and EC90 values: i.e., the concentrations of test substance that reduce culture density by 10, 50 and 90%, respectively, as compared with the control. The test procedures performed during the biological portions of the study will meet or exceed the standard procedures described in the U.S. EPA Toxic Substance Control Act (TSCA) Test Guidelines 797.1050 (U.S. EPA 1985) as amended in the Federal Register on 20 May 1987 (U.S. EPA 1987) and will meet the primary technical objectives of The Shell Research Limited/Sittingbourne Research Centre guidelines (SBT SOP No. 169, Edition No. 8).

## 2.0 MATERIALS AND METHODS

### 2.1 TEST ORGANISM.

- 2.1.1. **Justification for Test System.** Characteristics which make this test organism suitable for acute toxicity testing are their ease of culturing and handling, their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater algal species.
- 2.1.2. **Species.** *Selenastrum capricornutum* will be the alga used in this test. The particular strain and supplier of the test species will be identified in the final report.
- 2.1.3. **Source.** Culture conditions will be similar to the testing conditions (i.e., 24 ± 2 °C, continuous lighting at 3200 - 5400 lux (300 - 500 footcandles) and agitation rate at 100 ± 10 rpms). Cultures will be maintained in an environmental chamber and transfers will be made regularly into fresh medium to provide two- to seven-day-old cultures for test inoculations. Cultures used to inoculate the test solutions will be in logarithmic phase growth. Cultures will be maintained under the above conditions for at least the period of time from the last transfer.
- 2.1.4. **Reference Test.** In an effort to monitor the general health of the test organism culture, reference test will be conducted with *S. capricornutum* using copper nitrate as the toxicant. Results of this test will be evaluated based on measured test concentrations. A reference test will be conducted within 30 days of the definitive test (i.e., either 30 days prior to or 30 days following the definitive exposure).

## 2.2 PHYSICAL SYSTEM.

- 2.2.1. **Test Containers.** Test vessels will be 250-mL Erlenmeyer flasks containing 100 mL of test solution and will be covered with stainless steel caps which permit gas exchange. Flasks and caps will be autoclaved before use.
- 2.2.2. **Glassware Preparation.** All glassware used in testing will be thoroughly washed with detergent and rinsed with tap water. This will be followed by sequential rinsing with a 10% solution of nitric acid, acetone, distilled deionized water, isopropanol and finally distilled deionized water. The cleaned glassware will be stored in closed cabinets.
- 2.2.3. **Dilution Water.** Stock solutions used in the preparation of algal growth medium will be prepared by adding appropriate amounts of nutrients to sterile, deionized water. The stocks solutions will be stored in amber glass bottles in the dark at approximately 4°C to minimize photochemical changes, and will be renewed every six months. The test medium, Algal Assay Procedure (AAP) medium (Table I), will be prepared by adding appropriate volumes of stock solutions to sterile, deionized water. The medium will be allowed to equilibrate to test temperature before use. Each batch of medium will be adjusted to pH  $7.5 \pm 0.1$  with dilute hydrochloric acid or sodium hydroxide before use. Periodic analysis of representative samples of dilution water source will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the test organism. In addition, a sample of AAP medium will be analyzed monthly for total organic carbon (TOC) content.
- 2.2.4. **Replication and Control of Bias.** Three replicate flasks will be established for each treatment and control. Test flasks will be randomly positioned based on computer-generated random numbers, on an orbital shaker table in an environmentally-controlled chamber. Exposure flasks will be labelled by replicate and concentration or control.

## 2.3 CHEMICAL SYSTEM.

- 2.3.1. **Test Substance.** Upon arrival at Springborn Laboratories, Inc., the external packaging of the test substance will be inspected for damage. The packaging will be removed and the primary storage container will also be inspected for leakage or damage. The sample identity will be recorded and the material will be stored in the dark at approximately 20°C until used, unless specified differently by the test Sponsor.
- 2.3.2. **Toxicant Concentration Selection.** A 96-hour preliminary test will generally be conducted using five widely spaced test concentrations beginning with 1000 mg/L or the water saturation concentration. If less than 50% reduction in cell density occurs at 1000 mg/L or the water saturation concentration, a single concentration definitive test will be conducted at that maximum test concentration. If greater than or equal to 50% reduction in cell density is observed at 1000 mg/L or the water saturation

concentration, then five or more definitive test concentrations will be used. The definitive test concentrations will be selected in consultation with the Sponsor. A control, untreated algal growth medium, will be included in the test. Except for the control, the nominal concentration of test substance in each treatment will be approximately 50% of the next higher one. Definitive test concentrations will be selected to produce a sufficient response to determine an EC50. Additionally, a No-Observed-Effect Concentration (NOEC) will be defined.

- 2.3.3. **Stock Solution Preparation.** A primary stock solution at a final concentration of 1 - 2% active surfactant will be formulated on the day of test initiation in distilled deionized water. The surfactant will be heated to 50 - 60°C in a glass container until completely melted. The material will be stirred to ensure homogeneity and a glass pipet will be used to transfer the liquified material for weighing. The test substance will be weighed on an analytical balance for which a calibration log will be maintained. The stock solution will be mixed for several minutes. A Chemical Usage Log will also 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. Secondary stocks, if necessary, may be prepared in distilled water or AAP medium (if volume displacement of dilution water during test solution preparation will be greater than 1%).
- 2.3.4. **Test Solution Preparation.** Test vessels will be conditioned by rinsing with the appropriate test solution. Appropriate volumes of the test solutions will then be placed into the flasks.
- 2.3.5. **Sampling.** Samples (approximately 300-mL) will be collected from each test solution at test initiation and termination for analyses of test substance concentration. Additionally, the primary stock solution will be analyzed. Samples removed at test initiation will be collected from the freshly prepared exposure solutions before the algae is added. Test termination samples will be a composite of the three replicate solutions of each treatment level and control. The analytical samples collected at test termination will be centrifuged prior to preservation to remove algal cells. The supernatant of each centrifuged sample will be transferred to a borosilicate glass container and covered with a Teflon<sup>®</sup>-lined cap. All samples will be preserved with 1% formalin (i.e., 3 mL formalin/300 mL sample in a 700 mL bottle) and shipped within 24 hours to Battelle Ocean Sciences, Duxbury, Massachusetts for analysis. All glassware used during the sampling process will be prepared as described in Section 2.2.2.
- 2.3.6. **Analytical Chemistry.** Analyses of analytical samples will be conducted by Battelle Ocean Science, Duxbury, Massachusetts, using a Shell Development Company analytical method entitled "Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) and HPLC/ELSD (Evaporative Light Scattering Detection) in Dilute Aqueous Solutions".

## 2.4 EXPERIMENTAL CONDITIONS

- 2.4.1. **Inoculation.** Algae will be transferred aseptically from the stock cultures to each test vessel within 30 minutes of test solution preparation to provide an initial culture density of approximately  $1.0 \times 10^4$  cells/mL.
- 2.4.2. **Monitoring.** Culture density in each test vessel will be monitored at 24, 48, 72 and 96 hours after the start of the test. Algal density will be determined by cell counts using a Neubauer Improved hemacytometer and a compound microscope. One sample will be taken from each test vessel, and one count will be made on each sample. One or more hemacytometer fields, each 0.1 X 0.1 cm in surface area and 0.01 cm deep, containing 0.0001 mL of culture, will be counted for each sample until at least 400 cells or four fields are counted.
- At the time of each culture density determination, visual observations will be made of any unusual appearances of the algal cells including cell size, shape, color, occurrence of flocculation, adherence to glass walls, and/or aggregation.
- 2.4.3. **Measurement of Water Quality Variables.** Conductivity and pH in each test concentration will be measured at the start and finish of the test. At test initiation, water quality variables will be measured in the test solution remaining in the preparation vessels subsequent to filling the individual test flasks. At test termination, pH is measured in each replicate test and control solution. Conductivity will be measured at test termination in a composite solution of the three replicate solutions for each test concentration and control.
- 2.4.4. **Photoperiod.** The tests will be continuously illuminated at a light intensity of 3200 - 5400 lux (300 - 500 footcandles) using Duro-Test, Inc., Vita Lite<sup>®</sup> fluorescent bulbs. Light intensity of the test area will be measured daily.
- 2.4.5. **Temperature.** The water temperature of the test solutions will be maintained at  $24 \pm 2$  °C by controlling the air temperature within the environmental chamber. Test solution temperature will be continuously monitored with a minimum/maximum thermometer in an additional vessel containing water placed adjacent to the test vessels.
- 2.4.6. **Agitation.** The agitation rate of the orbital shaker will be monitored daily and maintained at  $100 \pm 10$  rpms.
- 2.4.7. **Test Duration.** The test will be initiated when all test and control solutions have been inoculated with algae. The test will be terminated following the 96-hour observation interval.
- 2.4.8. **Acceptance Criterion.** The control cultures must be in log phase growth throughout the 96-hour exposure period or the test will be considered unacceptable.

- 2.4.9. Evaluation of Algicidal/Algistatic Effects. At test termination the following procedure is used to differentiate between algistatic and algicidal effects. A sample of known volume of the culture is taken from the composite of the three replicate solutions of each test concentration which completely inhibited algal growth. If algal growth is not completely inhibited at any test concentration, the highest test concentration that inhibited growth is used. Sufficient fresh algal growth medium is added to dilute the test chemical to a concentration that does not affect growth. This subculture is incubated under test conditions for up to 9 days, during which time, it is examined microscopically every other day to determine whether growth has resumed. As soon as growth is observed (i.e., 10X) the subculture test is discontinued.

### 3.0 DATA EVALUATION

Cell densities will be calculated as means and standard deviations for each group of control and exposure replicates.

For a single concentration test, cell densities in the single treatment concentration will be compared with cell densities in the control, using a t-Test. Cell density in the treatment group will also be expressed in terms of percent reduction or stimulation from the control.

Data from a five concentration test will be subjected to Williams' Test (Williams 1971, 1972) to determine the No-Observed-Effect Concentration (NOEC). The NOEC is defined as the highest test concentration which causes no significant reduction in cell density when compared to the control data. Williams' Test will be preceded by Shapiro-Wilk's and Bartlett's Tests which test for normality and homogeneity of the data set. If necessary, replicate values will be transformed using square root, arcsine square root or log conversion procedures. If either Shapiro-Wilk's or Bartlett's Tests continue to fail after these conversions, Dunn's Test, a non-parametric procedure, will be used to establish the NOEC. All comparisons will be made at 95% level of certainty ( $P \leq 0.05$ ).

For each observation interval, EC10, EC50 and EC90 values (the concentrations of test substance which reduced culture density by 10, 50 and 90%, respectively) and 95% confidence limits will be determined by linear regression of response (percent reduction of culture density, as compared with control) vs. mean measured exposure concentration. Four linear regression curves will be computed based on (a) untransformed data, (b) untransformed response vs. logarithm-transformed concentration, (c) probit-transformed response vs. untransformed concentration, and (d) probit-transformed response vs. logarithm-transformed concentration. The regression line which provides the best fit of the untransformed or transformed data will be selected based on the highest coefficient of determination, i.e.,  $r^2$ . This regression equation will then be applied to calculate the EC10, EC50 and EC90 values and their 95% confidence limits, using the method of inverse prediction (Sokal and Rohlf, 1981). A computer program developed and validated at SLI will be used to assist in these computations. The concentration-response data generated during this test will be provided to the Study Sponsor in Lotus<sup>®</sup> format.

#### 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 generated at Springborn Laboratories, Inc., and draft and final report will be reviewed by the SLI Quality Assurance Unit and Study Director. All measurements (e.g., water quality) will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. A single copy of the draft report will initially be submitted to the study Sponsor for review. Upon acceptance by the Sponsor, three (3) copies of the final report will be submitted. All reports include, but will not be limited to, the following information:

- \* Springborn Laboratories, Inc., report and project numbers.
- \* Laboratory and site, the dates of testing and personnel involved in the study, e.g., Quality Assurance Unit, Program Coordinator (if applicable), Study Director.
- \* All information pertaining to the test substance which appears on the sample bottle, e.g., its source, percent active ingredient, physical properties, Sponsor's test substance I.D., and sample number.
- \* Scientific name of the test organism, strain, source, and culturing information.
- \* Results (i.e., 96-hour LC50 value and 95% confidence limits) of applicable copper nitrate reference test.
- \* A copy of the periodic analysis of the dilution water source for concentrations of toxic metals and pesticides.
- \* Range-finding study results.
- \* Test container volume, test solution volume, and inoculum culture density.
- \* Description of exposure solution preparation scheme.
- \* Description of test conditions.
- \* Criteria for determination of toxic effects and general observations on nonquantifiable effects.
- \* A table of culture density measurements for each 24-hour interval.

- \* Data on test temperatures, specific conductivity, pH, illumination and agitation.
- \* The EC10, EC50 and EC90 values and 95% confidence limits for 24, 48, 72 and 96 hours of exposure, when possible, and calculation methods used. All calculations will be based on mean measured concentrations.
- \* Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study, signed by the Study Director.
- \* Good Laboratory Practice (GLP) compliance statement (for the biological portion of the study) signed by the Study Director.
- \* Dates of Quality Assurance reviews, signed by the QA Unit.
- \* Location of raw data and final report.

#### 6.0 PROTOCOL CHANGES

All amendments to the approved protocol will be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. All deviations will be documented by the Study Director. Protocol amendments and deviations will include the reasons for the change and the impact of the change on results of the study, if any. Amendments should be authorized by the Sponsor's contact or the Study Monitor in advance of initiation of definitive test. If necessary, amendments initially may be in the form of verbal authorization, followed by Springborn's written documentation of the amendment. In such a case, the effective date of the amendment will be the date of verbal authorization.

#### 7.0 SPECIAL PROVISIONS

**GOOD LABORATORY PRACTICES (GLP):** All test procedures, documentation, records, and reports related to the biological portion of this study will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substance Control Act (FEDERAL REGISTER, Part IV, 17 August, 1989)

**TEST MATERIAL DISPOSAL:** After 60 days of the issuance of the final report, the test substance will be returned to the Sponsor's project officer, at Sponsor expense, unless different arrangements are made.

**DATA ARCHIVAL:** All raw data and the final report will be archived at Shell Development Company unless different arrangements are made.

**8.0 REFERENCES**

- Miller W.E., J.C. Green and T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. EPA 600/9-78-018. U.S. Environmental Protection Agency, Washington, D.C.
- Sokal, R.R., and F.J. Rohlf. 1981. *Biometry, 2nd Ed.* W.H. Freeman and Co., New York. 859 pp.
- U.S. Environmental Protection Agency. 1985. *Toxic Substances Control Act Test Guidelines*. Federal Register 50(188):39252-39516, September 27, 1985. Amended May 20, 1987, July 1, 1991 and July 1, 1992.
- U.S. Environmental Protection Agency 1989. *Toxic Substances Control; Good Laboratory Practice Standards; Final Rule*. (40 CFR, Part 792). Federal Register, Part III, 48(230):53922-53944, August 17, 1989.
- Williams, D.A. 1971. A test for differences between treatment means when survival 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 control. *Biometrics* 28: 519-531.

TABLE I  
COMPOSITION OF ALGAL ASSAY  
PROCEDURE (AAP) MEDIUM

Compound	Final Concentration
NaNO <sub>3</sub>	25.5 mg/L
MgCl <sub>2</sub> · 6H <sub>2</sub> O	12.16 mg/L
CaCl <sub>2</sub> · 2H <sub>2</sub> O	4.41 mg/L
MgSO <sub>4</sub> · 7H <sub>2</sub> O	14.7 mg/L
K <sub>2</sub> HPO <sub>4</sub> (K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O)	1.044 mg/L (1.368 mg/L)
NaHCO <sub>3</sub>	15.0 mg/L
H <sub>3</sub> BO <sub>3</sub>	185.52 µg/L
MnCl <sub>2</sub> · 4H <sub>2</sub> O	415.4 µg/L
ZnCl <sub>2</sub>	3.270 µg/L
CoCl <sub>2</sub> · 6H <sub>2</sub> O	1.43 µg/L
CuCl <sub>2</sub> · 2H <sub>2</sub> O	0.012 µg/L
Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O	7.26 µg/L
FeCl <sub>3</sub> · 6H <sub>2</sub> O	159.8 µg/L
Na <sub>2</sub> SiO <sub>3</sub> · 9H <sub>2</sub> O <sup>a</sup>	20 mg/L
Na <sub>2</sub> EDTA · 2H <sub>2</sub> O	300 µg/L
Na <sub>2</sub> SeO <sub>4</sub> <sup>b</sup>	1.88 µg/L

<sup>a</sup> Na<sub>2</sub>SiO<sub>3</sub> is included in medium for the diatom *Navicula pelliculosa* only.

<sup>b</sup> Additional nutrient required, personal communication, Dr. R.R.L. Guillard, June 1991.

SOURCE: Miller W.E., J.C. Greene and T. Shiroyama. 1978. The *Selenastrum capricornutum* Printz algal assay bottle test. EPA 600/9-78-018. U.S. Environmental Protection Agency, Washington, DC.

**Springborn Laboratories, Inc.**

Environmental Sciences Division

790 Main Street • Wareham, Massachusetts 02571-1075 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

**PROTOCOL AMENDMENT****AMENDMENT #:** 1**DATE:** 17 March 1994**PROTOCOL TITLE:** Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with the Freshwater Green Alga, *Selenastrum capricornutum*, Following TSCA Guideline 797-1050**SPECIES:** *Selenastrum capricornutum***STUDY SPONSOR:** Shell Development Company**TEST MATERIAL:** Neodol 45-13**SLI STUDY NO:** 777.0294.6111.430**AMENDMENT(S):**

1. The protocol requires that the following information be provided by protocol amendment.

Nominal Definitive Test Concentrations: 0.013, 0.025, 0.050, 0.10 and 0.20 mg/L

Solvent Used: Algal Assay Procedure (AAP) medium

CAS# or Lot#: Not Applicable

Proposed Schedule: (Start) 28 March 1994 (Completion) 1 April 1994

Proposed Draft Report Date: 15 May 1994

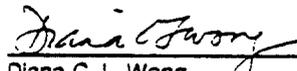
2. The protocol states that 250 mL Erlenmeyer flasks containing 100 mL of test solution will be used as the exposure system during the definitive test. Due to the analytical sample requires for this study, the test vessel size will be increased to 500 mL Erlenmeyer flasks which will contain 250 mL of solution.

Approval Signatures:

  
James R. Hoberg  
SLI Study Director

3/17/94

Date

  
Diana C. L. Wong  
Sponsor Contact

4/4/94

Date

Protocol #072993/TSCA/SHELL/SEL

Page 1 of 1



**Springborn Laboratories, Inc.**

Environmental Sciences Division

790 Main Street • Wareham, Massachusetts 02571-1075 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

**PROTOCOL AMENDMENT****AMENDMENT #:** 2**DATE:** 6 April 1994**PROTOCOL TITLE:** Alcohol Ethoxylate Surfactants: Protocol for Conducting a 96-Hour Toxicity Test with the Freshwater Green Alga, *Selenastrum capricornutum*. Following TSCA Guideline 797-1050**SPECIES:** *Selenastrum capricornutum***STUDY SPONSOR:** Shell Development Company**TEST MATERIAL:** Neodol 45-13**SLI STUDY NO:** 777.0294.6111.430**AMENDMENT(S):**

1. The proposed test start, completion and draft report dates provided in Protocol Amendment #1 have been revised to the following:

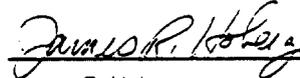
Proposed Start Date: 4 April 1994

Proposed Completion Date: 8 April 1994

Proposed Draft Report Date: 27 May 1994

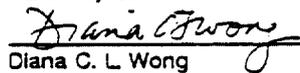
2. Protocol Amendment #1 states the test vessel size was increased to 500 mL Erlenmeyer flasks containing 250 mL of solution. In addition to the increased solution volume, an addition replicate, vessel D, will be initiated for the two lowest test concentrations, 0.013 and 0.025 mg/L, and the control to provide a sufficient analytical sample volume at test termination. The additional vessels will be maintained under the same conditions as the exposure vessels, but cell density and water quality parameters will not be monitored in the additional replicate. The sample containers for the control, 0.013 and 0.025 mg/L will be 1-L glass bottles and contain a sample volume of approximately 950 mL.

Approval Signatures:

James R. Hoberg  
SLI Study Director

4/6/94

Date

Diana C. L. Wong  
Sponsor Contact

4/13/94

Date

Protocol #072993/TSCA/SHELL/SEL

Page 1 of 1



**5.0 APPENDIX II - CERTIFICATE OF ANALYSIS**

## Shell Development Company

A Division of Shell Oil Company

Westhollow Research Center  
P. O. Box 1380  
Houston, TX 77251-1380

February 24, 1994

Pamela M. Lincoln  
Springborn Laboratories, Inc.  
790 Main Street  
Wareham, MA 02571

Dear Ms. Lincoln:

The missing information, pertaining to test substance NEODOL 45-13, that you requested of me in your letter dated February 21, is as follows:

Lot Number:	TANK TM 996
% Active Ingredient:	100%
Net Amount Shipped:	1 1/4 qt.
Molecular Weight:	Avg. 777
Expiration Date:	February 1995

Analytical characterization acquired in support of test substance NEODOL 45-13 was performed at Westhollow Research Center (WRC). Methods and procedures used follow all applicable government regulations regarding Good Laboratory Practices as stated in 40 CFR 792. All records and raw data generated by these analyses will be retained in the WRC Analytical Special Collection of Files.

Analytical methods used to characterize the test substance were Hydroxy Number (mg KOH/gm), % Water (%wt), Cloud Point, Ethylene Oxide Distribution, Polyethylene Glycol (%wt) and Carbon Number Distribution.

If you have any further questions, please feel free to contact me at (713) 544-8410

Sincerely,

A handwritten signature in cursive script that reads "Harriet Smith".

Harriet Smith

**6.0 APPENDIX III - DILUTION WATER ANALYSIS**

Well <sup>1</sup> Water Sample*		
Date Collected: 5/18/94 Date Reported: 6/10/94		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 µg/l	0.01
Beta BHC	< 0.01 µg/l	0.01
Gamma BHC - Lindane	< 0.01 µg/l	0.01
Delta BHC	< 0.01 µg/l	0.01
Heptachlor	< 0.01 µg/l	0.01
Aldrin	< 0.01 µg/l	0.01
Heptachlor Epoxide	< 0.01 µg/l	0.01
DDE	< 0.01 µg/l	0.01
DDD	< 0.01 µg/l	0.01
DDT	< 0.01 µg/l	0.01
HCB	< 0.01 µg/l	0.01
Mirex	< 0.01 µg/l	0.01
Methoxychlor	< 0.05 µg/l	0.05
Dieldrin	< 0.01 µg/l	0.01
Endrin	< 0.01 µg/l	0.01
Telodrin	< 0.01 µg/l	0.01
Chlordane	< 0.3 µg/l	0.3
Toxaphene	< 4. µg/l	4.
PCBs	< 1. µg/l	1.
Ronnel	< 0.01 µg/l	0.01
Ethion	< 0.02 µg/l	0.02
Trithion	< 0.05 µg/l	0.05
Diazinon	< 0.1 µg/l	0.1
Methyl Parathion	< 0.02 µg/l	0.02
Ethyl Parathion	< 0.02 µg/l	0.02
Malathion	< 0.05 µg/l	0.05
Endosulfan I	< 0.01 µg/l	0.01
Endosulfan II	< 0.01 µg/l	0.01
Endosulfan Sulfate	< 0.03 µg/l	0.03
<sup>1</sup> Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		

Well <sup>1</sup> Water Sample*		
Date Collected: 5/18/94 Date Reported: 6/10/94		
Analysis	Result As Received	Limit of Quantitation
Arsenic	< 0.10 mg/l	0.10
Selenium	< 0.20 mg/l	0.20
Boron	< 0.040 mg/l	0.040
Thallium	< 0.50 mg/l	0.50
Aluminum	< 0.20 mg/l	0.20
Antimony	< 0.20 mg/l	0.20
Barium	< 0.10 mg/l	0.10
Beryllium	< 0.010 mg/l	0.010
Cadmium	< 0.010 mg/l	0.010
Calcium	9.63 mg/l	0.20
Chromium	< 0.030 mg/l	0.030
Cobalt	< 0.050 mg/l	0.050
Copper	< 0.025 mg/l	0.025
Iron	< 0.10 mg/l	0.10
Lead	< 0.10 mg/l	0.10
Magnesium	2.75 mg/l	0.10
Manganese	< 0.010 mg/l	0.010
Molybdenum	< 0.050 mg/l	0.050
Nickel	< 0.050 mg/l	0.050
Potassium	1.03 mg/l	0.50
Silver	< 0.020 mg/l	0.020
Sodium	17.3 mg/l	0.40
Titanium	< 0.015 mg/l	0.015
Vanadium	< 0.015 mg/l	0.015
Zinc	< 0.025 mg/l	0.025
Total Organic Carbon***	< 1. mg/L	1.
Total Suspended Solids	< 5. mg/L	5.
<sup>1</sup> Well water supplemented by Town of Wareham water		
* Analyzed by Lancaster Laboratories, Inc.		
*** Represents "non-purgeable TOC"		

## **7.0 APPENDIX IV - ANALYTICAL METHODOLOGY**

---

**FINAL DATA REPORT**

**Study Title**

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Selenastrum capricornutum*

**Data Requirements**

United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA),  
Good Laboratory Practice Standards (40 CFR, Part 792)

**Submitted To**

Shell Development Company  
Westhollow Research Center  
Houston, TX 77251-1380

**Performing Laboratory**

Battelle Ocean Sciences  
397 Washington Street  
Duxbury, MA 02332

**Author**

Gregory S. Durell

**Study Initiation Date**

March 2, 1994

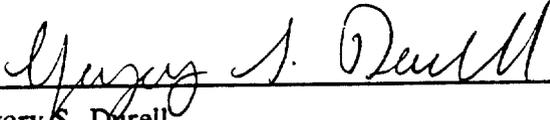
**Study Completion Date**

May 11, 1994

**Battelle Study Number**

SD-930120

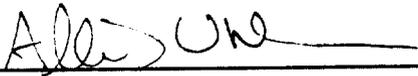
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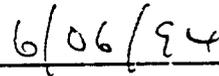
Gregory S. Durrell  
Analytical Chemistry Task Leader  
Battelle Ocean Sciences



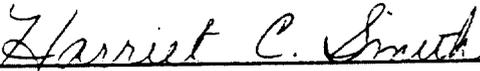
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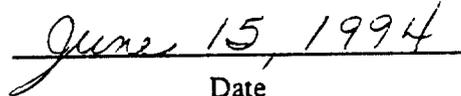
Allen D. Uhler  
Chemistry Department Manager  
Battelle Ocean Sciences



Date



Harriet C. Smith  
Project Monitor  
Shell Development Company



Date

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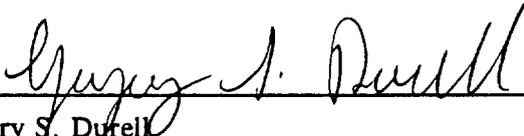
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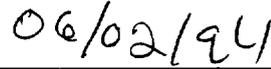
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**COMPLIANCE STATEMENT**

The work conducted at Battelle through Battelle Proposal/Agreement No. 882-H-0641 "Analysis of Non-ionic Surfactants in Water Samples by HPLC and ELSD" was performed in compliance with United States Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 CFR, Part 792), August 17, 1989.



\_\_\_\_\_  
Gregory S. Duffell  
Analytical Chemistry Task Leader  
Battelle Ocean Sciences



\_\_\_\_\_  
Date

**QUALITY ASSURANCE STATEMENT**

for

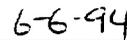
**Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Selenastrum capricornutum***

In accordance with Good Laboratory Practice Standards (40 CFR, Part 792) dated August 17, 1989, this study has been monitored by Battelle Ocean Science's Quality Assurance Unit. Study audit dates and dates when the results were reported to the Study Director and management are listed in the following table.

To the best of my knowledge, the analyses reported here accurately represent the data generated during this study.



Rosanna L. Buhl  
Quality Assurance Coordinator  
Battelle Ocean Sciences



Date

## QUALITY ASSURANCE AUDITS

Conducted for SD-930120

Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Selenastrum capricornutum*

Audit Type	Audit Date	Date of Report to Analytical Task Leader	Date of Report to Study Director	Date of Report to Management
Initiation	02/28/94 03/28/94	none	none	none
Lab Inspection	05/09/94 05/10/94 05/12/94 05/23/94	05/24/94	06/06/94	06/06/94
Data Package	06/01-02/94	06/02/94	06/06/94	06/06/94
Report Review	06/01-02/94	06/02/94	06/06/94	06/06/94

**STUDY PARTICIPANTS**

**SD-930120**

**Measurement of Neodol® 45-13 in Dilute Aqueous Samples in Support of  
Aquatic Toxicity Testing with *Selenastrum capricornutum***

---

Mr. Gregory S. Durell	Analytical Chemistry Task Leader; HPLC Analysts
Mr. Richard Restucci	Laboratory Technician, HPLC Analyst (in training)
Mr. Lyle Roberts	Laboratory Technician (in training)

---

## 1.0 INTRODUCTION

The work reported in this document is a component of aquatic toxicological testing that has been requested and initiated by the Sponsor of this study. The toxicological testing was conducted by Springborn Laboratories. Battelle Ocean Sciences was responsible for providing chemical analytical support to the toxicological testing by determining concentrations of the Test Substance in samples received from Springborn Laboratories. The results of these chemical analyses are presented in this Final Data Report.

The objective of the work reported in this document was to perform chemical analysis of aqueous samples and primary stock solutions, for the determination of concentrations of alcohol ethoxylate surfactants using the analytical method titled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*. This analytical procedure was approved by the Analytical Chemistry Task Leader on February 25, 1994, and by the Sponsor's Project Monitor on March 7, 1994.

### 1.1 Test Substance Identification

The Test Substance analyzed in this study was an alcohol ethoxylate (AE) surfactant (Neodol 45-13®). The Test Substance was provided by the Sponsor (Shell Development Company). The Sponsor was also responsible for providing Battelle with the lot number, analysis, purity, stability, storage requirements, and all other relevant chemical and physical characterization data for the Test Substance. The Test System and Test Substance identity and characterization information, and other relevant test information for this study, as provided by the Sponsor, is summarized below.

<b>Test System:</b>	<i>Selenastrum capricornutum</i>
<b>Test Substance:</b>	Neodol® 45-13
<b>Test Substance CAS#:</b>	68951-67-7
<b>Test Substance Lot#:</b>	Tank TM996 (01/23/92) 1209 (WRC Tox Sample Number)
<b>Test Substance Purity:</b>	100%

<b>Test Substance Composition:</b>	A mixture of C <sub>14</sub> -C <sub>15</sub> alcohol ethoxylates with an average of 13 moles of ethylene oxide per mole of alcohol. Traces of free ethylene oxide (less than or equal to 6 ppm) may be present in the neat Test Substance.
<b>Test Substance Analysis:</b>	The percent purity data is based on process knowledge, and research and development prior to manufacture of the Test Substance used.
<b>Test Substance Solubility:</b>	Completely soluble in water. May form gel.
<b>Test Substance Stability:</b>	Stable. A stability and holding time of two years from receipt at Battelle was assigned to the Test Substance.
<b>Test Substance Storage Requirements:</b>	Cool, dry.

## 2.0 MATERIALS AND METHODS

### 2.1 Analytical Method Description

The analytical procedure used was developed for the determination of total alcohol ethoxylate surfactants in aqueous samples. The method involves an extraction step to isolate the surfactant from a water sample and a high performance liquid chromatography (HPLC) analytical procedure to quantify the nonionic surfactant concentration. The results are reported as total alcohol ethoxylate surfactant concentrations. Concentrations of the surfactant were also determined in primary stock solutions received from the toxicology testing laboratory.

In order to analyze alcohol ethoxylate (AE) surfactants in aqueous matrices at low levels the surfactant must first be isolated from the water, concentrated, and analyzed using an appropriate method of detection to quantitate the amount of surfactant originally present in the aqueous sample. The method used employs solid phase extraction (SPE) with a C<sub>8</sub> reverse phase cartridge for isolating the analyte and concentrating the sample. After elution of the surfactant from the SPE cartridge with methyl and isopropyl alcohol it is analyzed using an HPLC procedure (octyl HPLC-column separation and methanol/water mobile phase gradient) that employs an evaporative light scattering detector

(ELSD) for analyte detection and quantification. This method quantifies total AE surfactant without distinguishing between the various individual AEs or carbon chain distributions.

The primary stock solution surfactant concentration was determined by simply diluting the sample to the appropriate concentration using methanol and analyzing it by HPLC/ELSD. No extraction step was needed.

The analytical procedure is described in detail in the document entitled *Analysis of Alcohol Ethoxylate Surfactants Using Solid Phase Extraction (SPE) Cartridges and High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC/ELSD) in Dilute Aqueous Solutions*, which was developed specifically for these analyses. This Test Substance specific document was prepared by Battelle and approved by the Sponsor on March 7, 1994. The analytical procedure document, and associated study-specific analytical information, is included in Battelle's data-package for this study. This data-package will be provided to the Sponsor and a copy maintained by Battelle Ocean Sciences.

## 2.2 Laboratory Quality Control

The water samples were processed in analytical batches of no more than 20 test samples. Each batch of test samples included four laboratory quality control (QC) samples: one procedural blank (PB), one matrix spike (MS), one matrix spike duplicate (MSD), and one blank spike (BS). The procedural blank (which consists of Milli-Q laboratory water carried through all steps and treated as other samples) sample was used to ensure that there were no significant levels of laboratory contamination. The matrix spike (test sample spiked with the target analyte), matrix spike duplicate, and blank spike (Milli-Q water spiked with the target analyte) samples were used to demonstrate laboratory accuracy and precision; these QC samples were also carried through all sample processing procedures and treated as the rest of the samples.

A portion of a non-fortified (control) test sample was used to prepare the matrix spike samples because it contained no background analyte levels yet had a sample matrix that was representative of the test samples. Milli-Q water fortified to 1% formaldehyde was used to complete the volume of matrix spike diluent needed. The blank spike was processed to determine if the accuracy/recovery of the analyte was affected by the sample matrix.

Each sequence of samples analyzed by the HPLC/ELSD instrument was initiated with a six-point multilevel calibration. Test samples followed the initial calibration in the analysis sequence, and a

calibration check standard was analyzed at least every 12 samples to verify the validity of the calibration.

Summarized below are the QC data quality objectives that applied for this study.

### Data Quality Objectives

QC Analysis	Criteria Objective
Blank spike analyte recovery	70%-120%
Matrix spike analyte recovery	70%-120%
Matrix spike/spike duplicate precision	≤30% RPD
Procedural blank	< 1 × limit of quantification (LOQ)
Instrument multilevel calibration	Correlation coefficient > 0.995
Instrument calibration check	15% RPD in determined and actual standard concentration

## 2.3 Calculations

### Sample Concentration Calculations

An external standard method of calibration and quantification was used. Sample extract concentrations were determined by applying the multilevel quadratic calibration equation using a chromatography data system on which the analytical data were acquired during the instrumental analysis. A six-point calibration curve which bracketed the expected concentration range of exposure samples was generated at the initiation of the HPLC analysis. Calibration standard concentrations were approximately 22, 44, 83, 122, 164, and 204  $\mu\text{g/mL}$ . Original water sample concentrations were subsequently determined by applying the water extraction volume (WEV) and pre-injection volume (PIV) information. The PIV of the PB, controls, and 13, 25 and 50 parts per billion (ppb) nominal concentration samples was 0.40 mL. For the BS, MS, and MSD samples and samples with nominal concentrations of 100 and 200 ppb the PIV was 1.00 mL. Analyte concentrations of the original water samples were determined in ppb. Analyte concentration of the primary stock solution samples were determined in parts per million (ppm).

$$\text{Determined Water Sample Concentration (ppb)} = \text{EC} \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$$\text{Primary Stock Solution Concentration (ppm)} = \text{EC} \times \text{DIL VOL}_1 \times (1/\text{DIL VOL}_2)$$

EC = Extract (HPLC sample) concentration ( $\mu\text{g}/\text{mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

DIL VOL<sub>1</sub> = Final volume of diluted Primary Stock subsample (mL)

DIL VOL<sub>2</sub> = Volume of Primary Stock subsample taken for the dilution (mL)

### Quality Control Sample Calculations

Two separate calculations were performed on the Quality Control (QC) sample data. Percent recoveries were determined for the blank spike and matrix spike samples, and the relative percent difference (%RPD) between the two percent recovery values was determined for the matrix spike/duplicate sample pair.

$$\% \text{ Recovery} = \text{WC}_D \times (1 / \text{WC}_S) \times 100\%$$

$$\% \text{ RPD} = [ \% \text{ REC}_{\text{MS}} - \% \text{ REC}_{\text{MSD}} ] \times (2 / (\% \text{ REC}_{\text{MS}} + \% \text{ REC}_{\text{MSD}})) \times 100\%$$

WC<sub>D</sub> = Determined water sample concentration (ppb) — calculated as shown above

WC<sub>S</sub> = Spiked water sample concentration (ppb) — prepared concentration

%REC<sub>MS</sub> = Percent recovery of the matrix spike sample

%REC<sub>MSD</sub> = Percent recovery of the matrix spike duplicate sample

### Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) was determined for the analyses. The target analyte had to provide a detector response of a minimum 3:1 signal:noise ratio to be considered detected.

The water sample equivalent LOD was calculated by comparing the peak height equivalent to a signal:noise ratio of 3:1 in the sample to the peak height of the analyte in the low-level calibration standard, and applying the water extraction volume (WEV) and the pre-injection volume (PIV), as shown previously. The LOD was determined using two samples with the lowest nominal concentration and averaging the values obtained in the two determinations.

The LOQ was determined as the water sample concentration equivalent to a final extract concentration that is the same as the low calibration standard. The LOQ was determined using the PIVs and WEVs used for the samples with the lowest nominal concentrations.

$$\text{LOD (ppb)} = H_{3x} \times (C_{LS}/H_{LS}) \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$$\text{LOQ (ppb)} = C_{LS} \times \text{PIV} \times (1/\text{WEV}) \times 1000$$

$H_{3x}$  = Peak height equivalent to  $3 \times$  the noise in the sample

$H_{LS}$  = Peak height of analyte in the low-level calibration standard

$C_{LS}$  = Concentration of analyte in the low-level calibration standard ( $\mu\text{g/mL} = \text{ppm}$ )

PIV = Pre-injection volume (mL)

WEV = Water extraction volume (mL)

### 3.0 RESULTS

#### 3.1 Analytical Results — Toxicological Test Samples

The results of the chemical analyses of the samples received from the toxicological testing laboratory are presented in Table 1. The analyses of the test samples was performed in one analytical batch. Table 1 also presents the data for the Primary Stock Solution analysis.

The measured analyte concentrations in the test samples that had been fortified with the Test Substance ranged from 6 ppb (for sample ND69 and ND 70; samples with a nominal concentrations of 13 and 25 ppb, respectively) to 195 ppb (for sample ND66, a sample with a nominal concentration of 200 ppb). The measured concentrations were between 16 percent (sample ND72) and 97 percent (sample ND66) of the nominal concentration. On an average, the concentrations in the  $t=96$  hour samples are lower than the  $t=0$  samples, suggesting that there may be a some loss of the analyte with time.

Some interference with the Neodol® 45-13 signal/peaks was evident in the HPLC/ELSD chromatogram of samples ND68, ND69, ND70, and ND71 — the extract of these samples were also visibly green. Additionally, a small amount of the analyte was detected in one of the two toxicological control samples; 4 ppb was measured in sample ND68, the  $t=96$  hr control. This measured concentration in ND68 may be a result of the coeluting matrix components that were evident in this sample (and in samples ND69, ND70, and ND71) and may not necessarily be test substance in the sample. The matrix components likely contributed approximately 5 to 10 ppb to the concentration data reported for the  $t=96$  hour samples.

**Table 1. Neodol® 45-13 Concentrations in Samples Received from the Toxicological Testing Laboratory**

Battelle Sample ID	Test Sample Time/Type	Nominal Conc. (ppb)	Measured Conc. (ppb)
<b>Batch #1</b>			
ND61	t=0	0	ND
ND62	t=0	13	8.9
ND63	t=0	25	22.6
ND64	t=0	50	37.8
ND65	t=0	100	85.4
ND66	t=0	200	195
ND68	t=96	0	4.0
ND69	t=96	13	5.6
ND70	t=96	25	6.4
ND71	t=96	50	13.8
ND72	t=96	100	15.7
ND73	t=96	200	45.2
<b>Primary Stock Solution</b>		(ppm)	(ppm)
NH13	t=0, stock	20,000	19,490

ND: Not detected; <LOD.

The concentrations measured for the Primary Stock Solution was slightly lower than the expected concentrations, with measured concentration of 19,490 ppm for the sample which had a nominal/expected concentrations of 20,000 ppm. The measured Primary Stock Solution concentration was 3 percent lower than the expected concentration.

### 3.2 Analytical Results — Quality Control Samples

All quality control objectives were met for this work. The six-point multi-level instrument calibration used had a correlation coefficient of 0.999213 for the quadratic equation, and the continuing calibration check analyses ranged from 5.4 to 11.6 relative percent difference between the determined and actual standard concentration.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as described in Section 2.3, and were as follows.

#### Limit of Detection and Limit of Quantitation

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---

Limit of Detection (LOD)	2.5 ppb
Limit of Quantitation (LOQ)	12 ppb

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The concentrations for all samples with anticipated analyte concentrations (i.e., all samples except the laboratory procedural blanks and toxicological test control samples) had measurable levels of analyte and were determined to be above the LOD. Four samples (ND62, ND68, ND69, and ND70) had a detectable (i.e., above the LOD) amount of target analyte/signal which was determined to be below the LOQ — 9, 4, 6, and 6 ppb, respectively, detected versus an LOQ of 12 ppb.

**Table 2. Laboratory Quality Control Sample Analysis Results**

<b>Battelle Sample ID</b>	<b>QC Sample Type</b>	<b>Recovery (%)</b>
<b>Batch #1</b>		
NH30PB	Procedural Blank	ND
NH31BS	Blank Spike	90.8
NH32MS	Matrix Spike	89.3
NH33MSD	Matrix Spike Duplicate	85.5
	MS/MSD %RPD:	4.3

ND: Not detected; &lt;LOD.

The results of the laboratory quality control (QC) sample analyses are presented in Table 2. The target analyte was not detected in the procedural blank sample. The analyte recovery in the blank spike (BS) sample was 91%. The analyte recovery in the two matrix spike (MS/MSD) samples were 89% and 86%, and these data suggest that there were no significant matrix effects on the analytical procedure. Acceptable precision was observed, with a relative percent differences, in the measured analyte recoveries for the MS/MSD duplicate analyses, of 4%.

The QC data indicate that the laboratory analysis was in control for this work. There were no identified circumstances or occurrences during the conduct of this work that may have affected the quality or integrity of the data.

#### 4.0 ARCHIVING OF DATA

Study records that will be maintained by Battelle include, but are not limited to:

- Verified copies of all raw data and documentation records
- Verified copy of the signed and approved Analytical Chemistry Method, and associated amendments and deviations
- All correspondence, memos, or notes pertaining to the study
- Verified copy of the signed Final Data Report
- Test Substance records, including receipt and inventory, and physical and chemical characterization data, as supplied by the Sponsor

All project files, including verified copies of the raw data and the Final Data Report, will be archived by Battelle after the submission of this Final Data Report. The Battelle Quality Assurance Unit manages the limited-access data archival. Additionally, a small amount of Test Substance will be archived by Battelle.

**APPENDIX A**

**Deviations to Analytical Method**

---

BATTELLE OCEAN SCIENCES  
Miscellaneous Documentation Form

Page 1 of 1

---

Project Title: Gap-Filling Project

Study Number: SD-930120

Battelle Project Number: N8328-0002

This is a Deviation to Analytical Method:

Yes X No       

Entered by: Gregory S. Durell

Date: May 26, 1994

---

Subject: Miscellaneous Deviations to Analytical Method

- The temperature recorded for Refrigerator #2, where standards and samples were stored, ranged from 1 to 10°C in March and April. Unextracted water samples were to be stored at approximately 4°C, as indicated in the Analytical Procedure Document. Although this temperature range is larger than what may be considered covered by "approximately 4°C", it is not expected to have impacted the integrity of the samples or results, because of the stability of the test substance.
- The nebulizer gas flow (nitrogen) for the HPLC detector (ELSD) was maintained at 9 L/min and not 11 L/min as specified in the Analytical Procedure Document. This analytical modification was requested by the Sponsor, based on method verification work performed at the Sponsor's laboratory, in order to achieve the necessary sensitivity. This method modification was requested by the Sponsor by telephone on 04/06/94 and confirmed by telephone on 04/08/94, as indicated in the Analytical Task Leader's telephone log.

Approved: Gregory S. Durell

Date: 06/03/94

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**BATTELLE OCEAN SCIENCES**  
Miscellaneous Documentation Form

Page 1 of 1

**Project Title:** Gap-Filling Project

**Study Number:** SD-930120

**Battelle Project Number:** N8328-0002

**This is a Deviation to Analytical Method:**

Yes X No \_\_\_\_\_

**Entered by:** Gregory S. Durell  
Richard P. Restucci

**Date:** May 26, 1994  
June 3, 1994

**Subject:** Miscellaneous Deviations

- The monthly calibration with a 70 g weight, as specified in SOP 3-073, was not performed on the Mettler AC100 balance that was used to prepare standards for this study. However, the balance was always calibrated on the day it was used with a weight of suitable for representing the weight actually being measured for this study.
- According to the Project-specific Analytical Information memo, seven calibration levels with an approximate concentration range of 20 - 380 ug/mL were to be analyzed prior to sample analysis. However, the actual concentration range analyzed with the Selenastrum study was 20 - 204 ug/mL.
- The Project-specific Analytical Information memo for the Selenastrum study specifies that 750 mL of sample be extracted for the procedural blank, control, and test levels 1-3. For two samples, ND64 and ND71, the actual volume extracted was 700 mL and 600 mL respectively, because the volume of water sample received from the toxicology laboratory was less than anticipated.

**Approved:** ADUW

**Date:** 6/06/94