STUDY TITLE

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach

DATA REQUIREMENT

OECD Guideline 203 and
U.S. EPA OPPTS 850.1075

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STUDY INITIATION DATE

24 February 2009

STUDY COMPLETION DATE

16 September 2010

SPONSOR

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005

PERFORMING LABORATORY

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STUDY IDENTIFICATION

ABC Study No. 64406

STATEMENT OF GLP COMPLIANCE

Compound: Naphthenic Acids

Study Title: Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach

The study described in this report, with the following exceptions, was conducted in compliance with the following Good Laboratory Practice Standards:

Organization for Economic Co-operation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act; Good Laboratory Practice Standards; Final Rule (40 CFR, Part 792).

1) The test substance characterization was not conducted in accordance to the stated Good Laboratory Practices.

2) The latest water characterizations performed in August 2009 were not performed in accordance to the stated Good Laboratory Practices.

3) Analyses conducted by the University of Alberta were not conducted in accordance to the stated Good Laboratory Practices.

These were the only exceptions to the stated GLP principles and they did not adversely affect the study integrity or the interpretation of the results generated from this study.

The original raw data and the study plan were provided to the American Petroleum Institute with the final report. Copies of all data in support of this report were retained at ABC Laboratories, Inc. along with original facility records and a copy of the final report and the study plan.

[redacted] 16Sept10
Date

[redacted]
ABC Laboratories, Inc.

[redacted] 16 Sept 2010
Date

[redacted]
ABC Laboratories, Inc.

Sponsor's Representative:

[redacted] 15 Nov 2010
Date

[redacted]
American Petroleum Institute

QUALITY ASSURANCE STATEMENT

ABC's Quality Assurance Unit reviewed Study No. 64406 entitled "Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach," for the American Petroleum Institute. The following inspections/audits were conducted on this study.

Date of Study-Based Inspection	Phase Inspected	Date Reported to Study Director	Date Reported to Management
27 Feb 09	Protocol	27 Feb 09	27 Feb 09
10 Dec 09	Procedure: 72 hr. Bio/Obs.	11 Dec 09	14 Dec 09
08 Jan 10	Raw Data & Draft Report	08 Jan 10	20 Jan 10
16 Sep 10	Final Report	16 Sep 10	16 Sep 10

These audits indicate that the report is an accurate reflection of the study as it was conducted by ABC Laboratories, Inc.

[redacted]

16 Sep 10
Date

ABC Laboratories, Inc.

STUDY PERSONNEL

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STUDY SUMMARY

Study Sponsor:	American Petroleum Institute
Study Title:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach
Location of Study:	ABC Laboratories, Inc. 7200 East ABC Lane Columbia, Missouri 65202 Department of Biological Sciences Z-207 Biological Sciences Centre 116th Street and 85th Avenue University of Alberta Edmonton, Alberta T6G 2R3 Canada
ABC Study No.:	64406
Test Substance:	Naphthenic Acids (CAS# 1338-24-5)
Test Species:	Fathead Minnow, *Pimephales promelas*
Definitive Dates (In-Life):	December 7 to 11, 2009
Length of Study:	96 Hours
Nominal Loading Rates:	0 (control), 1.3, 2.5, 5.0, 10, and 20 mg naphthenic acids/L.
Mean Measured Concentrations:	< MQL (control), 0.900, 2.08, 3.22, 6.04, and 13.8 mg naphthenic acids/L
Environmental Conditions:	Temperature: 21.6 to 23.6°C DO Concentrations: 7.1 to 9.3 mg/L (89 to 111% saturation) pH: 8.0 to 8.4 Total Alkalinity: 148 mg $CaCO_3$/L Total Hardness: 134 mg $CaCO_3$/L Specific Conductivity: 330 µS/cm Photoperiod: 16-hr light:8-hr dark Light Intensity: 523 lux

Results Based on Nominal Loading Rates of Naphthenic Acids:

24-Hour LL_{50} = >20 mg/L
48-Hour LL_{50} = 11 mg/L with 95% confidence limits (CL): 8.4 and 15 mg/L
72-Hour LL_{50} = 11 mg/L (95% CL: 8.4 and 15 mg/L)
96-Hour LL_{50} = 9.0 mg/L (95% CL: 6.6 and 12 mg/L)
96-Hour NOELR= 5.0 mg/L

Results Based on Mean Measured Concentrations of Naphthenic Acids :

24-Hour LC_{50} = >13.8 mg/L
48-Hour LC_{50} = 7.22 mg/L (95% CL: 5.53 and 9.43 mg/L)
72-Hour LC_{50} = 7.22 mg/L (95% CL: 5.53 and 9.43 mg/L)
96-Hour LC_{50} = 5.62 mg/L (95% CL: 2.47 and 10.6 mg/L)
96-Hour NOEC = 3.22 mg/L

1.0 INTRODUCTION

The American Petroleum Institute contracted ABC Laboratories, Inc., to conduct a 96-hour static-renewal toxicity test with fathead minnow exposed to water accommodated fraction (WAF) preparations of naphthenic acids (CAS# 1338-24-5). The criterion for effect was mortality (death). Results of the test are expressed as a 96-hour median lethal concentration (LC_{50}), the concentration of the test substance estimated to cause 50 percent mortality in the test population at 96 hours and as a 96-hour median lethal loading rate (LL_{50}), the WAF loading rate estimated to cause 50 percent mortality (4). A secondary objective was to determine the 96-hour no-observed-effect concentration (NOEC) and no-observed-effect loading rate (NOELR), if possible. The NOEC is defined as the highest concentration of test substance, among those tested, at which there is an absence of statistically significant mortality and sublethal effects. Likewise, the NOELR is the WAF loading rate that corresponds to the NOEC.

2.0 MATERIALS AND METHODS

2.1 Test and Reference Substance

A sample of the test substance, naphthenic acids (CAS# 1338-24-5); EPL P/A #1203-000 (collected from Drum #2), was received from EPL Archives, Inc. on January 20, 2009 and was stored at room temperature. An expiration date of the sample was not provided. The sample was assigned ABC reference number TS-22856. The Material Sample Safety Data Sheet (MSDS) described the test substance as an amber-colored liquid and stable under normal storage conditions. The MSDS and a profile of the physical chemical specifications of the test substance provided by the original supplier is given in Appendix A. This material was used to prepare all test solutions, matrix spiking solutions, and analytical standards. All solution preparations were based on total product.

2.2 Test Organism

Fathead minnows (*Pimephales promelas*) were obtained from an in-house culture. The fish were fed a commercially available fish food at least one time daily. A daily record of fish observations during the culture period, along with any prophylactic or therapeutic disease treatments, was maintained. Fathead minnows were maintained in the dilution water at the test temperature for eight days before the definitive test initiation. There was no mortality or treatments with prophylactics for two weeks prior to initiation of the study. The fish were not fed for 48 hours prior to initiation of the definitive test or during the subsequent test period. The control group of fish were measured and weighed at test termination. Fish ranged from 10 to 11 mm in standard length (mean and SD = 11 ± 0.55 mm) and 0.0065 to 0.0085 g in blotted wet weight (mean and SD = 0.0074 ± 0.00082 g). The instantaneous biomass loading rate did not exceed 0.026 g of fish tissue per liter of test solution.

2.3 Dilution Water

The dilution water was a laboratory freshwater prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis. These waters were blended to yield a total hardness of 130 to 160 mg/L as $CaCO_3$. The water was filtered through a sediment filter

prior to use. Chemical characterization of a representative sample of the base water, i.e., ABC well water, used to prepare the dilution water is presented in Appendix B.

2.4 Biological Test Methods

Test procedures followed the ABC test protocol entitled, "Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach," with amendments and deviation (Appendix C). This protocol was based on the step-down approach proposed by Jeram (1), OECD guideline 203 (2), and U.S. EPA OPPTS guideline 850.1075 (3). Modifications to the regulatory guidelines were made to address the testing of insoluble and complex mixtures (4, 5). This included adopting the WAF method of preparing exposure solutions, which is the preferred method when testing multi-component substances that are only partially soluble in water. By definition, the term WAF is applied to aqueous media containing only the fraction of multi-component substances that is dissolved and/or present as a stable dispersion or emulsion. A WAF equilibration study was done in advance of the toxicity tests to determine the optimum mixing time required to achieve equilibration of naphthenic acids dissolution in water. This is reported in ABC Study No. 64403 (10).

2.4.1 Exposure System

The range-finding test was conducted in 3.8-L glass jars containing approximately 2.8 L of control or test solution, and the limit test at the upper threshold concentration and definitive test were conducted in 3.8–L glass jars containing approximately 2 L of control or test solution. The test chambers were covered with plastic Petri dishes. A single test chamber was used for the control and all treatment levels for both the range-finding and definitive testing. The test chambers were labeled with study number, treatment, and replicate during the definitive test. The test chambers were placed in a temperature-controlled water bath with a target temperature range of 22 ± 1 ºC. Fluorescent lighting was maintained on a 16-hour daylight photoperiod with 30-minute simulated dawn and dusk periods. The light intensity during the definitive test was 523 lux as measured with a LI-COR Model LI-189 light meter equipped with a photometric sensor at test initiation.

2.4.2 Limit Test at the Upper Threshold Concentration

A limit test was initiated on November 9, 2009 at nominal WAF loading rates of 0 (control) and 24 mg naphthenic acids/L based upon the upper threshold concentration (UTC) determined from the studies with the water flea, *Daphnia magna*, (ABC Study No. 64404; 48-hr EL_{50} value of 24.0 mg/L from nominal naphthenic acid loading rates (7)) and the unicellular green alga, *Pseudokirchneriella subcapitata*, (ABC Study No. 64405; 72-hr EL_{50} value of 23.8 mg/L from nominal naphthenic acid loading rates (8)). The UTC was determined as the median effective loading rate (EL_{50}) for the most sensitive test species. At 24-hours, mortality was 0 and 100% in the 0(control) and 24 mg/L WAF treatments and the study was terminated. The 0-hour analytical measurement of the 24 mg/L WAF solution showed 21 mg/L in the dissolved phase. This equates to 88% recovery based on the loading rate, which was consistent with what was observed in the method validation and WAF equilibration trials. The measurement of the control solution was <MQL.

Because this testing indicated that the fathead minnows were more sensitive to the test substance exposure than the *Daphnia* or green algae, a range-finding and definitive testing were necessary to determine the 96-hour LC_{50} of the naphthenic acids. This is in agreement with the step-down testing approach described by Jeram, et al. (1).

2.4.3 Range-Finding Test

A static-renewal range-finding test was conducted from November 16 to 20, 2009 at nominal naphthenic acid loading rates of 0 (control), 1.0, 5.0, and 15 mg/L. One WAF at each loading rate was prepared by adding the appropriate amount of test substance to 4.0 L of dilution water in a clean 5-L glass carboy. The control WAF preparations consisted of dilution water only. Each carboy contained a 2-inch Teflon-coated stir bar and was covered with a screw cap. The WAF preparations were allowed to stir for 24 hours. The stirring speed was adequate so that the vortex was ~30 to 50% of the solution depth. After the stirring period, the stirring was stopped and each preparation was allowed to settle for approximately one hour before collection. To collect the WAF products, the solutions were drained from the carboys with a glass siphon. The first ~100 mL of solution from each WAF was drained into a waste container. The remaining solutions were collected into appropriately-labeled test chambers. This procedure for preparing test solutions was repeated to prepare fresh solutions at the 24, 48, and 72-hour time points of the exposure. The control and all test solutions were clear and colorless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

Five fish were added to each test chamber at the start of the test. The animals were observed after 24, 48, 72, and 96 hours. After 96 hours of exposure, mortality was 0, 0, 20, and 80% in the 0 (control), 1.0, 5.0, and 15 mg/L nominal naphthenic acid loading rate treatments. Sublethal effects included fish exhibiting a loss of equilibrium and were noted in the 15 mg/L treatment throughout the test.

2.4.4 Definitive Test

The definitive test was conducted from December 7 to 11, 2009 at nominal naphthenic acid loading rates of 0 (control), 1.3, 2.5, 5.0, 10, and 20 mg/L. A WAF was prepared at each loading rate in clean 5-L glass bottles. Approximately 0.0052, 0.0100, 0.0200, 0.0400, and 0.0800 g of naphthenic acid was added to 4.0-L volumes of dilution water for nominal naphthenic acid loading rates of 1.3, 2.5, 5.0, 10, and 20 mg/L, respectively. The control WAF preparations consisted of dilution water only. Each carboy contained a 2-inch Teflon-coated stir bar and was covered with a screw cap. The WAF preparations were allowed to stir for 24 hours. The stirring speed was adequate so that the vortex was ~30 to 50% of the solution depth. After the stirring period, the stirring was stopped and each preparation was allowed to settle for approximately 1 hour before collection. To collect the WAF products, the solutions were drained from the carboys with a glass siphon. The first ~100 mL of solution from each WAF was drained into a waste container. The remaining solutions were collected into appropriately-labeled test chambers. This procedure for preparing test solutions was repeated to prepare fresh solutions at the 24, 48, and 72-hour time points of the exposure.

The definitive test was conducted for 96 hours commencing when fish were added to the test chambers. The fish were impartially added one at a time proceeding from the control to the high

test substance treatment and repeating these steps until seven fish had been added to each test chamber. A total of seven fish were distributed to each test chamber resulting in a total of seven fish for each test substance treatment.

Test chambers for the control and all test treatments were 3.8-L glass jars with approximately 2.0 L of the appropriate solutions prepared at each solution renewal period. Fish were transferred daily from "old" to "new" solutions using a soft mesh net or by decanting off most of the old solution and adding the fish and remaining solution to the new solution. Transfers began with the control and then proceeded from lowest concentration to the highest concentration. Observations for mortality, moribundity, and sublethal responses were made every 24 hours (±1 hour) for the duration of the test.

Specific conductivity, total alkalinity, and total hardness were measured in a sample of the dilution water collected at test initiation. Specific conductivity was measured with a WTW Cond 330i conductivity/salinity meter. Total alkalinity and total hardness were measured using titrimetric methods adapted from Standard Methods (6). Temperature, dissolved oxygen concentration, and pH were measured in all test chambers daily throughout the test. The pH and temperature were measured with a WTW Model pH 330i meter. The dissolved oxygen was measured with a WTW OXi 330i dissolved oxygen meter. No aeration was provided to the test chambers. An electronic data logger and thermistor probe was placed in the control replicate and was used to make a continuous recording of the temperature during the test.

2.5 Analytical Test Method

Range-finding and definitive test solutions were analyzed for the concentration of naphthenic acid using Fourier transform infrared spectroscopy (FT-IR). Analysis was accomplished based on the method described by Jivraj et al. (9) and developed at ABC Laboratories (ABC study no. 64403 (10). Details of the sample preparation and method of analysis are described below.

2.5.1 Preparation of Analytical Standard and Matrix Spiking Solutions

A primary stock solution of the test substance was prepared on April 13, 2009 by weighing 10,001.0 mg of naphthenic acids into a 100-mL class A volumetric flask and bringing the flask to volume with acetonitrile for a concentration of 100 mg naphthenic acids/mL. Subsequent dilutions of this primary stock solution were prepared in acetonitrile. The primary stock and dilutions were used for quality control (QC) fortification samples during the definitive test. All solutions were stored at room temperature when not in use.

A primary stock solution of the test substance was prepared on March 19, 2009 by weighing 507.7 mg of naphthenic acids into a 100-mL class A volumetric flask and bringing the flask to volume with methylene chloride for a concentration of 5,080 mg naphthenic acids/L. Subsequent dilutions of this primary stock solution were prepared in methylene chloride. The dilutions were used to prepare analytical standards for this analyte. All solutions were stored at room temperature when not in use.

2.5.2 Test Solution Analysis

A volume of 500 mL was collected and transferred to 1,000-mL separatory funnel. Each sample was acidified with concentrated sulfuric acid to a pH level of 2.5 ± 0.1. A 100-mL volume of methylene chloride was added to each sample and the samples were shaken to mix. After approximately one minute of shaking, the sample phases were allowed to separate. The methylene chloride (lower layer) was filtered through anhydrous sodium sulfate and collected in a 500-mL flat-bottomed flask. The remaining aqueous sample was extracted a second time following the same procedure. The methylene chloride phase from the second extraction was filtered into the original flask containing the first filtrate. Each sample was then evaporated to dryness using a rotary evaporator and quantitatively transferred to 15-mL culture tubes using two separate 5-mL aliquots of methylene chloride. The samples were then evaporated to dryness under a gentle stream of nitrogen and then reconstituted with an appropriate volume of methylene chloride. Dilutions were made using methylene chloride, if necessary, to produce an analyte concentration that was within the range of the standard curve. The samples were vialed and analyzed by FTIR. QC fortifications were prepared in a similar manner after control medium had been fortified with the test substance.

2.5.3 Instrumentation Conditions

Sample analysis was performed using a FT-IR system equipped with the following analytical parameters:

Manufacturer: Thermo Nicolet
Model: Avatar 360
Software: Omnic 32
IR Cell: Thermo Scientific, KBr 1.0 mm sealed cell
Cell Holder: Thermo Scientific
Dry Nitrogen Gas Used to Protect the IR Cell Between Runs: Yes
Scan Times: 64
Scan Range: 4000-400 cm^{-1}
Scan Model: Absorbance
Resolution: 4 cm^{-1}
Wave Number of Interest: 1743 cm^{-1}
Solvent Used for Background Collection: Methylene chloride

2.5.4 Calculations

Naphthenic acid concentrations were determined directly from the standard curve by the following equation:

$$\frac{\left(\begin{array}{c}\text{µg/L or mg/L equivalents for}\\ \text{test substance from standard}\\ \text{curve equation}\end{array}\right)\left(\begin{array}{c}\text{sample volume}\\ \text{in mL for}\\ \text{chromatography}\end{array}\right)}{\left(\text{sample volume in mL before preparation}\right)} = \begin{array}{c}\text{µg/L or}\\ \text{mg/L}\end{array} = \begin{array}{c}\text{ppb or}\\ \text{ppm}\end{array}$$

The standard curve equation is of the form: y = mx + b

where:

y = peak response
m = slope of the standard curve
x = mg/L
b = y-intercept

Example calculation for the 2.5 mg/L nominal naphthenic acid loading rate WAF sample at 0 hours during the definitive test:

Standard Curve: y = 0.000161x + 0.008528
Sample Peak Response: 0.0499
Concentration from standard curve: 257 mg/L

Volume for Analysis: 4 mL
Sample Volume: 500 mL

The concentration of naphthenic acid in the sample was calculated by the following equation:

$$\frac{(257\ \text{mg/L})(4\ \text{mL})}{500\ \text{mL}} = 2.06\ \ \text{mg/L}$$

Recovery was calculated as a percentage of the corresponding nominal concentration, as shown for the 2.5 mg/L WAF sample at 0 hours during the definitive test:

$$\frac{2.06\,\text{mg/L}}{2.5\,\text{mg/L}} \times 100 = 82\%$$

The minimum quantifiable limit (MQL) was determined from the following equation:

$$\frac{\left(\begin{array}{c}\text{low standard}\\ \text{concentration mg/L}\end{array}\right)\left(\begin{array}{c}\text{analysis}\\ \text{volume (mL)}\end{array}\right)}{\left(\begin{array}{c}\text{sample}\\ \text{volume (mL)}\end{array}\right)} = \text{MQL expressed as mg/L}$$

Lowest standard concentration: 75.0 mg naphthenic acids/L
Analysis volume: 4 mL
Sample volume: 500 mL
therefore:

$$\text{MQL} = \frac{(75.0\ \text{mg/L})(4\,\text{mL})}{(500\,\text{mL})} = 0.600\,\text{mg/L}$$

2.6 Statistical Analysis

All statistical analyses were performed with SAS software (version 9.1). Estimates of LL_{50} and LC_{50} values and their 95% confidence limits were calculated using the probit method and Trimmed Spearman-Karber method. When the P value for Goodness of Fit was >0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting. The no-observed-effect concentration and loading rate (NOEC and NOELR) was determined by using a Fisher's exact test. A Hochberg adjustment was used to control the experiment wise error rate for the Fisher's test at the same alpha level.

2.7 Characterization and Stability of Naphthenic Acids WAFs by Analysis of Z-number and Carbon Number Distribution

As part of the characterization of naphthenic acids in the WAF solutions, Dr. Phillip M. Fedorak (Department of Biological Sciences, University of Alberta, Edmonton, Alberta Canada) was retained by the Study Sponsor to perform Gas Chromatography-Mass Spectrometry (GC-MS) analysis of a representative WAF preparation. At the initiation of the definitive test, samples of the Day 0 WAF solutions were collected and shipped to the University of Alberta, Department of Biological Sciences for analyses.

While not a quantitative technique as employed here, the GC-MS analyses allowed the discrimination of the naphthenic acids in the WAFs into relative abundances of each ion corresponding to the general formula for naphthenic acids, $C_nH_{2n+z}O_2$, where n is the carbon number and Z is zero or a negative even number defining the hydrogen deficiency due to cyclization. Although no analytical method exists whereby each individual naphthenic acid molecule is identified, the GC-MS method results in a distribution of families of molecules having similar carbon numbers and Z-numbers. Details of these analyses are provided in Appendix D.

3.0 RESULTS AND DISCUSSION

3.1 Analytical Results

3.1.1 FTIR Analyses – Range-Finding Test

Measured concentrations of naphthenic acids in the test substance WAF solutions at test initiation of the range-finder were 0.760, 3.98, and 12.0 mg naphthenic acids/L, which represented recoveries of 76 to 80% of the nominal loading rates. The measured concentrations in old test substance WAF solutions at 24 hours were 0.745, 3.83, and 11.4 mg naphthenic acids/L, which represented recoveries of 75 to 77% of the nominal loading rates. Recoveries from QC fortifications ranged from 81 to 110% of the nominal concentrations, with the exception of the low spike for the 0-hour analysis (421%), which was believed to be contaminated during sample preparation. The analytical results for the range-finder test are summarized in Table 1.

3.1.2 FTIR Analyses – Definitive Test

Measured concentrations of naphthenic acids in the test substance WAF solutions at test initiation of the definitive test were 0.795, 2.06, 3.15, 5.61, and 14.0 mg naphthenic acids/L, which represented recoveries of 56 to 82% of the nominal loading rates. The measured concentrations in old test substance WAF solutions at 24 hours were 0.805, 1.88, 2.98, 5.66, and 13.6 mg naphthenic acids/L, which represented recoveries of 57 to 75% of the nominal loading rates. The measured concentrations in new test substance WAF solutions at 72 hours were 1.06, 2.20, 3.48, and 6.62 mg naphthenic acids/L, which represented recoveries of 66 to 88% of the nominal loading rates. The measured concentrations in old test substance WAF solutions at 96 hours were 0.939, 2.18, 3.26, and 6.27 mg naphthenic acids/L, which represented recoveries of 63 to 87% of the nominal loading rates. Due to 100% mortality in the 20 mg/L WAF loading rate at 48 hr, the 20 mg/L WAF was not prepared at subsequent solution renewal interval. Recoveries from QC fortifications ranged from 75 to 86% of the nominal concentrations. The analytical results are summarized in Table 2.

3.1.3 GC/MS Analyses

Results of the analysis of Z-number and C-number families indicated a predominance of naphthenic acids contained 10 to 16 carbon atoms. Approximately 82-90% of the dissolved constituents fell within this range of carbon numbers. The dissolved fractions also showed a prevalence of one and two ring naphthenic acids isomers. These isomers made up approximately 68-75% of the dissolved fraction. Typically, the third highest group of naphthenic acids was the acyclic carboxylic acids. The detailed report of these analyses is presented in Appendix D.

3.2 Biological Results

After 96 hours, mortality was 14, 0, 0, 14, 57, and 100% in the 0 (control), 1.3, 2.5, 5.0, 10, and 20 mg/L nominal naphthenic acids loading rate WAF solutions, respectively. Sublethal effects included loss of equilibrium and were observed in the 10 and 20 mg/L nominal naphthenic acids loading rate WAF solutions throughout the test (Table 3). Based on nominal loading rates, the 24-hour LL_{50} value was estimated to be >20 mg naphthenic acids/L, the highest concentration tested. The 48- and 72-hour LL_{50} values were estimated to be 11 mg naphthenic acids/L with 95% confidence limits of 8.4 and 15 mg naphthenic acids/L. The 96-hour LL_{50} value was estimated to be 9.0 mg naphthenic acids/L with 95% confidence limits of 6.6 and 12 mg naphthenic acids/L. The 96-hour no-observed-effect-loading rate (NOELR) was determined to be 10 mg naphthenic acids/L based on the lack of statistically significant (p = 0.05) mortality and sublethal effects at this and all lower test substance concentrations. Although there was not a statistically significant effect at the 10 mg naphthenic acids/L treatment level, it was determined that the number of impacted fish (71%) in the 10 mg naphthenic acids/L treatment level were biologically significant. Therefore, the reported NOELR is 5.0 mg naphthenic acids/L.

Based on mean measured concentrations, the 24-hour LC_{50} value was estimated to be >13.8 mg naphthenic acids/L, the highest concentration tested. The 48- and 72-hour LC_{50} values were estimated to be 7.22 mg naphthenic acids/L with 95% confidence limits of 5.53 and 9.43 mg naphthenic acids/L. The 96-hour LC_{50} value was estimated to be 5.62 mg naphthenic

acids/L with 95% confidence limits of 2.47 and 10.6 mg naphthenic acids/L. The slope of the 96-hour concentration-response was 6.4. The reported 96-hour no-observed-effect-concentration (NOEC) is 3.22 mg naphthenic acids/L.

3.3 Environmental Conditions

Test solution temperature during the 96-hour exposure ranged from 21.6 to 23.6ºC (Table 4). The results from the continuous temperature recording of the water bath confirmed the temperature exceeded the 22 ± 1ºC range specified in the protocol for approximately 18 hours. The temperature deviation was due to the temperature sensor being bumped outside of the waterbath, causing the heater to stay on. The temperature sensor was discovered, placed back in the waterbath, and the temperature returned to the specified range through the remainder of the study. This minor temperature deviation did not adversely affect the fish survival and therefore did not adversely affect the study integrity or the interpretation of the biological results. Dissolved oxygen concentrations ranged from 7.8 to 8.5 mg/L (96 to 104% saturation) in new test solutions and from 7.1 to 9.3 (92 to 111% saturation) in old test solutions (Table 5). Test solution pH values ranged from 8.0 to 8.4 throughout the test (Table 6). Specific conductivity, total alkalinity, and total hardness values from the dilution water were 330 µS/cm, 148 mg $CaCO_3$/L, and 134 mg $CaCO_3$/L, respectively.

The control and test treatment solutions remained clear and colorless with no visible particulates, surface film, undissolved test substance, or precipitate throughout the test.

4.0 CONCLUSIONS

Based on nominal loading rates, the 24-hour LL_{50} value was estimated to be >20 mg naphthenic acids/L, the highest concentration tested. The 48- and 72-hour LL_{50} values were estimated to be 11 mg naphthenic acids/L with 95% confidence limits of 8.4 and 15 mg naphthenic acids/L. The 96-hour LL_{50} value was estimated to be 9.0 mg naphthenic acids/L with 95% confidence limits of 6.6 and 12 mg naphthenic acids/L. The 96-hour no-observed-effect-loading rate (NOELR) was determined to be 10 mg naphthenic acids/L based on the lack of statistically significant (p = 0.05) mortality and sublethal effects at this and all lower test substance concentrations. Although there was not a statistically significant effect at the 10 mg naphthenic acids/L treatment level, it was determined that the number of impacted fish (71%) in the 10 mg naphthenic acids/L treatment level were biologically significant. Therefore, the reported NOELR is 5.0 mg naphthenic acids/L.

Based on mean measured concentrations, the 24-hour LC_{50} value was estimated to be >13.8 mg naphthenic acids/L, the highest concentration tested. The 48- and 72-hour LC_{50} values were estimated to be 7.22 mg naphthenic acids/L with 95% confidence limits of 5.53 and 9.43 mg naphthenic acids/L. The 96-hour LC_{50} value was estimated to be 5.62 mg naphthenic acids/L with 95% confidence limits of 2.47 and 10.6 mg naphthenic acids/L. The slope of the 96-hour concentration-response was 6.4. The reported 96-hour no-observed-effect-concentration (NOEC) is 3.22 mg naphthenic acids/L.

This study met the EPA OPPTS 850.1075 and OECD 203 validity criteria for tests of acute toxicity to fish. This study is classified as acceptable and satisfies the guideline requirements for a fathead minnow acute study.

PROTOCOL DEVIATIONS

1. Protocol Section 10.1.4 – Temperature and Lighting

   The waterbath temperature was not maintained at 22 ± 1°C during the entire definitive test.

   Reason: For approximately 18 hours on study days 2 and 3, the waterbath temperature exceeded the range stated in the protocol by 1.1°C. This is due to the temperature sensor being bumped outside of the waterbath, causing the heater to stay on. The temperature sensor was discovered, placed back in the waterbath, and the temperature returned to the specified range through the remainder of the study.

   Effect on Study Integrity: None. The minor temperature deviation did not adversely affect the fish survival.

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10. Leak, A.T. 2010. Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies. ABC Laboratories, Inc. Study Number 64403.

Table 1. Measured Concentrations of Naphthenic Acids and Biological Responses During the Static-Renewal Range-Finding Test with the Fathead Minnow, *Pimephales promelas*

Nominal WAF Loading Rate (mg/L)	Measured Concentration of Naphthenic Acids in mg/L (Percent Nominal)		Cumulative Number Dead after 96-Hours (Percent Mortality)
	0-Hour Solutions	24-Hour Solutions	
Control (0)	< MQL [a]	< MQL [a]	0 (0)
1.0	0.760 (76)	0.745 (75)	0 (0)
5.0	3.98 (80)	3.83 (77)	1 (20)
15	12.0 (80)	11.4 (76)	4 (80) [c]
	QC Fortification Spikes (% Recovery)		
Low Spike (0.800)	3.37 (421) [b]	0.645 (81)	
High Spike (20.0)	22.0 (110)	17.8 (89)	

[a] Minimum Quantifiable Limit (MQL) = 0.600 mg naphthenic acids/L.
[b] Result may be due to contamination during sample preparation.
[c] One fish exhibited a loss of equilibrium.

Table 2. Measured Concentrations of Naphthenic Acids During the Static-Renewal Acute Toxicity Test with the Fathead Minnow, *Pimephales promelas*

Nominal WAF Loading Rate (mg/L)	Measured Concentration of Naphthenic Acids in mg/L (Percent Nominal/Initial Measured [a])			
	0-Hour	24-Hour (old)	72-Hour (new)	96-Hour (old)
Control (0)	< MQL [b]	< MQL [b]	< MQL [b]	< MQL [b]
1.3	0.795 (61/--)	0.805 (62/101)	1.06 (82/--)	0.939 (72/89)
2.5	2.06 (82/--)	1.88 (75/91)	2.20 (88/--)	2.18 (87/99)
5.0	3.15 (63/--)	2.98 (60/95)	3.48 (70/--)	3.26 (65/94)
10	5.61 (56/--)	5.66 (57/101)	6.62 (66/--)	6.27 (63/95)
20	14.0 (70/--)	13.6 (68/97)	---[d]	---[d]
QC Fortification Spikes (% Recovery)				
Low Spike (1.00)	---	0.835 (84)	0.795 (80)	0.746 (75)
Low Spike (5.00)	3.78 (76)[c]	---	---	---
High Spike (25.0)	20.1 (80)	21.5 (86)	19.2 (77)	19.1 (76)

[a] Initial measured value in corresponding freshly prepared solutions at 0 and 72 hours.
[b] Minimum Quantifiable Limit (MQL) = 0.600 mg naphthenic acids/L.
[c] Low spike was fortified with the wrong spiking solution.
[d] No analysis performed due to 100% mortality at this level.

Table 3. Mortality of Fathead Minnow, *Pimephales promelas* , Exposed to Water Accommodated Fractions of Naphthenic Acids for 96 Hours Under Static-Renewal Test Conditions

Nominal WAF Loading Rate (mg/L)	REP	Cumulative Number Dead (Percent Mortality)			
		24 Hours	48 Hours	72 Hours	96 Hours
0 (control)	A	0 (0)	1 (14)	1 (14)	1 (14)
1.3	A	0 (0)	0 (0)	0 (0)	0 (0)
2.5	A	0 (0)	0 (0)	0 (0)	0 (0)
5.0	A	1 (14)	1 (14)	1 (14)	1 (14)
10	A	1 (14) [a]	2 (29) [c]	2 (29) [b]	4 (57) [a Δ]
20	A	3 (43) [b]	7 (100)	7 (100)	7 (100) *

Note: Each test chamber contained seven fish at test initiation.

* Statistically significant difference in mortality and sublethal effects as compared to the controls (Fisher's Exact Test; $p = 0.05$)

[Δ] Although there was not a statistically significant difference in mortality and sublethal effects as compared to the controls, it is determined to be biologically significant. The NOELR is reported as 5.0 mg naphthenic acids/L.

[a] One fish exhibited a loss of equilibrium.

[b] Four fish exhibited a loss of equilibrium.

[c] Two fish exhibited a loss of equilibrium.

Table 4. Test Solution Temperature Measurements During a 96-Hour Exposure of Fathead Minnow, *Pimephales promelas,* to Water Accommodated Fractions of Naphthenic Acids

Nominal WAF Loading Rate (mg/L)	REP	Temperature (ºC)							
		0 Hr	24 Hr (old)	24 Hr (new)	48 Hr (old)	48 Hr (new)	72 Hr (old)	72 Hr (new)	96 Hr (old)
0 (control)	A	22.8	21.6	22.5	23.5	23.2	22.0	22.5	21.9
1.3	A	22.9	21.7	22.5	23.6	23.2	22.0	22.5	21.9
2.5	A	23.0	21.7	22.8	23.6	23.3	22.0	22.5	21.9
5.0	A	23.0	21.7	22.8	23.6	23.5	22.0	22.5	21.9
10	A	23.0	21.6	22.8	23.6	23.5	22.0	22.6	21.9
20	A	23.0	21.6	22.8	23.5	---	---	---	---

"---" indicates no measurement taken due to 100% mortality.

Table 5. Test Solution Dissolved Oxygen Concentration Measurements During a 96-Hour Static-Renewal Exposure of Fathead Minnow, *Pimephales promelas*, to Water Accommodated Fractions of Naphthenic Acids

Nominal WAF Loading Rate (mg/L)	REP	Dissolved Oxygen Concentration as mg/L							
		0 Hr	24 Hr (old)	24 Hr (new)	48 Hr (old)	48 Hr (new)	72 Hr (old)	72 Hr (new)	96 Hr (old)
0 (control)	A	8.2	8.2	8.0	8.0	8.1	8.2	8.5	9.3
1.3	A	8.1	8.1	8.0	7.7	8.1	8.1	8.4	9.1
2.5	A	8.1	7.9	8.0	7.5	8.0	7.7	8.3	8.8
5.0	A	8.1	7.8	7.9	7.5	7.9	7.8	8.2	9.1
10	A	8.1	7.7	7.9	7.3	7.8	8.0	8.2	9.1
20	A	8.1	7.7	8.0	7.1	---	---	---	---

"---" indicates no measurement taken due to 100% mortality.

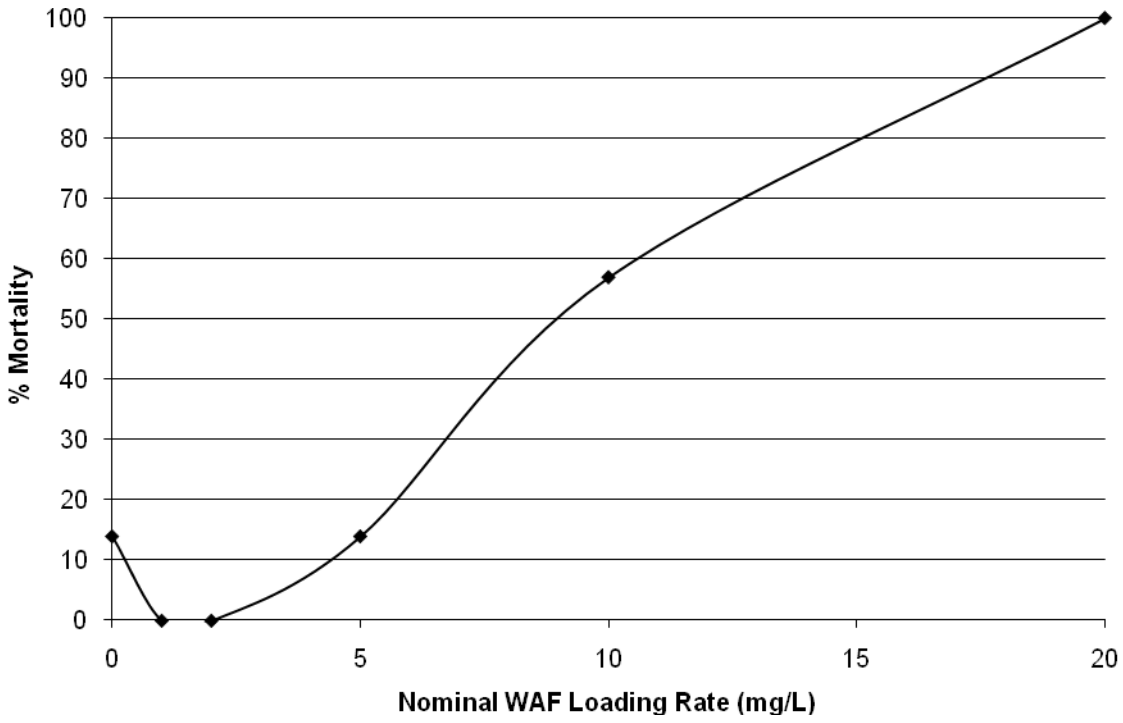
Note: At 22, 23, and 24°C, 100% saturation equals 8.4, 8.2, and 8.1 mg/L, respectively.

Table 6. Test Solution pH Measurements During a 96-Hour Static-Renewal Exposure of Fathead Minnow, *Pimephales promelas*, to Water Accommodated Fractions of Naphthenic Acids

Nominal WAF Loading Rate (mg/L)	REP	pH							
		0 Hr	24 Hr (old)	24 Hr (new)	48 Hr (old)	48 Hr (new)	72 Hr (old)	72 Hr (new)	96 Hr (old)
0 (control)	A	8.0	8.2	8.3	8.4	8.1	8.3	8.2	8.3
1.3	A	8.1	8.2	8.3	8.4	8.2	8.3	8.2	8.3
2.5	A	8.1	8.3	8.3	8.3	8.2	8.3	8.2	8.3
5.0	A	8.2	8.2	8.3	8.3	8.2	8.3	8.2	8.3
10	A	8.1	8.2	8.2	8.3	8.1	8.2	8.1	8.2
20	A	8.1	8.1	8.1	8.2	---	---	---	---

"---" indicates no measurement taken due to 100% mortality.

Figure 1. Cumulative Mortality of Fathead Minnow, *Pimephales promelas*, Exposed to Water Accommodated Fractions of Naphthenic Acids for 96 Hours Under Static-Renewal Test Conditions



APPENDIX A. TEST SUBSTANCE PHYSICAL-CHEMICAL SPECIFICATIONS FROM SUPPLIER

MERICHEM
CHEMICALS & REFINERY SERVICES LLC

REVISION 4: March 28, 2008 — MATERIAL SAFETY DATA SHEET — Page 1/6

1 IDENTIFICATION OF THE PRODUCT AND OF THE COMPANY

1.1	Identification of the Product:	**Naphthenic Acids (Carboxylic Acids, Fatty Acids)**
1.2	Product Code:	NAP ACID
1.3	Company:	Merichem Chemicals & Refinery Services LLC 5455 Old Spanish Trail Houston, TX 77023, U.S.A.
1.4	Transportation Emergency:	USA 1-800-424-9300 (CHEMTREC)
1.5	Product Information:	1-205-556-1556 1-205-556-0568 (Fax)
1.6	Intended Use :	For industrial use only. No other use is intended.

2 HAZARDS IDENTIFICATION

2.1	Classification:	Irritant (Xi). Harmful (Xn).
2.2	Warning Statements:	Causes eye and skin irritation. Harmful if swallowed – may enter lungs if swallowed or vomited. High vapor concentrations may cause drowsiness and irritation of the eyes or respiratory tract.
2.3	Hazard Symbol(s):	
2.4	Risk Phrase(s):	R36/38 (Irritating to eyes and skin). R65 (Harmful: May cause lung damage if swallowed).

2.3 Potential Health Effects:

Eye Contact:	Causes eye irritation. Exposure may cause irritation, redness and tearing.
Skin Contact:	Causes skin irritation. Exposure may cause redness, itching and inflammation.
Ingestion:	Expected to be a low ingestion hazard. Aspiration hazard. If swallowed, can enter lungs and cause damage.
Inhalation:	High vapor concentrations may cause drowsiness, respiratory tract irritation, coughing, asthmatic breathing and breathlessness.
Chronic Effects:	No known deleterious effects.

2.4 Other Hazards: Target organs: Eyes, skin and central nervous system.

3 COMPOSITION/INFORMATION ON INGREDIENTS

Substance	CAS No.	EC No.	% Present
Naphthenic Acid	1338-24-5	215-662-8	70 - 99
Petroleum Distillates	8008-20-6	232-366-4	1 - 30

4 FIRST-AID MEASURES

In case of contact with eyes: Promptly flush eyes with running water for 15 minutes, including under eyelids. Seek medical assistance if irritation develops.

In case of contact with skin: Wash affected area well with soap and water. Seek medical assistance if irritation develops.

In case of ingestion: Do not induce vomiting. Give 2-3 glasses of milk or water to dilute. Contact physician or poison control center promptly for instructions. If vomiting occurs, keep head lower than hips to help prevent aspiration. Never give anything by mouth to an unconscious person.

In case of inhalation: Remove to fresh air. Seek medical assistance if irritation develops.

5 FIRE-FIGHTING MEASURES

5.1 Suitable extinguishing media: Water fog, fire fighting foam, dry chemical or carbon dioxide.

5.2 Unsuitable extinguishing media: None

5.3 Specific hazards: Combustion products are Carbon Oxides.

5.4 Personal protective equipment: Wear Self Contained Breathing Apparatus and protective clothing appropriate for fire-fighting.

5.5 Other precautions: Non-emergency personnel should be removed from the area immediately. Cool fire-exposed containers with water spray. Prevent water runoff from reaching drains, surface water and ground water.

6 ACCIDENTAL RELEASE MEASURES

6.1 Personal precautions: Avoid unnecessary exposure by wearing personal protective equipment specified in Section 8. Remove material from eyes, skin and clothing.

6.2 Spill cleanup: Suction up free liquids using non-sparking equipment. Liquid unable to be suctioned may be absorbed with a non-combustible material (vermiculite, sand, earth, etc.) and transferred to container(s) for later disposal. Remove contaminated sand, earth, etc. and transfer to container(s) for later disposal.

6.3 Environmental precautions: Keep away from drains, surface water and ground water.

7 HANDLING AND STORAGE

7.1 Handling: Wear appropriate personal protective equipment (see Section 8).

Avoid breathing vapors, mists or spray. Use with adequate ventilation.

Avoid contact with eyes, skin and clothing.

Wash thoroughly after handling.

Do not taste or swallow.

7.2 Storage: Store in a sealed container in a clean, dry, well-ventilated area away from oxidizers, strong bases, heat and flame.

Avoid use of copper and brass alloys in storage and transfer equipment and process equipment.

8 EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Engineering controls: Use local exhaust ventilation to control emissions at source.

8.2 Eye/face protection: Wear safety glasses with side shields as minimum protection. Wear goggles or faceshield if a risk of splashing exists.

8.3 Skin protection: Wear chemical resistant gloves. Heavy PVC, butyl rubber or Viton are recommended.

8.4 Respiratory protection: An approved respirator must be worn if engineering controls do not maintain airborne concentrations below established exposure limits or, when limits have not been established, below irritant levels. Respirator selection must be based upon the airborne concentration. Consult a health and safety professional or manufacturer for specific recommendations.

8.5 Thermal hazards: None

8.6 Occupational Exposure Levels

Chemical Name	Source	Type	Exposure Limits	Notes
Cherosene (Non-Aerosol), Come Vapore totale Dell'Idrocarburo	Italy OEL's	TWA	200 mg/m^3	Skin Total Hydrocarbon Vapor
Kerosene	Poland MAC's	TWA	100 mg/m^3	
	Poland MAC's	STEL	300 mg/m^3	
Kerosine	Russian Federation MAC's	Ceiling	300 mg/m^3	As C
	Russian Federation MAC's	TWA	600 mg/m^3	As C
Kerosene (Non-Aerosol), As Total Hydrocarbon Vapor	ACGIH	TWA	200 mg/m^3	Irritation, CNS, Skin
Kerosene	NIOSH	REL	100 mg/m^3	

9 PHYSICAL AND CHEMICAL PROPERTIES

9.1 Appearance:	Amber color	9.2 Odour:	Hydrocarbon
9.3 pH:	5.2 (Saturated Solution)	9.4 Boiling Pt./range:	268°C (515°F)
9.5 Freezing Pt./Range:	Not established		
9.6 Flash point:	>149°C (300°F)		
9.7 Flammability:	See 9.6	9.8 Autoflammability:	See 9.6
9.9 Explosive properties:	Not applicable	9.10 Oxidizing properties:	Not an oxidizer
9.11 Vapor pressure:	0.005 mm Hg (37.8°C/100°F)	9.12 Relative density (H_2O = 1):	0.960 – 0.982 (15.6°C/60°F)
9.13 Apparent density:	Not applicable	9.14 Vapor density (Air = 1):	6.5

9.14 Solubility:

Water	-	<1% by weight (15.6°C/60°F)
Fat (type)	-	Not determined
Other solvents	-	Not determined

9.15 Partition coefficient: Log $P_{o/w}$ (Octanol/water) - Not determined

9.16 Other data: Not Applicable

10 STABILITY AND REACTIVITY

10.1 Reactivity: Not reactive under specified conditions of storage, shipment and use.

10.2 Stability: Stable under specified conditions of storage, shipment and use.

10.3 Conditions to avoid: Heat and ignition sources.

10.4 Incompatible materials: Strong oxidizers and strong bases.

10.5 Hazardous decomposition products: Carbon Oxides.

11 TOXICOLOGICAL INFORMATION

11.1 Acute: Eye and skin irritant. Not established as a respiratory tract irritant. May cause lung damage if aspirated.

Specified Substances

Chemical Name	Test Results
Kerosene	Dermal LD_{50} (Rabbit): >2000 mg/kg
	Oral LD_{50} (Rat): >5000 mg/kg
	Inhalation LC_{50}: >5000 mg/m^3, 4 H
	Skin (Rabbit): 500 mg (Severe Irritation)
	Skin (Rabbit): 100%/24 H (Moderate Irritation)
Naphthenic Acid	Oral LD_{50} (Rat): 3000 mg/kg
	Oral LD_{50} (Rat): 5880 mg/kg
	Dermal LD_{50} (Rabbit): >3160 mg/kg
	Eye (Rabbit): Moderate
	Skin Occluded (Rabbit): Moderate to Severe
	Skin (Rabbit): Slight

11.2 Chronic: Neither ingredient is listed by NTP, IARC or OSHA as a carcinogen. Kerosene (Non-Aerosol), as total hydrocarbon vapor, is listed by ACGIH as A3 (Confirmed Animal Carcinogen).

12 ECOLOGICAL INFORMATION

12.1 Ecotoxicity: No data available.

12.2 Persistence and degradability: No data available.

12.3 Bioaccumulation potential: No data available.

12.4 Mobility in soil: No data available.

12.5 Other adverse effects: No data available.

13 DISPOSAL CONSIDERATIONS

Generators of waste material are responsible for evaluating materials for compliance with all applicable procedures and regulations. Disposal of unused materials must be in accordance with all local, state and federal regulations. Containers should be cleaned of residual product and rinsed according to all local, state and federal regulations prior to disposal.

14 TRANSPORT INFORMATION

	UN Number	Proper Shipping Name	Hazard Class(es)	Packing Group
ADR/RID:	Not regulated			
IMO/IMDG:	Not regulated	Fatty Acids (Saturated C13+)		
IATA:	Not regulated			
DOT:	NA3082	Other Regulated Substances, Liquid, n.o.s., (Naphthenic Acid)	9	III

Note: Material is regulated by DOT only if shipped in a container containing an amount equal to or greater than the Reportable Quantity (RQ) of 100-pounds.

15 REGULATORY INFORMATION

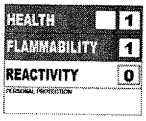
Warning symbol:

Warning words: Harmful. Irritant.

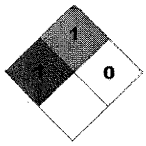
Risk phrases: R36/38: Irritating to eyes and skin
R65: May cause lung damage if swallowed

Safety phrases: S23: Do not breathe vapor
S24/25: Avoid contact with eyes and skin
S62: If swallowed, do not induce vomiting. Seek medical advice immediately and show this container or label

HMIS ratings (estimated):



NFPA ratings (estimated):



SARA : Section 302: None
Section 311/312: Immediate Health Hazard
Section 313: None

WHMIS: D2B

Inventories:

CAS Number	TSCA	DSL	EINECS
1338-24-5	Yes	Yes	Yes
8008-20-6	Yes	Yes	Yes

16 OTHER INFORMATION

Revision Date:	March 28, 2008
Supercedes Revision Date:	November, 2004
Revisions:	The latest informational changes are indicated by 20% shading.

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2701 Warrior Road
Tuscaloosa, Al 35404

SAMPLE SUMMARY: SAMPLE FOR API/HPV TESTING

Specification	Results	Procedure
Acid number	**235mg KOH/gm**	ASTM D664-59
Unsaponifiables (Total)	**4.9%**	ASTM D322
Viscosity @ 40	**32cst**	ASTM D445-88
Specific Gravity @ 20C	**0.969**	ASTM D1298-85
Color (Gardner), GI	**4.5**	ASTMD1544-80
Water Content	**0.07%**	ASTM D95-83
Phenolic Content (acid)	**0.31%**	Standard Methods for the Examination of Water and Wastewater, 14th Edition (1975); Method 510, pp 574-592, APHA-AWWA-WPCF
Total Sulfur	**0.34**	ASTM D4294-83
CP - Flash Point °F (COC)	**343**	ASTM D92

APPENDIX B. ABC WELL WATER CHARACTERIZATION

Chemical Characteristics of ABC Well Water Used by
ABC Laboratories' Chemical Services Group

August 2009 ABC Well Water Screen (non-GLP)

Organophosphate (µg/L)	2009	Historical Range 1998-2009 [1]
Azinphos ethyl	---	<1.0[5]
Azinphos-methyl	<0.200	<0.200[3]
Bolstar	<0.200	<0.200[4]
Chloropyrifos	<0.200	<0.200[4]
Coumaphos	<0.400	<0.400[4]
Demeton, Total	<0.200	<0.200-<1.0
Diazinon	<0.200	<0.200-<1.0
Dichlorvos	<0.200	<0.200[4]
Dimethoate	<1.00	<1.00[4]
Disulfoton	<0.200	<0.200-<1.0
EPN	<0.200	<0.200[4]
Ethion	---	<1.0[5]
Ethoprop	<0.200	<0.200[4]
Fensulfothion	<1.00	<1.00[4]
Fenthion	<0.200	<0.200[4]
Malathion	<0.200	<0.200-<1.0
Merphos	<0.200	<0.200[4]
Mevinphos	<1.00	<1.00[4]
Monocrotophos	<1.00	<1.00[4]
Naled	<2.00	<2.00[4]
Parathion:	---	<1.0[5]
Parathion, ethyl	<0.200	<0.200[3]
Parathion, methyl	<0.200	<0.200[3]
Phorate	<0.200	<0.200[4]
Ronnel	<0.200	<0.200[4]
Stirophos	<0.200	<0.200[4]
Sulfotepp	<0.200	<0.200[4]
TEPP	<0.200	<0.200[4]
Tokuthion	<0.200	<0.200[4]
Trichloronate	<0.200	<0.200[4]

Polychlorinated Biphenyls (µg/L)		
Aroclor 1016	<1.00	<1.00
Aroclor 1221	<1.00	<1.00
Aroclor 1232	<1.00	<1.00
Aroclor 1242	<1.00	<1.00
Aroclor 1248	<1.00	<1.00
Aroclor 1254	<1.00	<1.00
Aroclor 1260	<1.00	<1.00

Elements (mg/L)	2009	Historical Range 1998-2009 [1]
Aluminum	<0.0500	<0.0500[4]
Antimony	<0.0500	<0.0500[4]
Arsenic	<0.0250	<0.0250-<0.050
Barium	0.0189	0.0189[4]
Beryllium	<0.0010	<0.0010[4]
Boron	0.400	0.37-0.415
Cadmium	<0.0020	<0.0020-<0.0050
Calcium[2]	76.3	52-83.1
Chromium	<0.0100	<0.010
Cobalt	<0.0100	<0.0100[4]
Copper	<0.0100	<0.010
Iron	0.020	<0.0059-0.16
Lead	<0.0400	<0.0065-0.0400
Magnesium[2]	30.7	27-33.1
Manganese	<0.0050	<0.0050[4]
Molybdenum	<0.0100	<0.0100[4]
Mercury	---	<0.00060[5]
Nickel	<0.0100	<0.0100-<0.020
Potassium[2]	7.51	6.6-7.93
Selenium	<0.0500	<0.050
Silver	<0.0100	<0.010
Sodium[2]	29.0	27-32.2
Thallium	<0.0500	<0.0500[4]
Tin	<0.0200	<0.0200[4]
Vanadium	<0.0100	<0.0100[4]
Zinc	0.0197	0.0118-0.078

Chlorinated Hydrocarbons (µg/L)		
4,4'-DDD	<0.04	<0.040
4,4'-DDE	<0.04	<0.040
4,4'-DDT	<0.04	<0.040
Aldrin	<0.04	<0.040
α-BHC	<0.04	<0.040
β-BHC	<0.04	<0.040
Δ-BHC	<0.04	<0.040
Dieldrin	<0.04	<0.040
Endosulfan I	<0.04	<0.040
Endosulfan II	<0.04	<0.040
Endosulfan sulfate	<0.04	<0.040
Endrin	<0.04	<0.040
Endrin aldehyde	<0.04	<0.040

Chemical Characteristics of ABC Well Water Used by
ABC Laboratories' Chemical Services Group (continued)

August 2009 ABC Well Water Screen (non-GLP)

Miscellaneous (mg/L)	2009	Historical Range 1998-2009 [1]
Nitrite N	<0.01	<0.01-≤0.050
Nitrate N	0.328	<0.11-0.328
Total Phosphorus as P	0.12	<0.050-0.64
Chlorinated Herbicides (µg/L)		
2,4,5-TP (Silvex)	<0.200	<0.200-<50
2,4-D	<0.200	<0.200-<250

Chlorinated Hydrocarbons (µg/L) (continued)	2009	Historical Range 1998-2009 [1]
Endrin ketone	<0.04	<0.040
γ-BHC	<0.04	<0.040
Heptachlor	<0.04	<0.040
Heptachlor epoxide	<0.04	<0.040
Methoxychlor	<0.04	<0.04-<0.095
Toxaphene	<0.50	<0.50-<3.8
Chlordane	<0.05	<0.05-<0.48

[1] Data supporting these values are on file at ABC Laboratories. Less than (<) values indicate recovery was below the greatest limit of detection during these analyses.
[2] Historical Range is from 2003.
[3] Historical Range is from 2008.
[4] Historical Range is from 2009.
[5] Historical Range does not include 2009.

Note: ABC well water is the base water for ABC Blended Water.

APPENDIX C. PROTOCOL, AMENDMENTS, AND DEVIATION

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach

ABC Study No. 64406

This protocol is based on a proposed step-down approach and OECD Guideline 203 and U.S. EPA Ecological Effects Test Guideline OPPTS 850.1075

1.0 STUDY TITLE

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach

2.0 OBJECTIVE

The objective of this test is to determine with a step-down approach if the 96-hour EL_{50} and LC_{50}, if possible, of the test substance to fathead minnow under static-renewal test conditions at the upper threshold concentration (UTC) is greater than or less than the UTC. Mortality will be used to evaluate the acute toxicity of the test substance. Any sublethal effects of the test substance on the test species will also be noted.

3.0 STUDY SPONSOR

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005
Phone: (202) 682-8480 Fax: (202) 682-8270
Sponsor Representative: [redacted]
Email: [redacted]

Study Monitor: [redacted]
EcoTox Assessments LLC
506 Tennant Circle, Suite 100
St. Michaels, Maryland 21663
Tel: 410-745-6172 Fax: 410-745-9161
E-mail: [redacted]

4.0 TESTING FACILITY AND STUDY DIRECTOR ADDRESS

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202
Study Director: [redacted]
TEL: (573) 777-6341 FAX: (573) 777-6089
Email: [redacted]

5.0 PROPOSED SCHEDULE

PROPOSED EXPERIMENTAL START DATE: February 2009
PROPOSED EXPERIMENTAL COMPLETION DATE: April 2009

6.0 TEST PROTOCOL

The test protocol which follows is based on the step-down approach proposed by Jeram (1), OECD guideline 203 (2), and U.S. EPA OPPTS guideline 850.1075 (3). Modifications to the regulatory guidelines were made to address the testing of insoluble and complex mixtures (4, 5).

7.0 TEST AND REFERENCE SUBSTANCES

7.1 Test Substance

The test substance will be Naphthenic Acids (CAS # 1338-24-5). The following sample information and chemical/physical properties should be provided with the test substance sample or before its shipment: batch/lot number, sample expiration date, physical description, purity (including certificate of analysis), stability, suggested storage conditions, water and organic solvent solubility, vapor pressure, available toxicity information, a Material Safety Data Sheet (MSDS) or its equivalent, and handling precautions.

7.2 Reference Substance

The reference substance will be Naphthenic Acids (CAS# 1338-24-5). The same information specified for the test substance sample in section 7.1 should be provided for the reference substance sample.

7.3 Sample Characterization and Retention

Characterization, stability, and solubility studies will be the responsibility of the Sponsor unless otherwise contracted to ABC Laboratories, Inc. The test and reference substances will be returned to the Sponsor following completion of their use at ABC Laboratories, Inc. Archival of a retention sample will also be the responsibility of the Sponsor.

7.4 Test Substance Preparation/Addition

Test solutions will be prepared as water-accommodated fractions (WAF), with each WAF being independently prepared. The WAFs will be prepared by adding the test substance to dilution water on a weight/volume basis and will be reported as the loading rate of test substance per volume of dilution water. The maximum loading of the test substance in a WAF preparation will not exceed 1,000 mg/L. Trials of the WAF preparations and equilibration will be performed as part of ABC study 64403 to

determine the optimum mixing duration to maximize the soluble fraction of test substance in dilution water.

WAF preparations will be prepared by adding a calculated amount of test substance to the preparation container(s) containing dilution water to achieve the nominal loading rates. Since no volatilization is expected with the test substance constituent components, efforts to minimize the head space in each WAF preparation container are not required, but may be used if requested by the Sponsor (WAF preparation containers partially filled with dilution water, dosed with the test substance, and dilution water added such that there is a minimal head space, then covered with a glass lid or plate). The size and type of the WAF preparation container and specifications for collection will be documented in the raw data and summarized in the report.

Each container will be equipped with a Teflon-coated stir bar. The stirring speed will be adjusted so that the vortex in each bottle is within the range of 30 to 50% of the container depth. Stirring will take place at ambient room temperature and lighting. After the prescribed time of stirring, stirring will be stopped and the mixture allowed to sit undisturbed for approximately 1 hour before initiating drawing/siphoning of the WAF solution. The solution from each WAF will be drained/siphoned into a clean container for sample collection and subsequent analysis. If multiple WAF solutions are prepared, each WAF solution will be placed directly into the replicate test chambers for that treatment level. Portions of the WAF will also be drained/siphoned from the bottle for analytical and initial water quality measurements

8.0 TEST SYSTEM

8.1 Species

The test species will be the fathead minnow, *Pimephales promelas*. Species identification will be provided by the supplier or confirmed at ABC Laboratories using an appropriate taxonomic key.

8.2 Justification

Fathead minnow were chosen for this testing because they are representative of freshwater fish species and are one of the species recommended by U.S. EPA and EU regulations.

8.3 Source

Fathead minnow will be obtained from cultures maintained at ABC Laboratories or from a commercial supplier.

8.4 Size/Age

Fish will be uniform in size; that is, the longest fish will be no more than twice the standard length of the shortest fish. Juvenile fish (<3 g) will be used and all fish used will be approximately the same age. Length and wet weight measurements will be made on the dilution water or vehicle control group of fish at test termination to document size of the test fish population.

8.5 Holding/Acclimation

Fish will be maintained in the laboratory for a minimum of 14 days before starting the definitive test. During the holding period, the fish will receive a standard commercial fish food that may be supplemented with live brine shrimp nauplii (*Artemia* sp.) and/or other aquatic invertebrates. The test lot will be acclimated gradually to the test temperature and dilution water for at least 48 hours prior to definitive testing. The test fish will not be fed during the 48-hour acclimation period immediately prior to test initiation. A group of fish will not be used if individuals appear to be diseased or if mortality exceeds 5 percent during the 48-hour period immediately before initiation of the definitive test.

9.0 DILUTION WATER

The dilution water will be laboratory freshwater with a total hardness of 130-160 mg/L as $CaCO_3$ and a pH of approximately 8.0. This water is chemically characterized per ABC SOP to verify that it is free of contaminants that might interfere with test results.

10.0 TEST PROCEDURES

10.1 Definitive Test

10.1.1 Experimental Design

The definitive test will consist of a limit test at the upper threshold concentration (UTC). The UTC is defined as the lower concentration between the EL_{50} of *Daphnia magna* and IL_{50} of *Pseudokirchneriella subcapitata*. The UTC will be specified by protocol amendment. A negative control consisting of exposure to test water only will be tested

concurrently. All test chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, level 1, etc.), and replicate (e.g., A, B, etc.).

The test chambers will be glass and of a size sufficient to meet the fish loading restriction (i.e., loading will not exceed 0.8 grams of fish per liter of test solution in a test chamber at any one time). For the limit testing, a single test chamber containing five fish will be tested. The test fish will be impartially placed in each of the test chambers. One fish will be added to each test chamber, sequentially starting with the control(s) then the treatment, until all chambers contain their full complement of fish. This method of fish addition will be used to minimize bias due to organism selection. The test will be conducted for 96 hours commencing when the fish are first exposed to the test substance. Fish will be added to the test chambers within 30 minutes of the completion of the WAF preparation. This route of administration was selected in order to comply with OECD and U.S. EPA testing guidelines.

10.1.2 Renewals

All control and test substance treatment solutions will be freshly prepared and renewed daily. At each renewal period, all surviving fish will be transferred from old solutions to new solutions using a soft net.

10.1.3 Feeding

The test fish will not be fed during the test.

10.1.4 Temperature and Lighting

Temperature will be regulated to maintain 22 ± 1°C. A 16-hour light and 8-hour dark photoperiod will be maintained. Light intensity will be measured at the level of the test solutions once during the test.

10.1.5 Water Quality - Chemical/Physical

Specific conductivity, total alkalinity, and total hardness of the dilution water will be measured at the start of the test. Temperature will be measured at least hourly in one replicate throughout the test. Temperature, dissolved oxygen concentrations, and pH will be measured in all replicate test chambers daily throughout the test. If 100-percent mortality is observed in a test concentration, water quality data will be recorded at that

time and the concentration will be discontinued. TOC of the dilution water will be measured and should be ≤2.0 mg/L. Additional water quality measurements may be made at the discretion of the Study Director.

10.1.6 Dissolved Oxygen Concentration

The dissolved oxygen concentration in the dilution water at test initiation should be ≥90 percent of saturation. The dissolved oxygen concentrations in the control(s) and all exposure solutions should remain above 60% of saturation during the exposure. If dissolved oxygen concentrations fall below 60% of saturation, test solutions may be aerated during the test.

10.1.7 Biological Data

Observations of mortality, moribundity, and behavior will be recorded and reported for all WAF loading rates. Observations will be made at 24, 48, 72, and 96 hours (±1 hour from test initiation). Upon termination of the study, all fish will be euthanized according to one of the appropriate procedures detailed in ABC SOP CD-TS 1.7 "Euthanasia of Aquatic Vertebrates". The euthanasia procedure will be selected based upon study specific sampling requirements.

10.1.8 Analytical Confirmation

The concentrations of the total dissolved naphthenic acids in the WAFs will be measured in all control and test substance concentrations at 0, 24, 72, and 96 hours. Unless otherwise specified, time 0-hour and 72-hour fresh solution samples will be collected from parent solutions. Time 24-hour and 96-hour spent solution samples will be collected after combining replicate solutions by treatment. If fresh test solutions are prepared independently for the replicates, samples should be collected from each replicate test chamber and analyzed. Additional samples may be collected and analyzed at the discretion of the Study Director. A minimum of two fortification spikes (quality control samples) will be prepared and analyzed with each sample set.

The analytical method will be validated before the definitive test is started and will be described by protocol amendment to this protocol after validation.

10.2 Additional Testing

If mortality of the fish exposed at the UTC exceeds 50%, an additional definitive test will be done. The conditions, exposure concentrations, and observation procedures of the additional definitive test will be defined by amendment if additional testing is needed.

11.0 ANALYSIS OF RESULTS

The response of organisms to the test substance treatment will be compared to that of the control organisms using a chi-square or other appropriate procedure to determine statistical difference. If no mortality or other adverse effects are observed, statistical analysis is not necessary.

If additional definitive multiconcentration testing is performed, the results will be statistically analyzed for 24-, 48-, 72-, and 96-hour LL_{50} values and their corresponding 95% confidence limits, if possible. These values will be determined with an LC_{50} computer program using the probit model and/or Trimmed Spearman-Karber procedure or other appropriate statistical procedure. If possible, the slope of the 96-hour concentration-response line will be calculated for the 96-hour observation period. The slope will be calculated by a computer program that transforms percent immobility to probit values versus log of the concentration. A 96-hour no-observed-effect loading rate (NOELR) and concentration (NOEC) will be determined, if possible, based on the absence of any mortality.

12.0 TEST ACCEPTABILITY CRITERIA

The definitive test will not be valid if mortality in any control treatment exceeds 10 percent during the 96-hour test.

13.0 REPORT

A report will be submitted to the Sponsor and will include, but not be limited to, the following:

- Study dates, name, and address of test facility.
- Objectives and test procedures as stated in the approved protocol.
- A description of the experimental design along with a description of and reference to any statistical methods used for data analysis.

- Description of test substance (e.g., date of receipt, storage conditions, purity, physical characteristics, and method of preparing stock and/or test solutions) and identification of the reference substance, if applicable.
- Description of test conditions during the study (e.g., dilution water, test temperature, lighting, dissolved oxygen and pH).
- Description of methods used during the study.
- Description of test system (e.g., source, culture conditions, etc.).
- Summary of the data and a statement of the conclusions drawn from any data analyses, if appropriate.
- Description of any protocol deviations.
- Location of raw data.
- List of all study personnel.
- GLP compliance statement by the Study Director and a statement by ABC Laboratories' Quality Assurance Unit.

14.0 PROTOCOL AMENDMENTS AND DEVIATIONS

The Study Director, upon approval of the Sponsor Representative, may make amendments to this protocol. All amendments will describe the change(s), the reason(s) for the amendment, and the effect on the study, if any. All amendments will be signed and dated by at least the Study Director, and maintained with the protocol.

In the event of a protocol deviation, a written description of the deviation, including the reason for the deviation and any impact on the study as a result of the deviation, will be submitted to the Sponsor Representative. All deviations will be signed and dated by at least the Study Director.

15.0 QUALITY ASSURANCE

ABC's Quality Assurance Unit will inspect one or more critical phases to assure that equipment, personnel, procedures, and records conform to the guidelines listed in this protocol. The results of these inspections will be reported to the Study Director and ABC management. The draft and final reports will be reviewed for protocol and GLP compliance, as well as to assure that the methods and standard operating procedures used were followed.

A signed statement will be included in the report specifying types of inspections made, the dates inspections were made, and the dates inspections were reported to the Study Director and management.

16.0 GLP COMPLIANCE

All test procedures, documentation, records, and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the OECD Principles of Good Laboratory Practice (6) and Toxic Substances Control Act (7). The report will contain a statement attesting to that fact.

17.0 RECORDS

Records to be maintained will include, but not be limited to, test substance receipt; solution preparations and dilutions; instrument logbooks detailing calibration and maintenance; facility records (kept at ABC); material control identification numbers for all instruments used; storage of test substance, solutions, and samples; and weights and volumes. All original raw data collected during this study will be maintained at ABC Laboratories until finalization of the study. Upon completion of the study, all original raw data will be submitted to the Sponsor along with the final report. A copy of the final report, copies of all raw data from the study, and all original facility records will be kept on file in ABC Laboratories' archives.

18.0 SPECIMEN DISPOSAL

Following finalization of the report, disposition of all specimens (i.e., any material derived from the test system for examination, analysis, or retention) generated during the conduct of the test will be completed in a timely manner. Retention specimens holding time will be based on stability information provided by the Sponsor or by stability data generated by ABC Laboratories. Retention specimens will be returned to the Sponsor unless archiving is contracted with ABC Laboratories. Documentation of specimen disposal will be retained with study records in ABC Laboratories' Archive.

19.0 ANIMAL WELFARE ACT COMPLIANCE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR). The Sponsor should make particular note of the following:

- The Sponsor signature on this protocol documents for the Study Director the Sponsor's assurance that the study described in this protocol does not unnecessarily duplicate previous experiments.

- Whenever possible, procedures used in this study have been designed to avoid or minimize discomfort, distress or pain to animals. All methods are described in this study protocol or in written laboratory standard operating procedures.

- By design, this study may kill and/or result in the pain and distress of test organisms. Euthanization of test organisms before completion of the test would interfere with study objectives. Upon completion of the test all distressed fish will be painlessly euthanized in a timely manner as described in Section 10.2.6.

- Methods of euthanasia used during this study are in conformance with the above referenced regulation.

20.0 REFERENCES

(1) Jeram, S., et al. 2005. A strategy to reduce the use of fish in acute ecotoxicity testing of new chemical substances notified in the European Union. Reg. Toxicol. Pharma. Col. 42:218-224.

(2) Organization for Economic Cooperation and Development (OECD). April 4, 1984. OECD Guidelines for Testing of Chemicals. Fish, Acute Toxicity Test, OECD Guideline No. 203, 12 pp.

(3) U.S. Environmental Protection Agency. 1996. Ecological Effects Test Guidelines, OPPTS 850.1075, Fish Acute Toxicity Test, Freshwater and Marine, 11 pp.

(4) Organization for Economic Cooperation and Development (OECD). 2000. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Series on Testing and Assessment, No. 23. ENV/JM/MONO(2000)6, OECD, Paris, France.

(5) Girling, A.E., F.G. Whale, and D.M.M. Adema. 1994. A Guideline Supplement for Determining the Aquatic Toxicity of Poorly Water-Soluble Complex Mixtures Using Water-Accommodated Fractions. Chemosphere 29(12):2645-2649.

(6) Organization for Economic Co-operation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

(7) U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act; Good Laboratory Practice Standards; Final Rule (40 CFR, Part 792).

PROTOCOL APPROVAL

Study Director

Name (signed): [redacted] Date: 24 Feb 09

Name/Title: [redacted]

Sponsor Representative

Name (signed): [redacted] Date: 23 Feb 2009

Name/Title: Paula Podhasky

Test Facility Management

Name (signed): [redacted] Date: 25Feb09

Name/Title: [redacted]

QAU Protocol Review for GLP Compliance

Name (signed): [redacted] Date: 27Feb09

Name/Title: [redacted]

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64406
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	1	**EFFECTIVE DATE:**	Oct. 23, 2009

1. Protocol Section: 7.4 – Test Substance Preparation/Addition

   Test solutions will be prepared as water-accommodated fractions (WAF), with each WAF being independently prepared. The WAFs will be prepared by adding the test substance to dilution water on a weight/volume basis and will be reported as the loading rate of test substance per volume of dilution water. The WAF preparation will be prepared in an appropriately sized vessel made of glass and will be stirred with a magnetic stir bar. WAF vessels will be covered with a screw cap. The stirring speed will be adjusted so that the vortex in each bottle does not extend greater than approximately 30-50% of the water column. Stirring will take place at ambient room temperature and lighting. After the prescribed time of stirring, stirring will be stopped and the mixture allowed to sit undisturbed for approximately 1 hour before initiating drawing/siphoning of the WAF solution.

   WAF preparations during the definitive test will be prepared at a volume of 4 L in clean 5-L glass carboys, each containing a 2 inch Teflon-coated stir bar. Each WAF will be stirred for 24 hours ± 1 hour before being allowed to sit undisturbed and settle for approximately 1 hour before collection. WAFs prepared for the definitive test will be collected by siphoning the prepared solution from each carboy with a glass tube into a clean collection vessel. The first approximately 100 mL of prepared solution from each WAF will be discarded as waste to avoid the collection of any insoluble test substance.

   Reason: To describe the containers and preparations used in range finding and definitive tests.

   Effect on Study Integrity: None.

2. Protocol Section: 10.1.1 – Experimental Design

   The definitive test will consist of a limit test at the upper threshold concentration (UTC). The UTC is defined as the lower concentration between the EL_{50} values of *Daphnia magna* and *Pseudokirchneriella subcapitata*. The UTC is 24 mg Naphthenic Acids/L. A negative control consisting of exposure to test water only will be tested concurrently. All test

chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, level 1, etc.), and replicate (e.g., A, B, etc.).

The test chambers will be glass and of a size sufficient to meet the fish loading restriction (i.e., loading will not exceed 0.8 grams of fish per liter of test solution in a test chamber at any one time). For the limit testing, a single replicate per treatment containing five fish will be tested. The test fish will be impartially placed in each of the test chambers. One fish will be added to each test chamber, sequentially starting with the control(s) then the treatment, until all chambers contain their full complement of fish. This method of fish addition will be used to minimize bias due to organism selection. The test will be conducted for 96 hours commencing when the fish are first exposed to the test substance. Fish will be added to the test chambers within 30 minutes of the completion of the WAF siphoning. This route of administration was selected in order to comply with OECD and U.S. EPA testing guidelines.

Reason: To identify the nominal concentration selected.

Effect on Study Integrity: None.

3. Protocol Section: 10.1.8 – Analytical Confirmation

The concentrations of the total dissolved naphthenic acids in the WAFs will be measured in all control and test substance concentrations at 0, 24, 72, and 96 hours. Unless otherwise specified, all analytical samples will be collected from replicate test chambers. Additional samples may be collected and analyzed at the discretion of the Study Director. A minimum of two fortification spikes (quality control samples) will be prepared and analyzed with each sample set.

A 500 mL sample will be collected and transferred to a 1,000-mL separatory funnel. Each sample will be acidified with concentrated sulfuric acid to a pH level of 2.5 ± 0.1. A 100-mL volume of methylene chloride will be added to each sample and the samples shaken to mix. After approximately one minute of shaking, the sample phases will be allowed to separate and the the methylene chloride (lower layer) filtered through anhydrous sodium sulfate and collected in a 500-mL flat-bottomed flask. The methylene chloride extraction will be repeated once for each sample. The samples will then be evaporated to dryness using a rotary evaporator and quantitatively transferred to 15-mL culture tubes using two separate 5-mL aliquots of methylene chloride. Each sample will then be evaporated to dryness under a gentle stream of nitrogen and reconstituted with 4 mL of methylene chloride. Each 4 mL sample will be diluted using methylene chloride, if necessary, to produce an analyte concentration that is within the range of the standard curve. The samples will be put into vials and analyzed by FT-IR. QC fortifications will be prepared in a similar manner after dilution water had been fortified with naphthenic acids. Sample analysis was performed using a FT-IR system equipped with the following analytical parameters:

Manufacturer: Thermo Nicolet
Model: Avatar 360

Software: Omnic 32
IR Cell: Thermo Scientific, KBr 1.0 mm sealed cell
Cell Holder: Thermo Scientific
Dry Nitrogen Gas Used to Protect the IR Cell Between Runs: Yes
Scan Times: 64
Scan Range: 4000-400 cm^{-1}
Scan Model: Absorbance
Resolution: 4 cm^{-1}
Wave Number of Interest: 1743 cm^{-1}
Solvent Used for Background Collection: Methylene chloride

Note: These instrument parameters may be changed or modified to optimize conditions or to suit the instrument used.

Reason: To provide analytical methodology detail to the protocol.

Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** 6 Nov 09
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 6 Nov 09

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64406
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	2	**EFFECTIVE DATE:**	Nov. 23, 2009

1. Protocol Section: 10.2 – Additional Testing

   Due to > 50% mortality of the fish exposed at the UTC, an additional range-finding and definitive test will be performed.

   Based on results from the range-finding test, the nominal loading rates of the WAFs prepared for the definitive test will be 0 (control), 1.3, 2.5, 5.0, 10, and 20 mg/L. The WAFs will be prepared in the same manner as described in protocol amendment 1.

   The experimental design is as follows:

   A negative control consisting of exposure to test water only will be tested concurrently. All test chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, level 1, etc.), and replicate (e.g., A, B, etc.).

   The test chambers will be glass and of a size sufficient to meet the fish loading restriction (i.e., loading will not exceed 0.8 grams of fish per liter of test solution in a test chamber at any one time). A single replicate per treatment containing seven fish will be tested. The test fish will be impartially placed in each of the test chambers. One fish will be added to each test chamber, sequentially starting with the control(s) then the treatment, until all chambers contain their full complement of fish. This method of fish addition will be used to minimize bias due to organism selection. The test will be conducted for 96 hours commencing when the fish are first exposed to the test substance. Fish will be added to the test chambers within 30 minutes of the completion of the WAF siphoning. This route of administration was selected in order to comply with OECD and U.S. EPA testing guidelines.

   Observations of mortality, moribundity, and behavior will be recorded and reported for all WAF loading rates. Observations will be made at 24, 48, 72, and 96 hours (±1 hour from test initiation). Upon termination of the study, all fish will be euthanized according to one of the appropriate procedures detailed in ABC SOP CD-TS 1.7 "Euthanasia of Aquatic Vertebrates". The euthanasia procedure will be selected based upon study specific sampling requirements.

Reason: To describe the definitive test to be performed.

Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** 23 Nov 09
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 23 Nov 09

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64406
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	3	**EFFECTIVE DATE:**	Dec. 8, 2009

1. Protocol Section: 10.1.2 – Renewals

   All control and test substance treatment solutions will be freshly prepared and renewed daily. At each renewal period, the test solutions will be renewed by either transferring surviving fish from old solutions to new solutions using a soft net or decanting off most of the old solutions and adding the fish and remaining solutions to the new solutions.

   Reason: Based upon the age of the fish (approximately 24 days old upon initiation of the definitive test), this amendment describes the method of transferring fish from old solutions to new solutions in a means to minimize the potential of damaging the fish.

   Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** 8 Dec. 09
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 8 Dec 09

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64406
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	4	**EFFECTIVE DATE:**	Nov. 23, 2009

1. Protocol Section: 12.0 – Test Acceptability Criteria

   The definitive test will not be valid if mortality in any control treatment exceeds 14 percent during the 96-hour test.

   Reason: The guideline allows one fish mortality in the control group if less than 10 fish are used per treatment level. Because seven fish were used per treatment level during the test, mortality in the control treatment cannot exceed 14 percent.

   Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** 19 Jan 10
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 19 Jan 10

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64406
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	5	**EFFECTIVE DATE:**	Sept. 24, 2009

1. Protocol Section: 4.0 – Testing Facility and Study Director Address

   Additional analytical chemistry identification work will be performed at:

   Department of Biological Sciences
   Z-207 Biological Sciences Centre
   116th Street and 85th Avenue
   University of Alberta
   Edmonton, Alberta T6G 2R3 Canada

   Reason: To identify the location where additional analytical work will be performed.

   Effect on Study Integrity: None. This is additional work being contracted by the sponsor.

2. Protocol Section: 10.1.8 – Analytical Confirmation

   Analytical samples will also be collected at study initiation and sent to Dr. Fedorak at the University of Alberta for analysis.

   Reason: To describe the additional analytical samples to be collected for analysis by Dr. Fedorak.

   Effect on Study Integrity: None. This is additional work being contracted by the sponsor.

3. Protocol Section: 13.0 – Report

   The report from Dr. Fedorak's analysis will be presented in an appendix to the final report. Because ABC is not the sponsor of this additional work, ABC is not responsible for its GLP compliance or noncompliance and the corresponding data. A statement regarding these analyses will be added to the Statement of GLP Compliance page in the final report.

   Reason: To describe how Dr. Fedorak's analyses will be reported.

Effect on Study Integrity: None. The analyses performed by Dr. Fedorak are additional work contracted by the sponsor.

STUDY DIRECTOR:	[redacted]	**DATE:**	20July10
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:**	20Jul10

PROTOCOL DEVIATION NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Fathead Minnow, *Pimephales promelas*, Determined Under Static-Renewal Test Conditions Using a Step-Down Approach
TEST FACILITY:	ABC Laboratories, Inc. **ABC STUDY NO.:** 64406
STUDY SPONSOR:	American Petroleum Institute
DEVIATION NO.:	1 **NOTIFICATION DATE:** Dec. 11, 2009

DEVIATION:

1. Protocol Section 10.1.4 – Temperature and Lighting

   The waterbath temperature was not maintained at 22 ± 1°C during the entire definitive test.

   Reason: For approximately 18 hours on study days 2 and 3, the waterbath temperature exceeded the range stated in the protocol by 1.1°C. This is due to the temperature sensor being bumped outside of the waterbath, causing the heater to stay on. The temperature sensor was discovered, placed back in the waterbath, and the temperature returned to the specified range through the remainder of the study.

   Effect on Study Integrity: None. The minor temperature deviation did not adversely affect the fish survival.

STUDY DIRECTOR: [redacted]	**DATE:** 11 Dec 09
TEST FACILITY MANAGEMENT: [redacted]	**DATE:** 14 Dec 09

APPENDIX D. CHARACTERIZATION OF NAPHTHENIC ACIDS IN WAF SOLUTIONS BY GC/MS

1.0 Toxicity test with fish

1.1 Samples and Methods

In December 2009, a shipment of six samples containing the dissolved aqueous fraction (WAF) of naphthenic acids was received in the Department of Biological Sciences at the University of Alberta, Alberta, Canada. The six samples were from a fish exposure experiment, and the samples were labeled as control (0 mg/L), Level 1 (1.3 mg/L), Level 2 (2.5 mg/L), Level 3 (5.0 mg/L), Level 4 (10 mg/L), and Level 5 (20 mg/L).

An appropriate subsample volume (10 to 200 mL) from each bottle was diluted to 1 L with distilled water and these were extracted individually as outlined by Merlin et al. (2007). Briefly, the diluted sample was acidified to pH 2 with concentrated HCl, and then 150 g NaCl was dissolved into the sample. The water sample was then extracted with three 60-mL portions of chloroform (HPLC grade). Free carboxylic acids were separated from lipids by extracting the combined chloroform phase with three 10-mL portions of an alkaline aqueous solution containing 4% sodium carbonate (pH 11.6). The free carboxylic (naphthenic) acids were recovered from the alkaline solution by acidifying it with concentrated HCl to pH 2 and extracting three times with 10-mL portions of DCM. The combined DCM extracts were dried under nitrogen.

The residue was dissolved in 50 µL of DCM and the naphthenic acids were derivatized by adding 50 µL of MTBSTFA derivatizing agent (without 1% *t*-BDMCS) to each vial and heating at 60°C for 20 min.

1.2 Results and Discussion

The tables of relative abundances of each ion (expressed as percentages) in the six extracted fish water samples are shown in Tables 1.1 to 1.6. The values of the percentages reported in these tables are rounded to the nearest 0.1.

The distributions of ions in selected samples were used to prepare a three-dimensional plots of the ion abundances for each n and Z value. These plots are shown in Figures 1.1 to 1.3. The three-dimensional plots for the fish water samples that contained 20 mg WAF/L and 1.3 mg WAF/L are given in Figure 1.1 and Figure 1.2, respectively. The ion distributions in these two samples are consistent with the distributions found in the neat naphthenic acids.

The three-dimensional plot for the fish water sample that contained 0 mg WAF/L (control) given in Figure 1.3 is markedly different from the naphthenic acids-containing water samples (Figures 1.1 and 1.2). The most abundant ions in Figure 1.3 correspond to n = 16, Z = 0 and n = 18, Z =0, and are likely palmitic and stearic acids, respectively.

Table 1.1

Fish water Control

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.2
7	0.8	1.5	0.0	0.0	0.0	0.0	0.0	2.3
8	0.2	0.8	0.0	0.0	0.0	0.0	0.0	1.0
9	0.3	0.6	0.0	0.0	0.0	0.0	0.0	0.9
10	0.6	0.8	0.1	0.0	0.0	0.0	0.0	1.5
11	0.6	0.5	0.4	0.0	0.0	0.0	0.0	1.5
12	3.8	0.5	0.5	0.1	0.0	0.0	0.0	5.0
13	0.6	0.4	0.3	0.1	0.0	0.0	0.0	1.4
14	4.9	0.4	1.3	0.8	0.0	0.0	0.0	7.3
15	1.9	0.2	0.1	0.1	0.0	0.0	0.0	2.2
16	40.2	1.8	0.1	0.1	0.0	0.0	0.0	42.2
17	3.2	0.4	0.0	0.0	0.0	0.1	0.0	3.8
18	15.4	4.2	0.4	0.3	0.1	0.2	0.4	20.9
19	1.6	0.3	0.2	0.0	0.0	0.2	1.3	3.5
20	0.5	0.3	0.0	0.0	0.1	1.3	0.3	2.6
21	0.3	0.1	0.0	0.0	0.0	0.3	0.1	0.9
22	0.2	0.2	0.0	0.0	0.1	0.8	0.1	1.4
23	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.3
24	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2
25	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	75.7	13.1	3.5	1.6	0.5	3.3	2.2	100.0

Table 1.2

Fish water Level 1

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.1
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.0	1.0	0.0	0.0	0.0	0.0	0.0	1.9
8	0.7	0.6	0.0	0.0	0.0	0.0	0.0	1.3
9	0.7	0.7	0.0	0.0	0.0	0.0	0.0	1.5
10	0.8	2.4	1.1	0.0	0.0	0.0	0.0	4.3
11	1.3	6.5	4.3	0.0	0.0	0.0	0.0	12.0
12	2.3	8.4	7.7	0.7	0.0	0.0	0.0	19.1
13	1.7	7.3	7.7	1.2	0.0	0.0	0.0	17.9
14	1.7	4.6	5.2	1.2	0.4	0.0	0.0	13.1
15	0.8	2.2	2.7	0.9	0.3	0.0	0.0	6.8
16	5.6	1.1	1.3	0.6	0.2	0.1	0.0	9.0
17	0.4	0.4	0.6	0.3	0.2	0.1	0.0	1.9
18	2.3	0.5	0.3	0.2	0.1	0.1	0.1	3.7
19	0.3	0.1	0.1	0.1	0.1	0.1	0.3	1.0
20	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.6
21	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.3
22	0.1	0.0	0.0	0.0	0.0	0.2	0.1	0.4
23	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.4
24	0.2	0.0	0.0	0.1	0.0	0.1	0.2	0.6
25	0.2	0.1	0.0	0.1	0.0	0.1	0.2	0.7
26	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.7
27	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.5
28	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.3
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	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
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	22.0	36.4	31.3	5.7	1.6	1.5	1.5	100.0

Table 1.3

Fish water Level 2

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.1
6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
7	1.0	0.9	0.0	0.0	0.0	0.0	0.0	1.9
8	0.7	0.5	0.0	0.0	0.0	0.0	0.0	1.2
9	0.7	0.5	0.0	0.0	0.0	0.0	0.0	1.2
10	0.7	2.1	1.0	0.0	0.0	0.0	0.0	3.8
11	1.5	6.8	4.5	0.0	0.0	0.0	0.0	12.8
12	3.0	9.8	8.7	0.8	0.0	0.0	0.0	22.3
13	2.4	9.0	8.9	1.3	0.0	0.0	0.0	21.7
14	2.4	5.8	6.2	1.4	0.4	0.0	0.0	16.3
15	1.3	2.8	3.1	1.0	0.3	0.0	0.0	8.5
16	1.5	1.2	1.5	0.6	0.2	0.1	0.0	5.0
17	0.2	0.4	0.6	0.3	0.1	0.1	0.0	1.7
18	0.7	0.2	0.2	0.2	0.1	0.1	0.0	1.4
19	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.4
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	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
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	17.7	40.1	34.8	5.6	1.1	0.4	0.2	100.0

Table 1.4

Fish water Level 3

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.1
6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
7	1.0	0.9	0.0	0.0	0.0	0.0	0.0	1.9
8	0.8	0.5	0.0	0.0	0.0	0.0	0.0	1.3
9	0.3	0.4	0.0	0.0	0.0	0.0	0.0	0.7
10	0.7	1.1	0.7	0.0	0.0	0.0	0.0	2.4
11	1.8	5.0	3.5	0.0	0.0	0.0	0.0	10.3
12	3.6	9.0	8.1	0.7	0.0	0.0	0.0	21.4
13	3.0	9.3	9.1	1.4	0.0	0.0	0.0	22.8
14	3.0	6.3	6.6	1.5	0.4	0.0	0.0	17.8
15	1.7	3.2	3.5	1.1	0.3	0.0	0.0	9.8
16	1.8	1.4	1.8	0.7	0.2	0.1	0.0	5.9
17	0.2	0.5	0.7	0.4	0.1	0.1	0.0	2.0
18	0.9	0.2	0.2	0.2	0.1	0.1	0.0	1.6
19	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.4
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	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
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	20.2	37.8	34.3	6.0	1.2	0.4	0.1	100.0

Table 1.5

Fish water Level 4

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
7	0.9	0.9	0.0	0.0	0.0	0.0	0.0	1.9
8	0.8	0.5	0.0	0.0	0.0	0.0	0.0	1.4
9	1.4	0.8	0.0	0.0	0.0	0.0	0.0	2.2
10	1.9	2.8	1.1	0.0	0.0	0.0	0.0	5.8
11	3.1	6.9	4.2	0.0	0.0	0.0	0.0	14.3
12	5.3	8.8	7.5	0.6	0.0	0.0	0.0	22.2
13	3.2	7.7	7.5	1.1	0.0	0.0	0.0	19.5
14	2.8	4.9	5.1	1.2	0.3	0.0	0.0	14.3
15	1.5	2.5	2.6	0.8	0.2	0.0	0.0	7.6
16	2.1	1.1	1.3	0.5	0.1	0.1	0.0	5.3
17	0.2	0.4	0.5	0.3	0.1	0.1	0.0	1.6
18	1.0	0.3	0.2	0.2	0.1	0.1	0.0	1.8
19	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.4
20	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
22	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.2
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	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
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	25.8	37.7	30.2	4.7	0.9	0.5	0.2	100.0

Table 1.6

Fish water Level 5

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
7	0.9	0.9	0.0	0.0	0.0	0.0	0.0	1.8
8	0.8	0.5	0.0	0.0	0.0	0.0	0.0	1.4
9	1.0	1.0	0.0	0.0	0.0	0.0	0.0	2.1
10	1.9	3.4	1.3	0.0	0.0	0.0	0.0	6.6
11	3.2	7.5	4.5	0.0	0.0	0.0	0.0	15.2
12	4.7	8.9	7.7	0.6	0.0	0.0	0.0	21.9
13	3.4	7.7	7.4	1.1	0.0	0.0	0.0	19.5
14	2.8	5.0	4.9	1.1	0.3	0.0	0.0	14.0
15	1.6	2.6	2.5	0.8	0.2	0.0	0.0	7.7
16	1.5	1.2	1.4	0.5	0.1	0.1	0.0	4.8
17	0.2	0.4	0.6	0.3	0.1	0.1	0.0	1.6
18	0.6	0.2	0.2	0.2	0.1	0.0	0.0	1.3
19	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.3
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	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
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	24.2	39.4	30.5	4.5	0.9	0.3	0.1	100.0

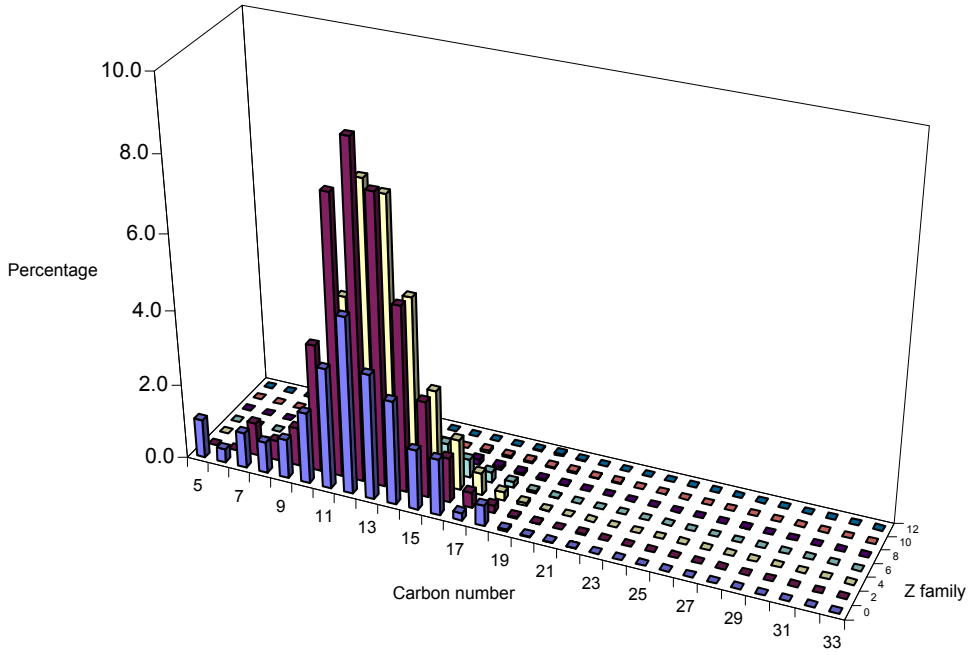

Figure. 1.1. Three-dimensional plot of naphthenic acids in the fish water that contained 20 mg WAF/L (Level 5). (Table 3.1.6). The sum of all bars equals 100%.

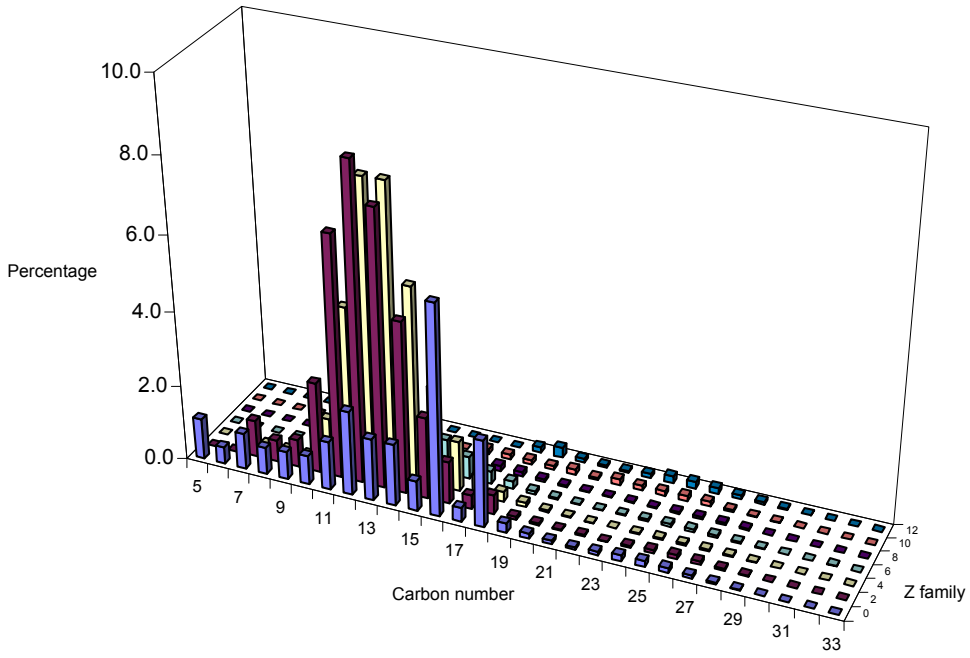

Figure. 1.2. Three-dimensional plot of naphthenic acids in the fish water that contained 1.3 mg WAF/L (Level 1). (Table 3.1.2). The sum of all bars equals 100%.

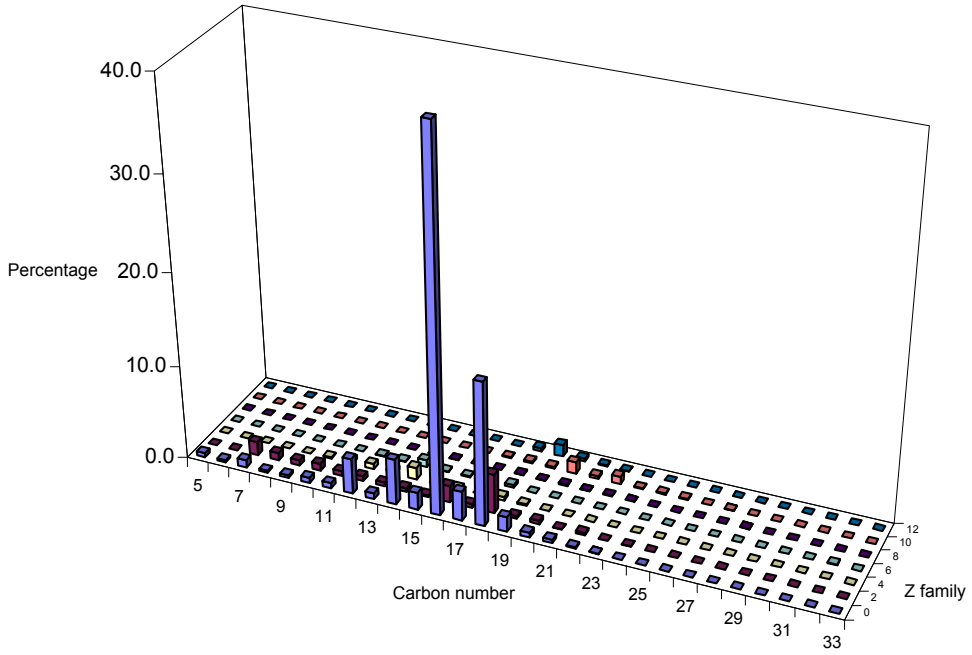

Figure. 1.3. Three-dimensional plot of naphthenic acids in the fish water that contained 0 mg WAF/L (Control). (Table 3.1.1). The sum of all bars equals 100%.

The most common fatty acids found in the phospholipids and glycolipids in cell membranes are C-16 (palmitic) and C-18 (stearic) acids (Stryer 1981). These fatty acids are also commonly found in the membranes of microorganisms (Lechevalier and Lechevalier 1988; O'Leary and Wilkinson 1988). Palmitic and stearic acids were observed by in bacterial cultures in which naphthenic acids had been removed by biodegradation (Clemente et al. 2004; Biryukova et al. 2007). In addition, these acids have also been found in river water samples (Fatoki and Vernon 1989; Scott et al. 2008). Thus, the appearance and predominance of palmitic and stearic acids in the 0 mg WAF/L (control) samples (Figure 1.3) is not unexpected because there were very likely microbial cells in these fish water samples.

The statistical method of Clemente et al. (2003) was also used to determine if there were any differences among the various WAF samples. This method allows two samples to be compared to one another. The Level 5 (20 mg/L) sample was arbitrarily chosen for each set of comparisons. Thus, each water sample was compared to the Level 5 (20 mg/L) sample. The results of these comparisons are summarized in Table 1.7.

Figures 1.1 and 1.3 show a marked difference between the distribution of ions in the 20 mg WAF/L fish water sample and the 0 mg WAF/L fish water sample. The statistical comparison of these two samples (Table 1.7) shows that (a) there is a difference between the Group 1 naphthenic acids in these two samples, and (b) there is a difference between the Group 3 naphthenic acids in these two samples.

Table 1.7. Statistical comparisons of distributions of naphthenic acids in various WAF fish water samples with the distributions of naphthenic acids in the Level 5 (20 mg/L) sample. "S" indicates a significant difference ($P < 0.05$); "NS" indicates no significant difference ($P > 0.05$).

Sample	Group 1 (C_5 to C_{13})	Group 2 (C_{14} to C_{21})	Group 3 (C_{22} to C_{33})
Control 0 (mg/L)	S	NS	S
Level 1 (1.3 mg/L)	NS	NS	S
Level 2 (2.5 mg/L)	NS	NS	NS
Level 3 (5.0 mg/L)	NS	NS	NS
Level 4 (10 mg/L)	NS	NS	NS

Surprisingly, the statistical method does not show a difference between the Group 2 naphthenic acids in the control (0 mg/L) and in the Level 5 (20 mg/L) samples (Table 1.7), despite the visual differences between Figures. 1.1 and 1.3.

As part of the statistical analysis developed by Clemente et al. (2003), each percent value in the matrix (e.g. Table 1.6) was divided by 100, and the arcsine of each of the quotient was taken as a variance stabilizing transformation. The mean of the arcsine-transformed data for each group from the GC-MS analysis of one sample was compared to the mean of the corresponding arcsine-transformed data of the corresponding group from a second sample by applying an independent two-sample t-test assuming equal variance.

The mean of the relative abundance of the ions in each group from one naphthenic acids sample is compared to the sum of the relative abundance of the ions in each group from another naphthenic acids sample by a two-sided t-te st. For example, the comparisons in Table 1.7 are based on the relative abundance of ions in one group of an individual sample being compared to the relative abundance of the ions in the corresponding group in the 20 mg WAF/L fish water sample.

The inability of this statistical test to detect a difference between the sample means of the Group 2 naphthenic acids in the control (0 mg/L) and in the Level 5 (20 mg/L) samples is because of the large variability in the abundances of the various ions observed in the GC-MS analysis. For example, from Table 1.1, the abundance of the n = 16, Z = 0 ion was 40.2% whereas the abundance of the n = 17, Z = -4 was 0.0% in the control water. Even after the arcsine-transformations, this large variability would yield a large standard deviation, making it difficult to distinguish between the sample means. This is a limitation of the statistical method of Clemente et al. (2003).

The statistical analyses discussed above are based on ion groupings according to carbon numbers. The data from the GC-MS analyses of the control (Table 1.1) and the 20 mg WAF/L (Level 5) (Table 1.6) have been analyzed by the same statistical approach with groupings based on Z numbers. These t-tests showed that the Z = -4 groups of the control and 20 mg WAF/L (Level 5) samples are statistically different and this result is consistent with results from an ongoing survey of river water samples and naphthenic acids-containing oil sands water samples from Alberta that have shown that the abundance of the Z = -4 group is an excellent indicator of the presence of naphthenic acids (Fedorak and co-workers, unpublished). Indeed, Merlin et al.

(2007) and Young et al. (2007; 2008) selectively monitored for ions corresponding to n = 13, Z = -4 to detect naphthenic acids in water and fish samples.

The only other statistical difference between samples shown in Table 1.7 is in the Group 3 (C_{22} to C_{33}) naphthenic acids of the 1.3 mg WAF/L (Level 1) fish water sample and the 20 mg WAF/L (Level 5) fish water sample. However, commercial naphthenic acids preparations typically lack Group 3 acids (Clemente et al. 2003). For example, only 0.12% of the ions detected in the Level 5 extract fell into the Group 3 acids. In contrast, 2.2% of the ions detected in the Level 1 extract fell into the Group 3 acids. Although this small difference was enough to be considered statistically different by the method of Clemente et al. (2003), the magnitude of the difference is not important.

2.0 References

Biryukova, O.V., P.M. Fedorak & S.A. Quideau. (2007) Biodegradation of naphthenic acids by rhizosphere microorganisms. *Chemosphere 67,* 2058-2064.

Clemente, J.S, N.G.N. Prasad, M.D. MacKinnon & P.M. Fedorak. (2003) A statistical comparison of naphthenic acids characterized by gas chromatography-mass spectrometry. *Chemosphere 50,* 1265-1274.

Clemente, J.S., M.D. MacKinnon & P.M. Fedorak. (2004) Aerobic biodegradation of two commercial naphthenic acids preparations. *Environ. Sci. Technol. 38,* 1009-1016.

Fatoki, O.S. & F. Vernon. (1989) Determination of free fatty acid content of polluted and unpolluted waters. *Water Res. 23*, 123–125.

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Scott, A.C., R.F. Young & P.M. Fedorak. (2008) Comparison of GC-MS and FTIR methods for quantifying naphthenic acids in water samples. *Chemosphere 73,* 1258-1264.

Stryer, L. (1981) Biochemistry 2nd Edition. W,H. Freeman and Company, New York. p. 207.

Young, R.F., E.A. Orr, G.G. Goss & P.M. Fedorak. (2007) Detection of naphthenic acids in fish exposed to commercial naphthenic acids or oil sands process-affected water. *Chemosphere 68,* 518-527.

Young, R.F., W.V. Wismer, & P.M. Fedorak. (2008) Estimating naphthenic acids in laboratory-exposed fish and in fish from the wild. *Chemosphere 73,* 498-505.

APPENDIX E. STATISTICS

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

PRINTOUT OF RAW DATA
BY EXPOSURE PERIOD

EXP_PERD=24 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.0	7	0
L1	1.3	7	0
L2	2.5	7	0
L3	5.0	7	1
L4	10.0	7	1
L5	20.0	7	3

EXP_PERD=48 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.0	7	1
L1	1.3	7	0
L2	2.5	7	0
L3	5.0	7	1
L4	10.0	7	2
L5	20.0	7	7

EXP_PERD=72 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.0	7	1
L1	1.3	7	0
L2	2.5	7	0
L3	5.0	7	1
L4	10.0	7	2
L5	20.0	7	7

EXP_PERD=96 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.0	7	1
L1	1.3	7	0
L2	2.5	7	0
L3	5.0	7	1
L4	10.0	7	4
L5	20.0	7	7

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	0	0.0000	0.0000
1.3000	7	0	0.0000	0.0038
2.5000	7	0	0.0000	0.0187
5.0000	7	1	0.1429	0.0725
10.0000	7	1	0.1429	0.2025
20.0000	7	3	0.4286	0.4175

Goodness of Fit Test:
Pearson Chi-Square = 0.8322 with 3
Prob>Chi-Square (P)= 0.8417 Degrees of Freedom

****
* Since the P value for Goodness of Fit is greater *
* than 0.05 and there is no other evidence of *
* questionable convergence, the probit model may be an *
* appropriate method for these data. *
****

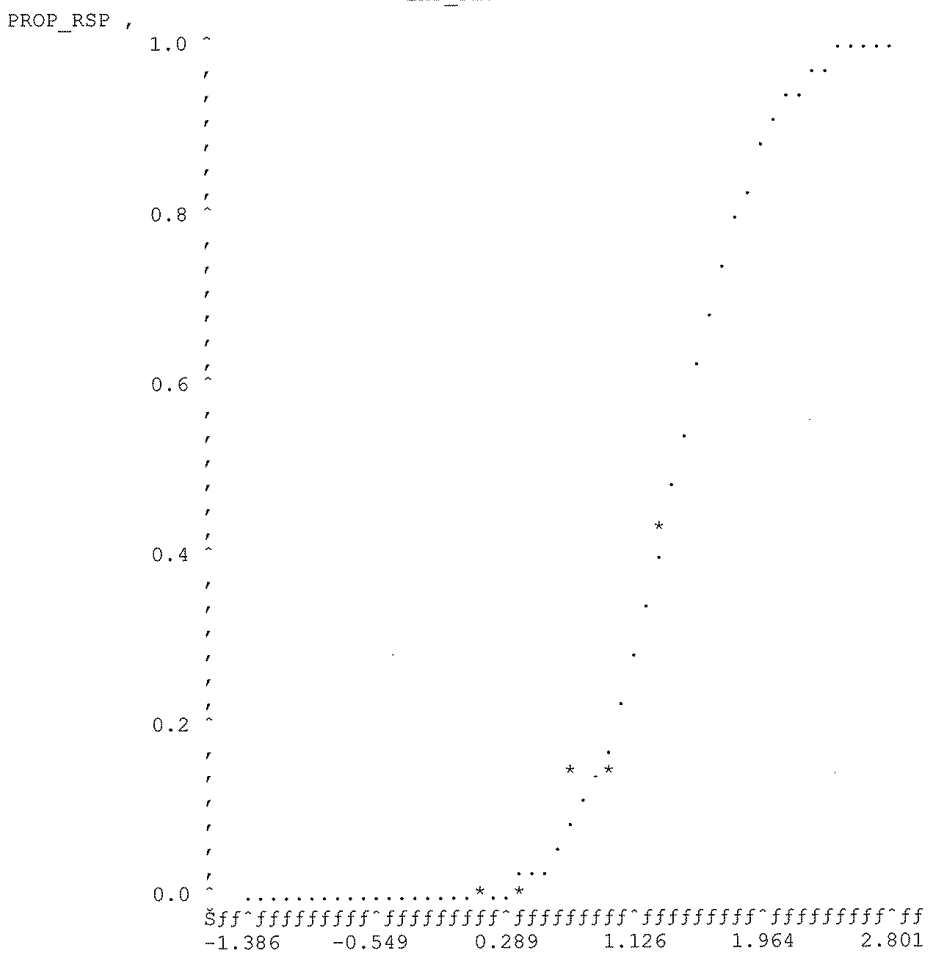
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	2.092898	1.036764	(0.06084, 4.124957)
Slope	2.074338	0.988697	(0.13649, 4.012185)
Threshold	0.000000		

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	25.203 (>Highest)	12.610	14791264

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 1 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	0	0.0000	0.0000
1.3000	7	0	0.0000	0.0000
2.5000	7	0	0.0000	0.0000
5.0000	7	1	0.1429	0.1429
10.0000	7	1	0.1429	0.1429
20.0000	7	3	0.4286	0.4286

TRIMMED SPEARMAN-KARBER ESTIMATE CANNOT BE MADE.

SMOOTHED/ADJUSTED PROPORTIONS DO NOT SPAN 50%.

ALL PROPORTIONS ARE LESS THAN 50%.

LC50 IS GREATER THAN THE
HIGHEST CONCENTRATION = 20.0000 MG/L

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0714
1.3000	7	0	0.0000	0.0714
2.5000	7	0	0.0000	0.0714
5.0000	7	1	0.1429	0.0714
10.0000	7	2	0.2857	0.2857
20.0000	7	7	1.0000	1.0000

Goodness of Fit Test:
Pearson Chi-Square = 2.1538 with 3
Prob>Chi-Square (P)= 0.5411 Degrees of Freedom

```
**********************************************************
*  Since either the P value for Goodness of Fit is less *
*  than 0.05 or there is other evidence of questionable *
*  convergence, the probit model may NOT be an          *
*  appropriate method for these data.                   *
**********************************************************
```

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-20.77969983613.45337		(-163903.148,163861.58890)
Slope	25.04338383613.45335		(-163857.325,163907.41195)
Threshold	0.071429	0.048670	(-0.02396, 0.166822)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	10.700	0	+INFINITY

```
                        ABC LABORATORIES, INC.
                SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
           STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
        FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

              ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
                  BASED ON NUMBER DEAD OF NUMBER EXPOSED
                       *'S ARE OBSERVED DATA POINTS
                  .'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
              INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL

------------------------------ EXP_PERD=48 HOUR -------------------------
PROP_RSP ,
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              -1.386    -0.549     0.289     1.126     1.964     2.801

                                       LOG_CONC
NOTE: 58 obs had missing values.  1 obs hidden.
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ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
1.3000	7	0	0.0000	0.0000
2.5000	7	0	0.0000	0.0000
5.0000	7	1	0.1429	0.1000
10.0000	7	2	0.2857	0.2500
20.0000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 11.096 MG/L
95% LOWER CONFIDENCE LIMIT: 8.419 MG/L
95% UPPER CONFIDENCE LIMIT: 14.623 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0714
1.3000	7	0	0.0000	0.0714
2.5000	7	0	0.0000	0.0714
5.0000	7	1	0.1429	0.0714
10.0000	7	2	0.2857	0.2857
20.0000	7	7	1.0000	1.0000

Goodness of Fit Test:
Pearson Chi-Square = 2.1538 with 3
Prob>Chi-Square (P)= 0.5411 Degrees of Freedom

```
**********************************************************
*  Since either the P value for Goodness of Fit is less *
*  than 0.05 or there is other evidence of questionable *
*  convergence, the probit model may NOT be an          *
*  appropriate method for these data.                   *
**********************************************************
```

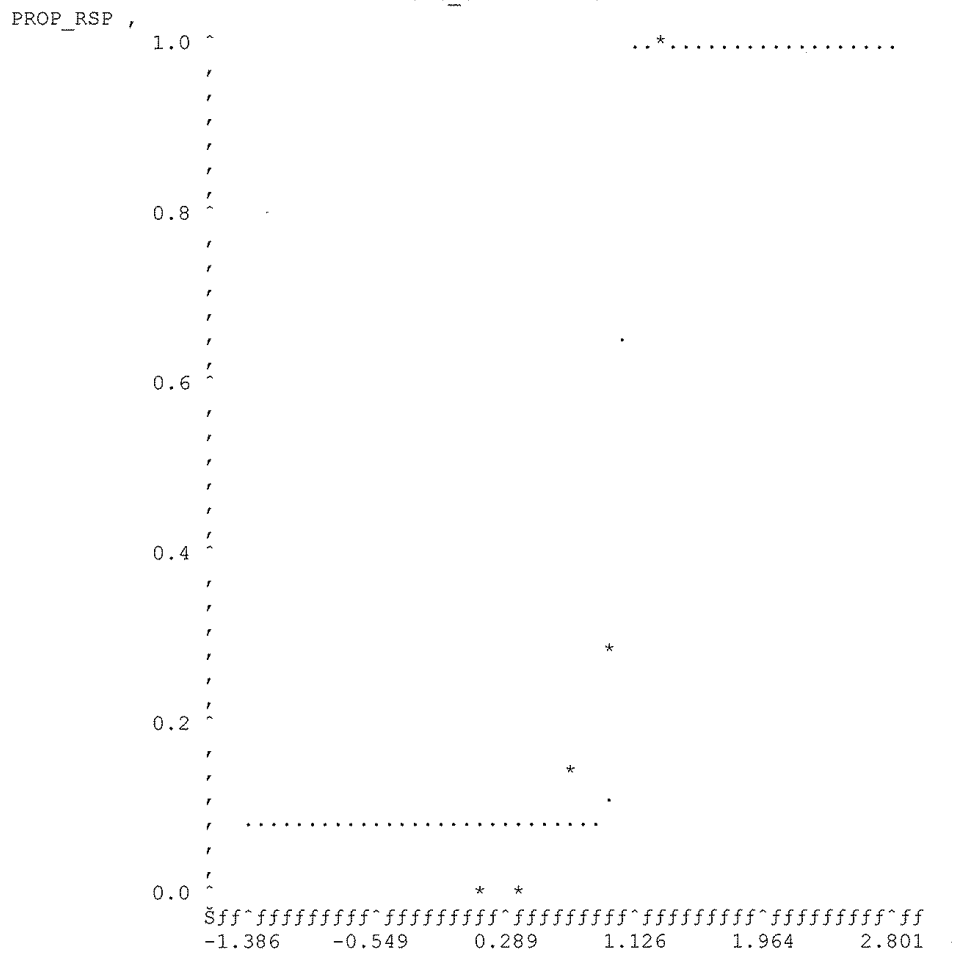
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-20.77969983613.45337		(-163903.148,163861.58890)
Slope	25.04338383613.45335		(-163857.325,163907.41195)
Threshold	0.071429	0.048670	(-0.02396, 0.166822)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	10.700	0	+INFINITY

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 1 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
1.3000	7	0	0.0000	0.0000
2.5000	7	0	0.0000	0.0000
5.0000	7	1	0.1429	0.1000
10.0000	7	2	0.2857	0.2500
20.0000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 11.096 MG/L
95% LOWER CONFIDENCE LIMIT: 8.419 MG/L
95% UPPER CONFIDENCE LIMIT: 14.623 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0528
1.3000	7	0	0.0000	0.0528
2.5000	7	0	0.0000	0.0530
5.0000	7	1	0.1429	0.1050
10.0000	7	4	0.5714	0.6242
20.0000	7	7	1.0000	0.9839

Goodness of Fit Test:
Pearson Chi-Square = 2.2228 with 3
Prob>Chi-Square (P)= 0.5275 Degrees of Freedom

```
**********************************************************
*  Since either the P value for Goodness of Fit is less *
*  than 0.05 or there is other evidence of questionable *
*  convergence, the probit model may NOT be an          *
*  appropriate method for these data.                   *
**********************************************************
```

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-0.913854	3.225583	(-7.23599, 5.408288)
Slope	6.175543	3.146299	(0.00879, 12.342288)
Threshold	0.052772	0.052348	(-0.04983, 0.155374)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	9.070	0	+INFINITY

```
                        ABC LABORATORIES, INC.
                SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
           STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
        FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

              ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
                  BASED ON NUMBER DEAD OF NUMBER EXPOSED
                       *'S ARE OBSERVED DATA POINTS
                  .'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
              INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL

------------------------------ EXP_PERD=96 HOUR -------------------------
PROP_RSP ,
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          0.0 ^                      *  *
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              -1.386    -0.549     0.289     1.126     1.964     2.801

                                       LOG_CONC
NOTE: 58 obs had missing values.
```

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 11DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
1.3000	7	0	0.0000	0.0000
2.5000	7	0	0.0000	0.0000
5.0000	7	1	0.1429	0.1000
10.0000	7	4	0.5714	0.5500
20.0000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 9.013 MG/L
95% LOWER CONFIDENCE LIMIT: 6.647 MG/L
95% UPPER CONFIDENCE LIMIT: 12.219 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

THIS COMPLETE ANALYSIS WAS CONDUCTED

BY: AMANDA GERKE ON: 11DEC09 AKG 11 Dec 09

THE ANALYSIS WAS REVIEWED

BY: AV ON: 11DEC09

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

PRINTOUT OF RAW DATA
BY EXPOSURE PERIOD

EXP_PERD=24 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.00	7	0
L1	0.90	7	0
L2	2.08	7	0
L3	3.22	7	1
L4	6.04	7	1
L5	13.80	7	3

EXP_PERD=48 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.00	7	1
L1	0.90	7	0
L2	2.08	7	0
L3	3.22	7	1
L4	6.04	7	2
L5	13.80	7	7

EXP_PERD=72 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.00	7	1
L1	0.90	7	0
L2	2.08	7	0
L3	3.22	7	1
L4	6.04	7	2
L5	13.80	7	7

EXP_PERD=96 HOUR

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
Con.	0.00	7	1
L1	0.90	7	0
L2	2.08	7	0
L3	3.22	7	1
L4	6.04	7	4
L5	13.80①	7	7

① SAS printout didn't include last digit wky 8Jan10

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	0	0.0000	0.0000
0.9000	7	0	0.0000	0.0043
2.0800	7	0	0.0000	0.0303
3.2200	7	1	0.1429	0.0690
6.0400	7	1	0.1429	0.1795
13.8000	7	3	0.4286	0.4311

Goodness of Fit Test:
Pearson Chi-Square = 0.9070 with 3
Prob>Chi-Square (P)= 0.8237 Degrees of Freedom

****
* Since the P value for Goodness of Fit is greater *
* than 0.05 and there is no other evidence of *
* questionable convergence, the probit model may be an *
* appropriate method for these data. *
****

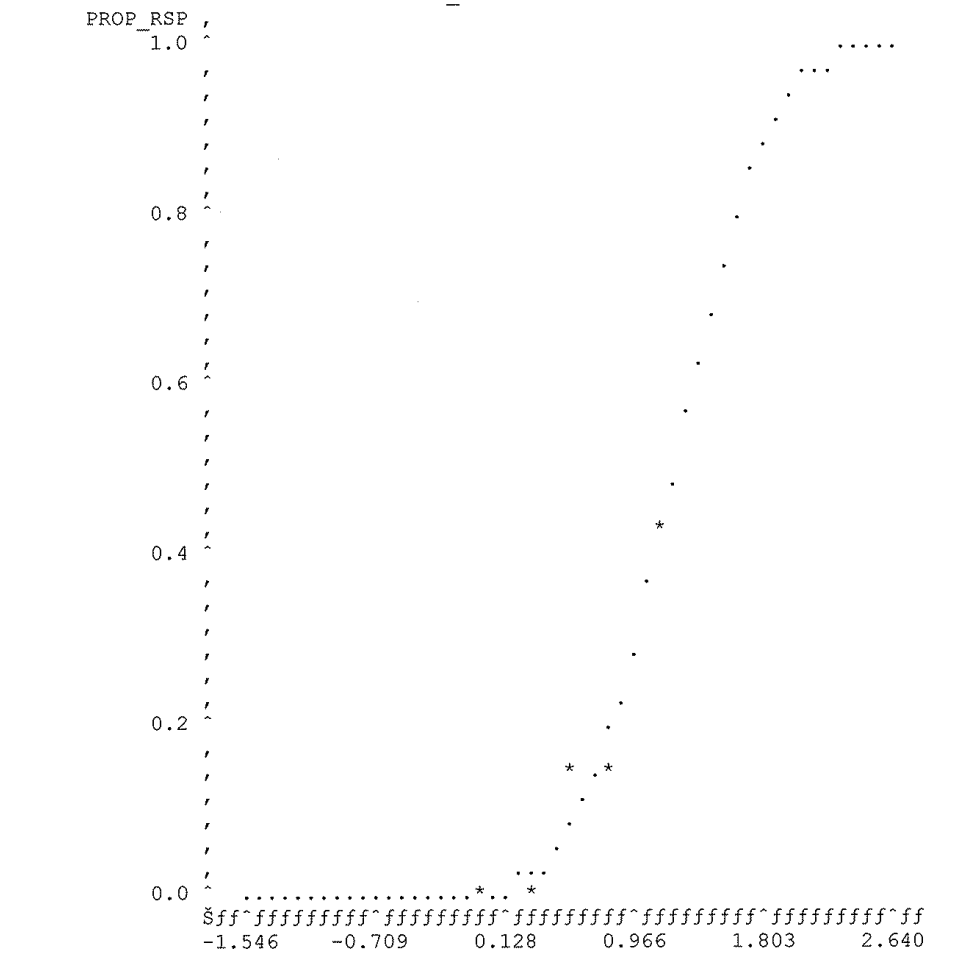
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	2.464404	0.833030	(0.83166, 4.097143)
Slope	2.072206	0.957512	(0.19548, 3.948931)
Threshold	0.000000		

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	16.735 (>Highest)	8.346	195333.7

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 2 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 24 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	0	0.0000	0.0000
0.9000	7	0	0.0000	0.0000
2.0800	7	0	0.0000	0.0000
3.2200	7	1	0.1429	0.1429
6.0400	7	1	0.1429	0.1429
13.8000	7	3	0.4286	0.4286

TRIMMED SPEARMAN-KARBER ESTIMATE CANNOT BE MADE.

SMOOTHED/ADJUSTED PROPORTIONS DO NOT SPAN 50%.

ALL PROPORTIONS ARE LESS THAN 50%.

LC50 IS GREATER THAN THE
HIGHEST CONCENTRATION = 13.8000 MG/L

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0714
0.9000	7	0	0.0000	0.0714
2.0800	7	0	0.0000	0.0714
3.2200	7	1	0.1429	0.0714
6.0400	7	2	0.2857	0.2857
13.8000	7	7	1.0000	1.0000

Goodness of Fit Test:
Pearson Chi-Square = 2.1538 with 3
Prob>Chi-Square (P)= 0.5411 Degrees of Freedom

```
**********************************************************
*  Since either the P value for Goodness of Fit is less *
*  than 0.05 or there is other evidence of questionable *
*  convergence, the probit model may NOT be an          *
*  appropriate method for these data.                   *
**********************************************************
```

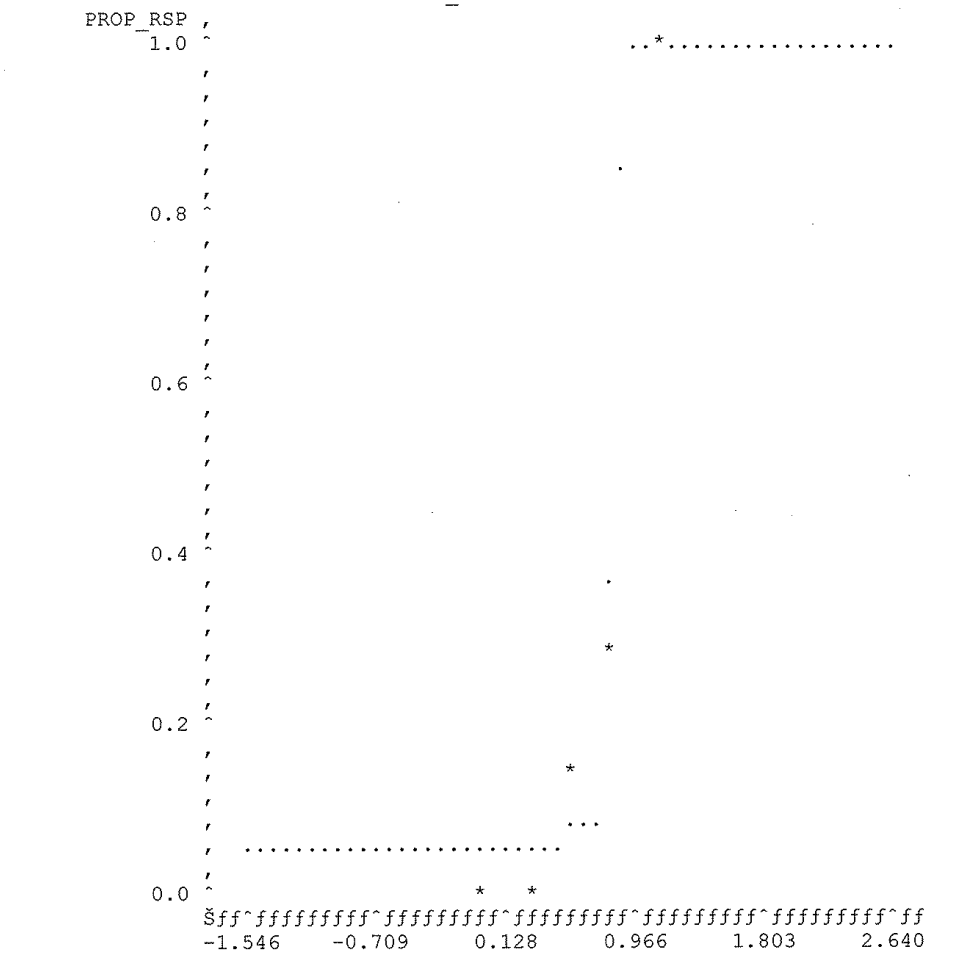
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-9.4866082	787.680588	(-5473.34056, 5454.367345)
Slope	17.6051753	569.201209	(-6978.02919, 7013.239545)
Threshold	0.071429	0.048670	(-0.02396, 0.166823)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	6.651	0	+INFINITY

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 1 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 48 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
0.9000	7	0	0.0000	0.0000
2.0800	7	0	0.0000	0.0000
3.2200	7	1	0.1429	0.1000
6.0400	7	2	0.2857	0.2500
13.8000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 7.216 MG/L
95% LOWER CONFIDENCE LIMIT: 5.525 MG/L
95% UPPER CONFIDENCE LIMIT: 9.425 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0714
0.9000	7	0	0.0000	0.0714
2.0800	7	0	0.0000	0.0714
3.2200	7	1	0.1429	0.0714
6.0400	7	2	0.2857	0.2857
13.8000	7	7	1.0000	1.0000

Goodness of Fit Test:
Pearson Chi-Square = 2.1538 with 3
Prob>Chi-Square (P)= 0.5411 Degrees of Freedom

```
**********************************************************
*  Since either the P value for Goodness of Fit is less *
*  than 0.05 or there is other evidence of questionable *
*  convergence, the probit model may NOT be an          *
*  appropriate method for these data.                   *
**********************************************************
```

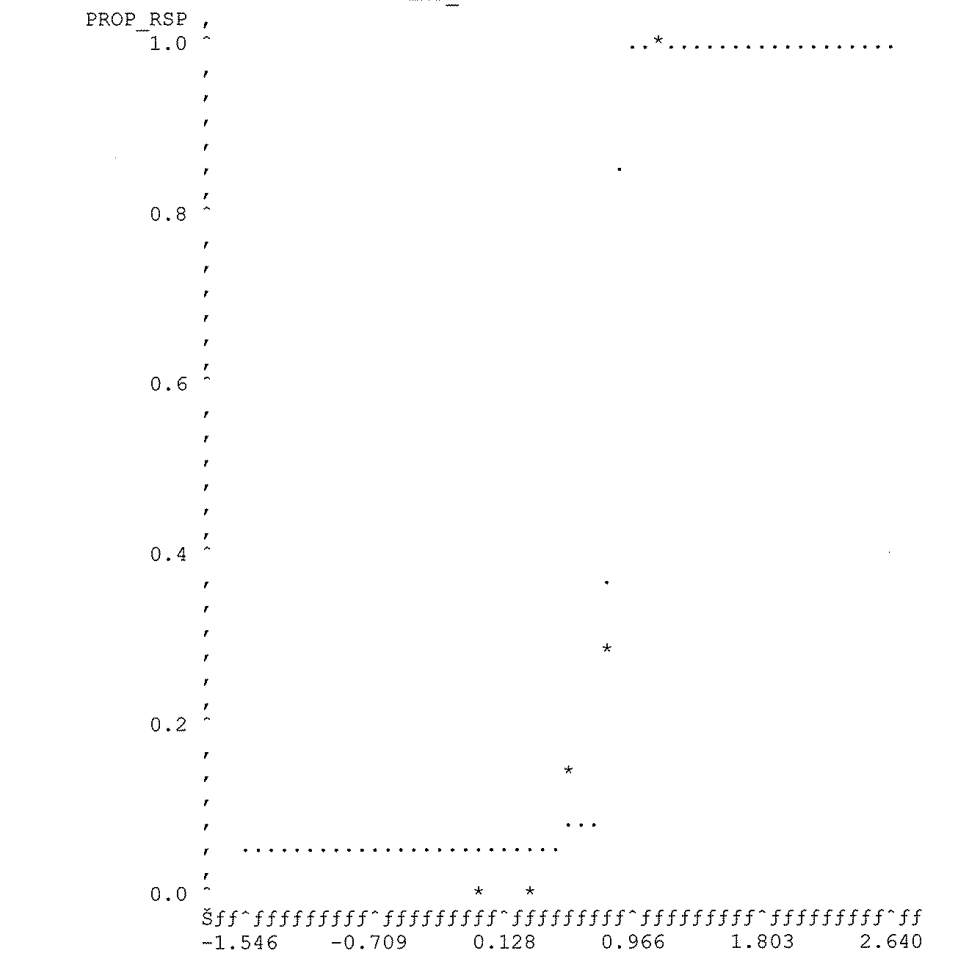
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-9.4866082	787.680588	(-5473.34056, 5454.367345)
Slope	17.6051753	569.201209	(-6978.02919, 7013.239545)
Threshold	0.071429	0.048670	(-0.02396, 0.166823)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	6.651	0	+INFINITY

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 1 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 72 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
0.9000	7	0	0.0000	0.0000
2.0800	7	0	0.0000	0.0000
3.2200	7	1	0.1429	0.1000
6.0400	7	2	0.2857	0.2500
13.8000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 7.216 MG/L
95% LOWER CONFIDENCE LIMIT: 5.525 MG/L
95% UPPER CONFIDENCE LIMIT: 9.425 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE PROBIT METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Predicted Proportion Responding
0.0000	7	1	0.1429	0.0519
0.9000	7	0	0.0000	0.0519
2.0800	7	0	0.0000	0.0548
3.2200	7	1	0.1429	0.1104
6.0400	7	4	0.5714	0.6013
13.8000	7	7	1.0000	0.9939

Goodness of Fit Test:
Pearson Chi-Square = 2.1077 with 3
Prob>Chi-Square (P)= 0.5504 Degrees of Freedom

```
**********************************************************
*  Since the P value for Goodness of Fit is greater      *
*  than 0.05 and there is no other evidence of           *
*  questionable convergence, the probit model may be an  *
*  appropriate method for these data.                    *
**********************************************************
```

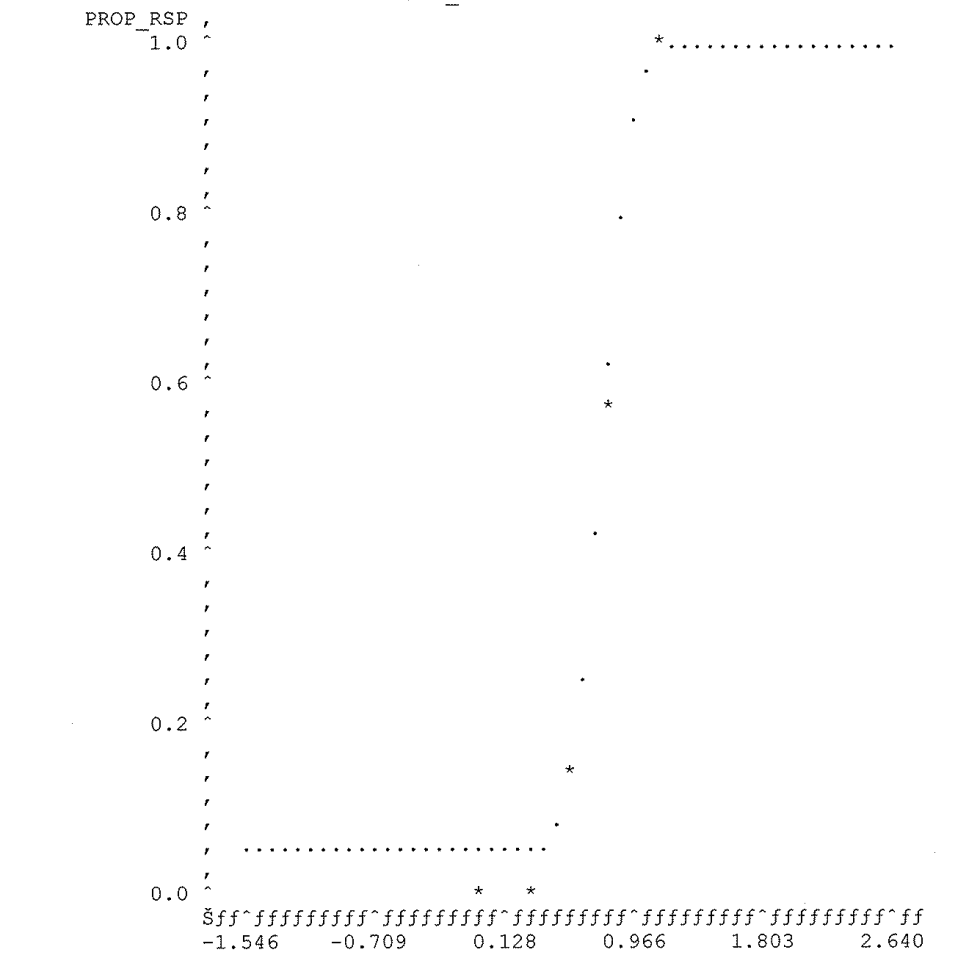
Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	0.222316	2.259130	(-4.20557, 4.650210)
Slope	6.373865	2.914668	(0.66111, 12.086615)
Threshold	0.051947	0.049939	(-0.04593, 0.149827)

Estimated LCx Values and Confidence Limits
For Specified Percentages (x):

Percentage (x)	Estimated Conc. (MG/L)	95% Confidence Limits Lower	Upper
50.00	5.618	2.468	10.639

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
*'S ARE OBSERVED DATA POINTS
.'S ARE PREDICTED VALUES FROM THE PROBIT MODEL
INSPECT THIS GRAPH FOR GOODNESS OF FIT OF DATA TO MODEL



NOTE: 58 obs had missing values. 2 obs hidden.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 31DEC09
STUDY NUMBER: 64406 WITH TEST MATERIAL: NAPHTHENIC ACIDS
FISH ACUTE STUDY USING DATA FILE: U:\GERKEA\SAS\64406LC50.PRN

ANALYSIS FOR FATHEAD MINNOW AT 96 HOUR
BASED ON NUMBER DEAD OF NUMBER EXPOSED
RESULTS CALCULATED USING THE SPEARMAN-KARBER METHOD

Conc. (MG/L)	Number Exposed	Number Resp.	Observed Proportion Responding	Smoothed/Adj. Proportion Responding
0.0000	7	1	0.1429	0.0000
0.9000	7	0	0.0000	0.0000
2.0800	7	0	0.0000	0.0000
3.2200	7	1	0.1429	0.1000
6.0400	7	4	0.5714	0.5500
13.8000	7	7	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

LC50: 5.801 MG/L
95% LOWER CONFIDENCE LIMIT: 4.301 MG/L
95% UPPER CONFIDENCE LIMIT: 7.824 MG/L

THE AUTOMATIC TRIM IS EQUAL TO ZERO.
UNTRIMMED AND TRIMMED SPEARMAN-KARBER ESTIMATES ARE THE SAME.

THIS COMPLETE ANALYSIS WAS CONDUCTED

BY: AMANDA GERKE ON: 31DEC09 AG 31Dec09

THE ANALYSIS WAS REVIEWED

BY: JH ON: 31Dec09

ABC LABORATORIES, INC.
SAS PROGRAM CH_TOX (VER 2.0) RUN ON 11DEC09
FATHEAD MINNOW FISH ACUTE STUDY OF NAPHTHENIC ACIDS
STUDY NUMBER: 64406 --- DATA FILE: U:\GERKEA\SAS\64406NOEC.PRN
ANALYSIS FOR NUMBER NORMAL
PRINTOUT OF RAW DATA

TREATMENT	REPLICATE	INITIAL NUMBER	FINAL NUMBER	PERCENT NORMAL
Con.	A	7	6	85.7
L1	A	7	7	100.0
L2	A	7	7	100.0
L3	A	7	6	85.7
L4	A	7	2	28.6
L5	A	7	0	0.0

N = 6

ABC LABORATORIES, INC.
SAS PROGRAM CH_TOX (VER 2.0) RUN ON 11DEC09
FATHEAD MINNOW FISH ACUTE STUDY OF NAPHTHENIC ACIDS
STUDY NUMBER: 64406 --- DATA FILE: U:\GERKEA\SAS\64406NOEC.PRN
ANALYSIS FOR NUMBER NORMAL
ALL VALUES IN THE DATA FILE ARE BEING PROCESSED
DESCRIPTIVE STATISTICS (N, MIN, MAX, MEAN, STANDARD DEVIATION, CV
AND UPPER AND LOWER 95% CONFIDENCE LIMITS)
BY TREATMENT GROUP

TREATMNT	NO_REPS	MINIMUM	MAXIMUM	AVERAGE	STD_DEV	CV	LOWER_CI	UPPER_CI
Con.	1	85.7	85.7	85.7	.	.	.	.
L1	1	100.0	100.0	100.0	.	.	.	.
L2	1	100.0	100.0	100.0	.	.	.	.
L3	1	85.7	85.7	85.7	.	.	.	.
L4	1	28.6	28.6	28.6	.	.	.	.
L5	1	0.0	0.0	0.0	.	.	.	.

ABC LABORATORIES, INC.
SAS PROGRAM CH_TOX (VER 2.0) RUN ON 11DEC09
FATHEAD MINNOW FISH ACUTE STUDY OF NAPHTHENIC ACIDS
STUDY NUMBER: 64406 --- DATA FILE: U:\GERKEA\SAS\64406NOEC.PRN
ANALYSIS FOR NUMBER NORMAL
ALL VALUES IN THE DATA FILE ARE BEING PROCESSED
COMPARING THE TREATMENT GROUPS TO THE CONTROL

RESULTS OF TESTS FOR NORMALITY & HOMOGENEITY OF VARIANCE

Shapiro-Wilk Test for Normality cannot be Conducted
When there are only 2 Reps for each Treatment.

Results of Bartlett's Test for Homogeneity of Variance
Conducted on Residuals for each Treatment.
P value less than 0.01 indicates Unequal Treatment Variances.
P value greater than 0.01 indicates Equal Treatment Variances.

VARIABLE	DEGREES OF FREEDOM	U	p VALUE
NORMAL	5	.	.
TRANSFORM	5	.	.

Conclusion:

Assumptions of Normality and Homogeneity of Variance
Are Not Met for the Raw or Transformed Values.
A Nonparametric Analysis is Performed on the Ranks of the Data.

ABC LABORATORIES, INC.
SAS PROGRAM CH_TOX (VER 2.0) RUN ON 11DEC09
FATHEAD MINNOW FISH ACUTE STUDY OF NAPHTHENIC ACIDS
STUDY NUMBER: 64406 --- DATA FILE: U:\GERKEA\SAS\64406NOEC.PRN
ANALYSIS FOR NUMBER NORMAL
ALL VALUES IN THE DATA FILE ARE BEING PROCESSED
COMPARING THE TREATMENT GROUPS TO THE CONTROL

DESCRIPTIVE STATISTICS AND RESULTS OF DUNNETT'S TEST

GROUP	MEAN	STD.DEV.	p	SIG.
Con.	85.700	.		
L1	100.00	.	.	
L2	100.00	.	.	
L3	85.700	.	.	
L4	28.600	.	.	
L5	0.000	.	.	

ABC LABORATORIES, INC.
SAS PROGRAM CH_TOX (VER 2.0) RUN ON 11DEC09
FATHEAD MINNOW FISH ACUTE STUDY OF NAPHTHENIC ACIDS
STUDY NUMBER: 64406 --- DATA FILE: U:\GERKEA\SAS\64406NOEC.PRN
ANALYSIS FOR NUMBER NORMAL
ALL VALUES IN THE DATA FILE ARE BEING PROCESSED

RESULTS OF FISHER'S ONE-TAILED EXACT TEST

GROUP	PERCENT NORMAL	FISHER'S 1-TAILED P	HOCHBERG SIGNIFICANCE
Con.	85.7		
L1	100.0	1.0000	
L2	100.0	1.0000	
L3	85.7	1.0000	
L4	28.6	0.0513	
L5	0.0	0.0023	*

Note: * indicates significant differences from control at the 0.05 level using a one-tailed Fisher's test with Hochberg's familywise adjustment for significance.

THIS COMPLETE ANALYSIS WAS CONDUCTED

BY: AMANDA GERKE ON: 11DEC09 AKJ 11 Dec 09

THE ANALYSIS WAS REVIEWED

BY: AV ON: 11Dec 09