STUDY TITLE

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions

DATA REQUIREMENT

U.S. EPA OPPTS 850.1010
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OECD Guideline 202

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SPONSOR

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STUDY IDENTIFICATION

ABC Study No. 64404

STATEMENT OF GLP COMPLIANCE

Test Stubstance: Naphthenic Acids

Study Title: Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions

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] 29SEPT2010
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Sponsor's Representative:

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QUALITY ASSURANCE STATEMENT

ABC Laboratories' Quality Assurance Unit reviewed Study No. 64404, entitled "Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions", 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
01 Oct 09	Procedure: Water Quality	05 Oct 09	05 Oct 09
14-15 Jan 10	Raw Data and Draft Report	15 Jan 10	27 Jan 10
27 Sept 10	Final Report	27 Sept 10	27 Sept 10

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

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29Sep2010
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STUDY SUMMARY

Study Sponsor:	The American Petroleum Institute
Protocol Title:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions
Location of Study:	ABC Laboratories, Inc. 7200 E. 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.:	64404
Test Substance:	Naphthenic Acids (CAS# 1338-24-5)
Test Species:	*Daphnia magna* (<24-hour old neonates)
Definitive Test Dates (in-life):	September 29 to October 1, 2009
Test Duration:	48 hours
Nominal Loading Rates:	0 (control), 5.0, 10, 20, 40, and 80 mg naphthenic acids/L
Mean Measured Concentrations:	< MQL (control), 3.90, 7.68, 17.0, 33.3, and 69.0 naphthenic acids/L
Environmental Conditions:	Temperature: 20.6 to 22.0°C DO Concentrations: 6.8 to 8.8 mg/L (81 to 104% saturation @22°C) pH: 7.5 to 8.6 Alkalinity: 148 mg $CaCO_3$/L Hardness: 150 mg $CaCO_3$/L Conductivity: 368 $\mu S \cdot cm^{-1}$ Photoperiod: 16-hr light: 8-hr dark Light Intensity: 521 lux
Results (Based on Nominal Loading Rates):	24-Hour EL_{50} = 28.3 mg naphthenic acids/L (95% confidence limits could not be calculated) 48-Hour EL_{50} = 24.0 mg naphthenic acids/L (95% confidence limits of 21.0 and 27.4 mg naphthenic acids/L) 48-Hour NOELR = 10 mg naphthenic acids/L

Results (Based on Mean Measured Concentrations):	24-Hour EC_{50} =	23.8 mg naphthenic acids/L (95% confidence limits could not be calculated)
	48-Hour EC_{50} =	20.0 mg naphthenic acids/L (95% confidence limits of 17.4 and 23.0 mg naphthenic acids/L)
	48-Hour NOEC =	7.68 mg naphthenic acids/L

1.0 INTRODUCTION

The American Petroleum Institute contracted ABC Laboratories, Inc. to perform a 48-hour static-renewal toxicity test to the freshwater invertebrate, *Daphnia magna*, exposed to water accommodated fraction (WAF) preparations of naphthenic acids (CAS# 1338-24-5). The criterion for effect was immobilization. Immobilization is defined as those animals that are not able to swim or show movement within 15 seconds, after gentle agitation of the test chamber or gentle disturbance of the daphnid itself. The primary objective of this test was to estimate the 48-hour median effect loading rate (EL_{50}) and concentration (EC_{50}) for the test substance. A secondary objective was to determine the 48-hour no-observed-effect loading rate (NOELR) and concentration (NOEC), if possible. The NOELR and NOEC are defined as the highest loading rate or concentration of test substance at which there is an absence of any abnormal effects or immobility.

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 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 Species

Daphnia magna neonates (<24-hours old) were obtained from an in-house daphnid culture. All daphnids were cultured in a temperature-controlled area at approximately 20°C. During the holding period, the daphnids were fed a suspension of the algal species, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) at least once a day supplemented by a commercially available artificial diet consisting of a wheat grass, salmon starter, and yeast suspension (YTC daphnid feed mixture; Aquatic Bio Systems Inc., Fort Collins, Colorado). Neonates for the definitive test were collected from a single culture containing adults that were approximately 13 days old. The adults were considered acceptable with no signs of stress, disease or physical damage. Since the culturing and testing environmental parameters were equivalent (i.e. temperature, dilution water, and lighting), no acclimation period was necessary. Test daphnids were not fed during the test.

2.3 Dilution Water

The dilution water was an aged 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 approximately 130 to 160 mg $CaCO_3$/L and biologically aged (held in a tank containing aquatic organisms). The water was then passed through a sediment filter, UV

irradiated, and aerated prior to use. Characterization of a representative sample of the base water, i.e., ABC well water, used to prepare the dilution water can be found 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 Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions" with amendments and deviation (Appendix C). The protocol was based on U.S. EPA OPPTS Guideline 850.1010 (1) and was intended to comply with the Organization for Economic Cooperation (OECD) Guideline 202 (2) and with U.S. EPA TSCA Guideline 797.1300 (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 (6).

2.4.1 Exposure System

The range-finding and definitive tests were conducted in 250-mL glass jars containing approximately 200 mL of control or test solution and covered with a plastic Petri dish. The jars were maintained at 20 ± 2°C in a temperature-controlled water bath. Fluorescent lighting was maintained on a 16-hour daylight photoperiod with 30-minute simulated dawn and dusk periods. The measured light intensity at the start of the definitive test was 521 lux as measured with a LI-COR Model LI-189 light meter equipped with a photometric sensor.

2.4.2 Range-Finding Test

Two range-finding tests were performed. A static range-finding test was conducted from July 28 to 30, 2009 at nominal loading rates of 0 (control), 5.0, 10, and 20 mg naphthenic acids/L. One water accommodated fraction (WAF) at each loading rate was prepared by adding the appropriate amount of test substance to 2 L of dilution water in a clean 2-L glass aspirator bottle. The control WAF preparation consisted of dilution water only. Each aspirator bottle contained a 2-inch Teflon-coated stir bar and was sealed with parafilm. The WAF preparations prepared for the first range-finding test were allowed to stir for 4 hours ±1 hour. 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 outlet of their respective aspirator bottle. The first ~100 mL of solution from each WAF was drained into a waste container. The remaining solutions were collected into separate clean glass containers, leaving a small portion of the WAF preparation in the aspirator bottles.

Five neonates were added to each of two test chambers per treatment at the start of the tests. The animals were observed after 24 and 48 hours. An additional replicate was prepared for each

treatment in order to achieve the necessary solution volume for analytical sampling. Five neonate daphnids were also added to these extra replicates, but were not biologically observed. After 48 hours of exposure in the first range-finding test (static), immobility was 0, 0, 0, and 30% in the 0 (control), 5.0, 10, and 20 mg naphthenic acids/L nominal loading rate treatments. Sublethal effects were noted in the 10 and 20 mg/L treatments (Table 1). 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.

Based on the dose response pattern and lower than expected analytical recoveries in the first range-finding test, a second range-finding test using static-renewal exposures was conducted from August 12 to 14, 2009 at nominal loading rates of 0 (control), 5.0, 10, 20, and 40 mg naphthenic acids/L. WAF preparations and test procedures for the second range-finding test were identical to those for the initial range-finding test, but WAF preparations were allowed to stir for 24 hours ±1 hour prior to collection. The procedure for preparing test solutions was repeated to prepare fresh solutions at the 24 hours time point of the second range-finding test.

After 48 hours of exposure in the second range-finding test (static-renewal), immobility was 0, 0, 0, 0, and 70% in the 0 (control), 5.0, 10, 20, and 40 mg naphthenic acids/L nominal loading rate treatments. Sublethal effects were noted in the 20 and 40 mg/L treatments (Table 2). 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.

2.4.3 Definitive Test

Based upon the results of the range-finding test, the definitive test was performed from September 29 to October 1, 2009 at nominal loading rates of 0 (control), 5.0, 10, 20, 40, and 80 mg Naphthenic Acids/L. One water accommodated fraction (WAF) at each loading rate was prepared by adding the appropriate amount of test substance to 4 L of dilution water in a clean 4-L glass carboy. The control WAF preparations consisted of dilution water only. Each carboy contained a 2-inch Teflon-coated stir bar and was sealed with parafilm. The WAF preparations were allowed to stir for 24 hours ±1 hour. 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 siphoned using a glass tube from their respective carboy. The first ~100 mL of solution from each WAF was drained into a waste container. The remaining solutions were collected into separate clean glass containers, leaving a small portion of the WAF preparation in the carboys. This procedure for preparing test solutions was repeated to prepare fresh solutions at the 24 hours time point 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.

Each treatment or control was replicated four times. All jars were labeled with the study number, treatment, and replicate. The jars were positioned by treatment in a water bath. No aeration was provided to any jar during the test.

Five neonates (<24-hours old) were added to each jar. Daphnids were impartially added to a set of labeled containers with each container representing one treatment replicate. Each container

was then randomly assigned to a treatment replicate by random number generator. The individuals within each container were then transferred into the corresponding test chamber below the surface of the solution using a pipet.

Renewal test chambers for the control and all remaining test treatments were 250-mL glass jars (the same as those used at initiation) with 200 mL of the appropriate solutions prepared as above. Organisms were transferred after 24 hours from "old" to "new" solutions using a glass pipet. Transfers began with the control and then proceeded from the lowest concentration to the highest concentration. Observations for immobility and sublethal responses were made every 24 hours (±1 hour) for the duration of the test. The 40 and 80 mg naphthenic acid/L treatments were not renewed at 24 hours due to 100% observed immobility in those treatments.

Total hardness, total alkalinity, and conductivity were measured in the dilution water at test initiation. Total hardness and alkalinity were measured using titrimetric methods adapted from Standard Methods (7). Conductivity was measured with a WTW Cond 330i conductivity/salinity meter. Temperature, dissolved oxygen concentration, and pH were measured daily in all replicates. Dissolved oxygen concentrations were measured with a WTW Oxi 330i dissolved oxygen meter. The test solution temperature and pH were measured with a WTW pH 330i meter. A thermistor probe was positioned in the water bath to continuously record temperature.

2.5 Analytical Test Method

Test solutions were analyzed for the concentration of naphthenic acids using Fourier transform infrared spectroscopy (FTIR). Analysis was accomplished based on the method described by Jivraj et al. (8) and developed at ABC Laboratories (6). 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

The concentration of total dissolved naphthenic acids was measured in test solution samples collected at 0 and 24 hours of the range-finding test and 0, 24, and 48 hours of the definitive test. Samples from freshly-prepared parent solutions were collected at 0 and 24 hours of the definitive

test, and composite samples comprised of equal volumes from replicate test chambers, were collected at 24 and 48 hours. The analyses were completed on August 19, 2009. Control and naphthenic acids -fortified samples were also prepared for analysis at each sample period.

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 FT-IR. QC fortifications were prepared in a similar manner after control medium had been fortified with the test substance.

2.5.3 Instrument 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:

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

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 5.0 mg/L nominal loading rate WAF sample at 0 hours of the range-finding test:

Standard Curve: y = 0.000168x +0.007968
Sample Peak Response: 0.0212
Concentration from standard curve: 78.6 mg/L

Volume for Analysis: 20 mL
Sample Volume: 500 mL

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

$$\frac{(78.6\text{ mg naphthenic acid})(20\text{ mL})}{500\text{ mL}} = 3.14\ \text{mg/L}$$

Recovery was calculated as a percentage of the corresponding nominal concentration, as shown for the 5.0 mg/L WAF sample at 0 hours:

$$\frac{3.14\text{ mg naphthenic acid/L}}{5.0\text{ mg naphthenic acid/L}} \times 100 = 63\%$$

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

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

Lowest standard concentration:	75.0 µg/mL
Analysis volume:	4 mL
Sample volume:	500 mL

therefore:

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

2.6 Statistical Analysis

All statistical analyses were performed with SAS software (version 9.1). Estimates of EL_{50} and EC_{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 loading rate (NOELR) and no-observed-effect concentration (NOEC) were determined based on the absence of any abnormal effects or immobility. All output from the statistical evaluations are presented in Appendix E.

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 with the FTIR analysis, 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 treatment solutions at test initiation of the first range-finding test (static) were 3.04, 7.38, and 15.0 mg naphthenic acids/L, which represented recoveries of 61 to 75% of the nominal loading rates. The measured concentrations in old test substance treatment solutions at 48 hours were 2.57, 5.34, 14.5 mg naphthenic acids/L, which represented recoveries of 51 to 73% of the nominal loading

rates and 72 to 97% of the measured concentrations at initiation. Recoveries from QC fortifications ranged from 90 to 95% of the nominal concentrations (Table 3).

Measured concentrations of naphthenic acids in the test solutions at test initiation of the second range-finding test (static-renewal) were 3.14, 6.24, 14.5, and 19.7 mg naphthenic acids/L, which represented recoveries of 49 to 73% of the nominal treatment concentrations. The measured concentrations in the 24-hour old test solutions were 3.29, 6.53, 14.5, and 31.7 mg naphthenic acids/L, which represented recoveries of 65 to 79% of the nominal treatment concentrations and 100 to 161% of the 0-hour concentrations. The mean measured concentrations in the test solutions were 3.22, 6.39, 14.5, and 25.7 mg naphthenic acids/L, which represented recoveries of 64 to 73% of the nominal treatment concentrations. Recoveries from quality control fortifications ranged from 82 to 93% of the nominal concentrations (Table 4).

3.1.2 FTIR Analyses - Definitive Test

Analytical confirmation of naphthenic acids in test solutions was performed at 0, 24, and 48 hours during the definitive test. Measured concentrations of naphthenic acids in the test solutions at test initiation were 4.23, 7.81, 16.7, 34.7, and 69.6 mg naphthenic acids/L, which represented recoveries of 78 to 87% of the nominal loading rates. The measured concentrations in the 24-hour old test solutions were 3.70, 7.51, 15.4, 31.8, and 68.4 mg naphthenic acids/L, which represented recoveries of 74 to 86% of the nominal treatment concentrations. The measured concentrations in the 24-hour new test solutions were 3.81, 7.51, and 17.5 mg naphthenic acids/L, which represented recoveries of 75 to 88% of the nominal loading rates. The measured concentrations in the 48-hour test solutions were 3.85, 7.90, and 18.4 mg naphthenic acids/L, which represented recoveries of 77 to 92% of the nominal loading rates. Analytical confirmation was not performed in the 24-hour new and 48-old test solutions for levels 4 and 5 (40 and 80 mg naphthenic acids/L nominal), due to 100% immobility in those treatments. When the concentrations of dissolved naphthenic acids in the old solutions were viewed on the basis of percent of initial measured values, the dissolved naphthenic acids appeared stable over each renewal period. For the 0-24 hour renewal, the percentage of total dissolved naphthenic acids in the old solutions ranged from 87% to 98% of the initial measured concentrations, while those for the 24-48 hour renewal period ranged from 101% to 105% of the initial measured concentrations (Table 5). The mean measured concentrations in the test solutions during the 48-hour study were 3.90, 7.68, 17.0, 33.3, and 69.0 mg naphthenic acids/L, which represented recoveries of 77 to 86% of the nominal loading rates. Recoveries from quality control fortifications ranged from 103 to 121% of the nominal concentrations (Table 5). The biological response results are based upon nominal loading rates and the mean measured concentrations.

3.1.3 GC/MS Analyses

Results of the analysis of Z-number and C-number families indicated a predominance of naphthenic acids containing 10 to 16 carbon atoms. Approximately 85-91% 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 64-80% of the dissolved fraction. Typically, the third highest group of naphthenic acids were the acyclic carboxylic acids. The detailed report of these analyses is presented in Appendix D.

3.2 Biological Results

After 48 hours of exposure, immobility was 5, 0, 0, 25, 100, and 100% in the 0 (control), 5.0, 10, 20, 40, and 80 mg naphthenic acids/L treatments (Table 6) (Figure 1). The 24-hour $EL_{50,}$ based on nominal loading rates, was estimated to be 28.3 mg naphthenic acids/L (95% confidence limits could not be calculated). The 24-hour $EC_{50,}$ based on mean measured concentrations, was estimated to be 23.8 mg naphthenic acids/L (95% confidence limits could not be calculated). The 48-hour EL_{50}, based on nominal loading rates, was estimated to be 24.0 mg naphthenic acids/L with 95% confidence limits of 21.0 and 27.4 mg naphthenic acids/L. The 48-hour EC_{50}, based on mean measured concentrations, was estimated to be 20.0 mg naphthenic acids/L with 95% confidence limits of 17.4 and 23.0 mg naphthenic acids/L. The slope of the 48-hour concentration-response line could not be calculated because of the lack of more than one partial response. Daphnids floating on the surface were observed in the 20 mg naphthenic acids/L treatment at 24 and 48 hours. No other sublethal effects were observed (Table 6). The 48-hour NOELR and NOEC values, based on nominal loading rates and mean measured concentrations, were 10 and 7.68 mg naphthenic acids/L, respectively, based upon an absence of any abnormal effects or immobility at these and all lower test substance concentrations.

3.3 Environmental Conditions

Test solution temperatures during the 48-hour exposure ranged from 20.6 to 22.0°C as measured in the individual test replicates (Table 7). The continuous temperature recording confirmed the test solution temperature was within the 20 ± 2°C during the exposure. Test solution pH ranged from 7.5 to 8.6 throughout the test (Table 8). Dissolved oxygen concentrations ranged from 6.8 to 8.8 mg/L (81 to 104% saturation) throughout the test (Table 9). The total alkalinity and total hardness of the dilution water at test initiation were 148 and 150 mg $CaCO_3$/L, respectively. The conductivity of the dilution water was 368 $\mu S \cdot cm^{-1}$. Total organic carbon (TOC) of the dilution water was less than 2.0 mg/L as measured in the monthly facility records.

The control and test solutions were clear and colorless with no visible signs of undissolved test substance, precipitate, or surface film throughout the study.

4.0 CONCLUSIONS

The test acceptability criteria were met for this study. Immobilization in the control treatment was 5%, below the acceptability limit of 10%. Since the study was not conducted as a limit test, there were at least one test concentration exhibiting less than 50% immobility and one concentration exhibiting greater than 50% immobility. This study is classified as acceptable and satisfies the guideline requirement for an acute toxicity test with *Daphnia magna*.

The 24-hour $EL_{50,}$ based on nominal loading rates, was estimated to be 28.3 mg naphthenic acids/L (95% confidence limits could not be calculated). The 24-hour $EC_{50,}$ based on mean measured concentrations, was estimated to be 23.8 mg naphthenic acids/L (95% confidence limits could not be calculated). The 48-hour EL_{50}, based on nominal loading rates, was estimated to be 24.0 mg naphthenic acids/L with 95% confidence limits of 21.0 and 27.4 mg naphthenic acids/L. The 48-hour EC_{50}, based on mean measured concentrations, was estimated to be 20.0 mg naphthenic acids/L with 95% confidence limits of 17.4 and

23.0 mg naphthenic acids/L. The slope of the 48-hour concentration-response line could not be calculated. Daphnids floating on the surface were observed in the 20 mg naphthenic acids/L treatment at 24 and 48 hours. No other sublethal effects were observed. The 48-hour NOELR and NOEC values, based on nominal loading rates and mean measured concentrations, were 10 and 7.68 mg naphthenic acids/L, respectively, based upon an absence of any abnormal effects or immobility at these and all lower test substance concentrations.

PROTOCOL DEVIATIONS

Protocol Section: 10.1 – Range-Finding Test

The initial range-finding test was performed as a static exposure instead of the static-renewal test design specified in the Protocol.

Reason: Technical oversight.

Effect on Study Integrity: None. A second range-finding exposure was performed using a static-renewal test design. The methods and concentrations for the definitive test were established based on the second range-finding test.

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Table 1. Biological Responses During the Static Range-Finding Test with *Daphnia magna* Exposed to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	24 Hours		48 Hours	
	Biological Observations	Cumulative % Immobile	Biological Observations	Cumulative % Immobile
0 (control)	10 N	0	10 N	0
5.0	10 N	0	10 N	0
10	7 N, 3 T	0	2 N, 3 F, 5 T	0
20	2 F, 8 T	0	2 F, 5 T, 3 I	30

Key to Abbreviations: N = normal; F = Floating; T = Trailing; I = Immobile

Table 2. Biological Responses During the Second Static-Renewal Range-Finding Test with *Daphnia magna* exposed to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	24 Hours		48 Hours	
	Biological Observations	Cumulative % Immobile	Biological Observations	Cumulative % Immobile
0 (control)	10 N	0	10 N	0
5.0	10 N	0	10 N	0
10	10 N	0	10 N	0
20	10 N	0	5 N, 5 F	0
40	5 N, 1 T, 4 I	40	1 T, 2 F, 3 I	70

Key to Abbreviations: N = normal; F = Floating; T = Trailing; I = Immobile

Table 3. Measured Concentrations of Naphthenic Acids During the Static Range-Finding Test with *Daphnia magna*

Nominal WAF Loading Rate (mg naphthenic acids/L)	Measured Concentration of Naphthenic Acids in mg/L (Percent Nominal)			
	0-Hour Fresh Solutions	48-Hours Old Solutions	48-Hours Percent of 0-Hour	Mean
Control (0)	< MQL[a]	< MQL[a]	NA	< MQL[a]
5	3.04 (61)	2.57 (51)	85%	2.81 (56)
10	7.38 (74)	5.34 (53)	72%	6.36 (64)
20	15.0 (75)	14.5 (73)	97%	14.8 (74)
QC Fortification Spikes (% Recovery)				
Low Spike (1.0)	0.952 (95)	0.902 (90)	---	---
High Spike (30)	27.9 (93)	27.7 (92)	---	---

[a] Minimum Quantifiable Limit (MQL) = 0.600 mg/L.

Table 4. Biological Responses During the Static-Renewal Range-Finding Test with *Daphnia magna* exposed to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	24 Hours		48 Hours	
	Biological Observations	Cumulative % Immobile	Biological Observations	Cumulative % Immobile
0 (control)	10 N	0	10 N	0
5.0	10 N	0	10 N	0
10	10 N	0	10 N	0
20	10 N	0	5 N, 5 F	0
40	5 N, 1 T, 4 I	40	1 T, 2 F, 3 I	70

Key to Abbreviations: N = normal; F = Floating; T = Trailing; I = Immobile

Table 5. Measured Concentrations of Naphthenic Acids During the Static-Renewal Range-Finding Test with *Daphnia magna*

Nominal WAF Loading Rate (mg naphthenic acids/L)	Measured Concentration of Naphthenic Acids in mg/L (Percent Nominal)			
	0-Hour Fresh Solutions	24-Hours Old Solutions	24-Hours Percent of 0-Hour	Mean
Control (0)	< MQL[a]	< MQL[a]	NA	< MQL[a]
5	3.14 (63)	3.29 (66)	105%	3.22 (64)
10	6.24 (62)	6.53 (65)	105%	6.39 (64)
20	14.5 (73)	14.5 (73)	100%	14.5 (73)
40	19.7 (49)[b]	31.7 (79)[c]	161%	25.7 (64)
QC Fortification Spikes (% Recovery)				
Low Spike (1.0)	0.881 (88)	0.824 (82)	---	---
High Spike (50)	46.5 (93)	42.3 (85)	---	---

[a] Minimum Quantifiable Limit (MQL) = 0.600 mg/L.
[b] Average of two injections.
[c] Average of three injections.

Table 6. Measured Concentrations of Naphthenic Acids During the Static-Renewal Acute Toxicity Test with *Daphnia magna*

Nominal WAF Loading Rate (mg naphthenic acids/L)	Measured Concentration of Naphthenic Acids in mg/L (Percent Nominal/Initial Measured [a])				
	0 Hour	24-Hour (old)	24-Hour (new)	48-Hour (old)	Mean
Control	< MQL[b]	< MQL[b]	< MQL[b]	< MQL[b]	NA
5.0	4.23 (85/--)	3.70 (74/87)	3.81 (76/--)	3.85 (77/101)	3.90 (78)
10	7.81 (78/--)	7.51 (75/96)	7.51 (75/--)	7.90 (79/105)	7.68 (77)
20	16.7 (84/--)	15.4 (77/92)	17.5 (88/--)	18.4 (92/105)	17.0 (85)
40	34.7 (87/--)	31.8 (80/92)	---	---	33.3 (83)
80	69.6 (87/--)	68.4 (86/98)	---	---	69.0 (86)
QC Fortification Spikes (% Recovery)					
Low Spike (3.00)	3.63 (121)	3.14 (105)	3.13 (104)	3.36 (112)	
High Spike (90.0)	102 (113)	92.7 (103)	96.0 (107)	98.3 (109)	

[a] Initial measured value in corresponding freshly prepared solutions at 0 and 24 hours.
[b] Minimum Quantifiable Limit (MQL) = 0.600 mg/L.

"---" indicates no analytical confirmation performed due to 100% immobility

Table 7. Immobility of *Daphnia magna* Exposed to Naphthenic Acids for 48 Hours Under Static-Renewal Test Conditions

Nominal WAF Loading Rate (mg naphthenic acids/L)	REP	Cumulative Percent Immobile (% Sublethal Observations)		
		24 Hours	48 Hours	Treatment Mean (48 Hours)
Control (0)	A	0 (0)	20 (0)	5
	B	0 (0)	0 (0)	
	C	0 (0)	0 (0)	
	D	0 (0)	0 (0)	
5.0	A	0 (0)	0 (0)	0
	B	0 (0)	0 (0)	
	C	0 (0)	0 (0)	
	D	0 (0)	0 (0)	
10	A	0 (0)	0 (0)	0
	B	0 (0)	0 (0)	
	C	0 (0)	0 (0)	
	D	0 (0)	0 (0)	
20	A	0 (100)[a]	20 (80)[b]	25
	B	0 (100)[a]	20 (80)[b]	
	C	0 (100)[a]	20 (80)[b]	
	D	0 (100)[a]	40 (40)[c]	
40	A	100 (--)	100 (--)	100
	B	100 (--)	100 (--)	
	C	100 (--)	100 (--)	
	D	100 (--)	100 (--)	
80	A	100 (--)	100 (--)	100
	B	100 (--)	100 (--)	
	C	100 (--)	100 (--)	
	D	100 (--)	100 (--)	

[a] Five daphnids floating on the surface.
[b] Four daphnids floating on the surface.
[c] Two daphnids floating on the surface.

Note: Five daphnids were placed in each replicate at test initiation, totaling 20 daphnids per treatment.

Table 8. Test Solution Temperature Measurements During a 48-Hour Static-Renewal Exposure of *Daphnia magna* to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	REP	Temperature (ºC)			
		0 Hour	24 Hour (old)	24 Hour (new)	48 Hour (old)
Control (0)	A	21.3	20.7	21.8	20.9
	B	21.4	20.7	21.8	20.8
	C	21.3	20.6	21.8	20.8
	D	21.3	20.6	21.7	20.8
5.0	A	21.7	20.6	21.8	20.7
	B	21.7	20.6	21.7	20.7
	C	21.6	20.6	21.6	20.7
	D	21.5	20.6	21.6	20.8
10	A	21.5	20.6	21.9	20.8
	B	21.7	20.6	21.8	20.8
	C	21.4	20.6	21.6	20.8
	D	21.5	20.6	21.5	20.7
20	A	21.5	20.6	22.0	20.8
	B	21.7	20.6	21.8	20.8
	C	21.6	20.6	21.7	20.8
	D	21.7	20.7	21.6	20.8
40	A	21.8	20.7	---	---
	B	21.7	20.6	---	---
	C	21.9	20.7	---	---
	D	21.9	20.6	---	---
80	A	21.8	20.6	---	---
	B	21.9	20.6	---	---
	C	21.8	20.6	---	---
	D	21.9	20.7	---	---

"---" indicates no measurement taken due to 100% immobility

Table 9. Test Solution pH Measurements During a 48-Hour Static-Renewal Exposure of *Daphnia magna* to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	REP	pH			
		0 Hour	24 Hour (old)	24 Hour (new)	48 Hour (old)
Control (0)	A	8.3	8.4	8.3	8.3
	B	8.3	8.3	8.3	8.4
	C	8.3	8.3	8.4	8.3
	D	8.3	8.3	8.4	8.4
5.0	A	8.3	8.3	8.3	8.4
	B	8.3	8.3	8.3	8.3
	C	8.3	8.3	8.3	8.3
	D	8.3	8.3	8.3	8.4
10	A	8.6	8.2	8.2	8.3
	B	8.4	8.2	8.1	8.3
	C	8.2	8.2	8.1	8.3
	D	8.2	8.2	8.2	8.3
20	A	8.1	8.2	7.8	8.1
	B	8.1	8.2	7.8	8.0
	C	8.0	8.1	7.8	8.1
	D	8.0	8.2	7.8	8.0
40	A	7.9	8.1	---	---
	B	7.8	8.0	---	---
	C	7.7	7.9	---	---
	D	7.8	8.0	---	---
80	A	7.6	7.9	---	---
	B	7.6	7.8	---	---
	C	7.5	7.8	---	---
	D	7.5	7.8	---	---

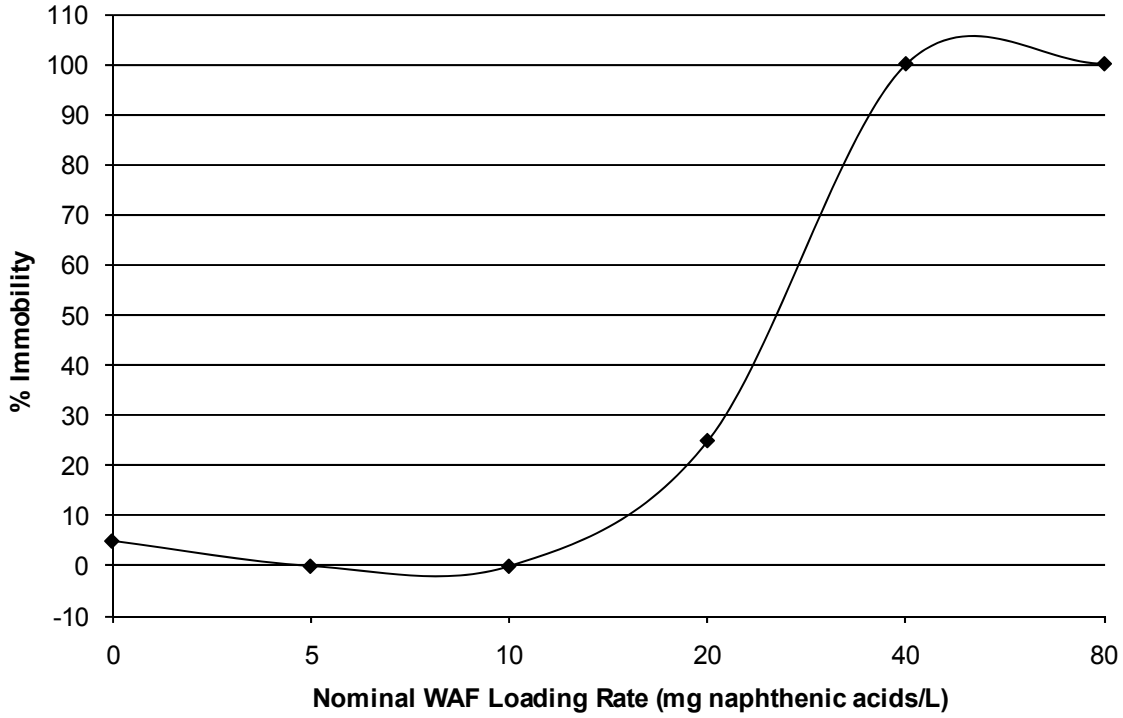
"---" indicates no measurement taken due to 100% immobility

Table 10. Test Solution Dissolved Oxygen Concentration Measurements During a 48-Hour Static-Renewal Exposure of *Daphnia magna* to Naphthenic Acids

Nominal WAF Loading Rate (mg naphthenic acids/L)	REP	Dissolved Oxygen (mg/L)			
		0 Hour	24 Hour (old)	24 Hour (new)	48 Hour (old)
Control (0)	A	8.1	8.8	7.8	8.1
	B	8.2	8.7	7.9	8.0
	C	8.2	8.8	7.9	8.0
	D	8.2	8.8	8.0	8.1
5.0	A	8.2	8.5	7.8	8.2
	B	8.1	8.7	7.8	8.0
	C	8.1	8.7	7.7	8.0
	D	8.0	8.8	7.8	8.1
10	A	8.0	8.3	7.8	8.0
	B	7.6	8.6	7.6	8.0
	C	7.7	8.5	7.5	8.0
	D	7.6	8.4	7.4	8.1
20	A	7.6	8.4	7.3	7.3
	B	7.7	8.3	7.1	7.3
	C	7.7	8.4	7.0	7.3
	D	7.6	8.4	7.0	7.3
40	A	7.4	8.2	---	---
	B	6.9	8.1	---	---
	C	6.8	8.1	---	---
	D	7.0	8.3	---	---
80	A	6.8	8.3	---	---
	B	6.9	8.1	---	---
	C	6.9	8.0	---	---
	D	6.8	8.8	---	---

Note: 100% saturation at 21 and 22°C is 8.5 and 8.4 mg/L, respectively.
"---" indicates no measurement taken due to 100% immobility

Figure 1. Cumulative Immobility of *Daphnia magna* , Exposed to Water Accommodated Fractions of Naphthenic Acids for 48 Hours Under Static-Renewal Test Conditions



APPENDIX A - TEST SUBSTANCE PHYSICAL-CHEMICAL SPECIFICATIONS FROM SUPPLIER

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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:	
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

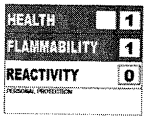
Warning symbol:

Warning words: Harmful. Irritant.

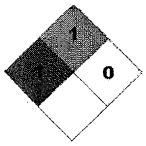
Risk phrases:
R36/38: Irritating to eyes and skin
R65: May cause lung damage if swallowed

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.

The information on this form is furnished solely for the purpose of enabling those who transport, handle or use our products to ensure the safety and health of their employees and to comply with various laws and regulations (federal, state and local). This information is offered in good faith and is believed to be accurate. however, makes no guarantee or warranty, expressed or implied, regarding the accuracy of these data or the results to be obtained from the use hereof.

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 - DILUTION 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	<0.0100
Cobalt	<0.0100	<0.0100[4]
Copper	<0.0100	<0.0100
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 AND AMENDMENTS

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions

ABC Study No. 64404

This protocol complies with U.S. EPA Ecological Effects Test Guideline OPPTS 850.1010, TSCA 797.1300, and OECD Guideline 202

This protocol is based upon ABC generic protocol G114.

1.0 STUDY TITLE

Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions

2.0 OBJECTIVE

The objective of this test is to determine the 48-hour EL_{50} and EC_{50}, if possible, of the test substance to water fleas under static-renewal test conditions. Immobilization and/or 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. In addition, the no-observed-effect loading rate (NOELR) and concentration (NOEC), at 48 hours will be reported, if possible.

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]

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) 777-6385 FAX: (573) 777-6089
Email: [redacted]

5.0 PROPOSED SCHEDULE

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

6.0 TEST PROTOCOL

The test protocol which follows is based on U.S. EPA OPPTS guideline 850.1010 (1) and OECD guideline 202 (2) and is intended to also comply with U.S. EPA TSCA guideline 797.1300 (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 Sponsor's responsibility.

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 to exceed 1,000 mg/L. The WAF preparation will be prepared in an appropriately sized vessel made of glass and will be stirred with magnetic stir bar. WAF vessels will be filled as to maintain minimum head space and covered with a rubber stopper or parafilm. 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. 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 the test substance in dilution water. The containers and preparations used in this study will be described by protocol amendment and summarized in the final report.

8.0 TEST SYSTEM

8.1 Species

The test species will be the freshwater cladoceran, *Daphnia magna*. The taxonomic key presented by Pennak (6) is used to identify to species the in-house culture daphnids.

8.2 Justification

Daphnia magna was chosen for this testing because it is representative of freshwater invertebrate species and is the species recommended under U.S. EPA and EU regulations.

8.3 Source

Adult *Daphnia magna* used to produce neonates for the test will be obtained from an established in-house culture.

8.4 Age

First instar *Daphnia magna* used to initiate the test will be neonates (<24 hours old) obtained by isolating gravid females from the culture water in dilution water <24 hours prior to beginning the test.

8.5 Culture

All test daphnids will be cultured in a controlled temperature area at 20 ± 2°C in dilution water. During the holding period, they will be fed algae (at least one species) at least every 3 days, which may or may not be supplemented with an additional nutrient food suspension. A test will not be started if (a) ephippia are being produced in the primary culture, (b) adults do not produce approximately three young per adult per day in the 7-day period before testing, (c) the brood stock do not produce young before 12 days old, or (d) more than 20% of the brood stock die during the 2 days preceding the test.

9.0 DILUTION WATER

The dilution water will be biologically aged laboratory freshwater with a total hardness of 130-160 mg/L as $CaCO_3$ and a pH of approximately 8.0. The base water, i.e., ABC well water, used to prepare the dilution water is chemically characterized as per ABC SOP to verify that it is free of contaminants that might interfere with test results.

10.0 TEST PROCEDURES

Generally two toxicity tests will be conducted, a range-finding and definitive test. The range-finding test is an abbreviated toxicity test employing widely spaced test concentrations to define the approximate range within which the test substance produces a gradient from nontoxic to toxic effects. The range-finding test is conducted using the same basic procedures and conditions as those used during the definitive test. Results of the range-finding test(s) guide selection of the test concentrations for the definitive test, the purpose of which is to provide a precise estimate of the 48-hour median effective loading rate (EL_{50}) of the test substance.

10.1 Range-Finding Test

The static-renewal range-finding test will be initiated by adding at least10 organisms to each test substance WAF concentration. WAF concentrations will typically cover several orders of magnitude (e.g., nominal loading rates of 1.0, 10, 100, and 1,000 mg/L). Unless otherwise specified, test condition parameters such as dissolved oxygen concentration, pH, and temperature will be typically measured only at test

initiation and termination. Additional or fewer water quality measurements may be made at the discretion of the Study Director. Survival of test organisms will be monitored daily during a 48-hour exposure. These results will be used to set the concentration range for the definitive test.

10.2 Definitive Test

10.2.1 Experimental Design

The definitive test will consist of one or more control treatments and a geometric series of at least five test substance WAF loading rates. The loading rate of each treatment, except the highest treatment and the control(s) will be at least 50 percent of the next higher one. Test substance loading rates will be selected with the desired goal of obtaining at least one loading rate that kills or affects more than 65 percent of the daphnids, and one treatment (not the control) that kills or affects less than 35 percent of the exposed water fleas. However, if no immobilization or adverse effects were noted in the range-finding tests, a limit test may be conducted with triplicate test chambers at a single loading rate (e.g., at the water solubility limits of the test substance). Triplicate dilution water control chambers will be also be utilized. Definitive test loading rates will be specified by protocol amendment. All test chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, level 1, level 2, etc.), and replicate (e.g., A, B, etc.).

The test chambers will be 250-mL glass beakers containing 200 mL of test water. Ten water fleas will be impartially assigned to each test chamber within 30 minutes after solution preparations, unless special, lengthy solution preparation (such as stirring overnight) is required. The method of preparation will be documented, if special preparation is needed. Treatments will be duplicated resulting in 20 water fleas per treatment. Impartial addition will be achieved through the addition of animals to clean vials such as scintillation vials. The same number of test organisms will be added to each vial as are to be placed in each test chamber. Each vial will be assigned to a specific test chamber via a randomization table. The animals will be transferred from the vial to the test chamber according to the randomization table. This will minimize bias due to test organism selection. Other sources of bias are not expected. The test will be conducted for 48 hours commencing when the animals are first exposed to the test substance. This route of administration will comply with U.S. EPA and OECD testing guidelines.

10.2.2 Daily Renewals

All control and test substance WAF solutions will be freshly prepared at 24 hours and all surviving daphnids will be transferred by pipet from old solutions to new solutions.

10.2.3 Feeding

Water fleas will not be fed during the test.

10.2.4 Temperature and Lighting

Temperature will be regulated to maintain 20 ± 2°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.

10.2.5 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 in at least one test chamber daily and continuously from a surrogate test chamber in the waterbath. Dissolved oxygen concentrations and pH will be measured at test initiation, at 24 hours, and termination in all replicate test solutions and the control(s). At 24 hours, measurements will be made of both old and new solutions. If 100-percent mortality occurs in a test chamber, water quality data will be recorded at that time and further measurements will be discontinued. TOC of the dilution water should be ≤2.0 mg/L. TOC may be measured concurrently or as part of the periodic facility measurements for this parameter. Additional water quality measurements may be made at the discretion of the Study Director.

10.2.6 Dissolved Oxygen Concentration

The dissolved oxygen concentration in the dilution water at test initiation and at renewal 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.

10.2.7 Biological Data

Observations of immobilization and/or mortality will be recorded and reported for all treatments. At a minimum, observations will be made at 24 and 48 hours (±1 hour from test initiation). Water fleas counted as dead will include those that are immobilized (i.e., those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel).

10.2.8 Analytical Confirmation

The concentrations of the total dissolved naphthenic acids in the WAFs will be measured in the range-finding and definitive tests. Test substance concentrations in the range-finding test will be measured in all control and test substance treatment levels at 0 and 48 hours. During the definitive test, test substance concentrations will be measured in all control and test substance treatments at 0, 24, and 48 hours. Unless otherwise specified, time 0-hour and 24-hour fresh solution samples will be collected from parent solutions. Time 24-hour spent solution and 48-hour samples will be collected after combining replicate solutions by treatment. If additional volume is necessary for chemical analysis, additional test chambers per concentration may be prepared. A minimum of two fortification spikes (quality control samples) will be prepared and analyzed with each sample set. Additional samples may be collected and analyzed at the discretion of the Study Director.

The analysis of the samples for the test substance will be based on an analytical method provided by the Sponsor and validated at ABC Laboratories. The analytical method will be described by protocol amendment to this protocol after validation.

11.0 ANALYSIS OF RESULTS

Results will be reported using nominal WAF loading rates and mean measured concentrations of total dissolved naphthenic acids. The results of the definitive study will be statistically analyzed for 24- and 48-hour EL_{50} and EC_{50} values and their corresponding 95% confidence limits, if possible. These values will be determined by a computer program using the probit model and/or Trimmed Spearman-Karber procedure or other appropriate statistical procedure. If possible, the slope of the 48-hour concentration-response line will be calculated for the 48-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 statistical test of goodness-of-fit will also be performed and reported. A 48-hour no-observed-effect loading rate (NOELR) and concentration (NOEC) will be determined, if possible, based on the absence of any abnormal effects or immobility.

If a limit test is performed, the response of organisms to the test substance treatment will be compared to that of the control organisms using a Fishers Exact test or similar procedure to determine statistical difference. If no immobility or other adverse effects are observed, statistical analysis is not necessary.

12.0 TEST ACCEPTABILITY CRITERIA

The test will not be valid if immobilization/mortality in any control treatment exceeds 10 percent during the 48-hour test. Unless the maximum loading rate of 1000 mg/L is tested, one test concentration should exhibit <50% immobility/mortality and one test concentration should exhibit ≥50% immobility/mortality.

13.0 REPORT

Upon completion of the range-finding test, a summary report will be submitted to the Sponsor. The summary report will briefly describe the test methods and test results. A Final report detailing all aspects of the study 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, 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 Toxic Substances Control Act (7) and with OECD Principles of Good Laboratory Practice (8). 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 SPECIMENT 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 REFERENCES

(1) U.S. Environmental Protection Agency. 1996. Ecological Effects Test Guidelines, OPPTS 850.1010, Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids, 8 pp.

(2) Organization for Economic Cooperation and Development (OECD). April 13, 2004. OECD Guidelines for Testing of Chemicals. *Daphnia* sp., Acute Immobilisation Test, OECD Guideline No. 202.

(3) U.S. Environmental Protection Agency. 1997. Code of Federal Regulations. Title 40 - Protection of Environment. Daphnid Acute Toxicity Test. 40 CFR 797.1300.

(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) Pennak, R.W. 1978. Freshwater Invertebrates of the United States, 2nd ed. 365 p.

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

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

PROTOCOL APPROVAL

Study Director

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

Name/Title: [redacted]

Sponsor Representative

Name (signed): [redacted] Date: 23 Feb 09

Name/Title: [redacted]

Test Facility Management

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

Name/Title: [redacted]

QAU Protocol Review for GLP Compliance

Name (signed): [redacted] Date: 25 Feb 09

Name/Title: [redacted]

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64404
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	1	**EFFECTIVE DATE:**	Sept 25, 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 maximum loading of the test substance in a WAF preparation will not to exceed 1,000 mg/L. The WAF preparation will be prepared in an appropriately sized vessel made of glass and will be stirred with magnetic stir bar. WAF vessels will be covered with a rubber stopper or parafilm. 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 range-finding test will be prepared at a volume of 2L in clean 2-L glass aspirator bottles, 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 range finding test will be collected by draining the solutions from the outlet of each aspirator bottle, after the first approximately 100 mL of prepared solution is discarded as waste.

   WAF preparations during the definitive test will be prepared at a volume of 4 L in clean 4-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: Describe the containers and preparations used in range finding and definitive tests.

   Effect on Study Integrity: None.

2. Protocol Section: 10.2.1 – Experimental Design

The definitive test will be performed at the target nominal loading rates of 0 (control), 5.0, 10, 20, 40, and 80 mg Naphthenic Acids/L. All test chambers will be labeled with the following information for identification purposes: ABC study number, treatment (e.g., control, level 1, level 2, etc.), and replicate (e.g., A, B, etc.).

The test chambers will be 250-mL glass jars or beakers containing 200 mL of test water. Five water fleas will be impartially assigned to each test chamber within 30 minutes after solution preparations, unless special, lengthy solution preparation (such as stirring overnight) is required. The method of preparation will be documented, if special preparation is needed. Treatments will be quadruplicated resulting in 20 water fleas per treatment. Impartial addition will be achieved through the addition of animals to clean vials such as scintillation vials. The same number of test organisms will be added to each vial as are to be placed in each test chamber. Each vial will be assigned to a specific test chamber via a randomization table. The animals will be transferred from the vial to the test chamber according to the randomization table. This will minimize bias due to test organism selection. Other sources of bias are not expected. The test will be conducted for 48 hours commencing when the animals are first exposed to the test substance. This route of administration will comply with U.S. EPA and OECD testing guidelines.

Reason: Identify the nominal concentrations selected, modify the test chamber description, and modify the number of replicates for the definitive test.

Effect on Study Integrity: None.

3. Protocol Section: 10.2.8 – Analytical Confirmation

The concentrations of the total dissolved naphthenic acids in the WAFs will be measured in the range-finding and definitive tests. Test substance concentrations in the range-finding test will be measured in all control and test substance treatment levels at 0 (parent solutions) and 24 (spent solutions) hours. During the definitive test, test substance concentrations will be measured in all control and test substance treatments at 0, 24, and 48 hours. Unless otherwise specified, time 0-hour and 24-hour fresh solution samples will be collected from parent solutions. Time 24-hour spent solution and 48-hour samples will be collected after combining replicate solutions by treatment. If additional volume is necessary for chemical analysis, additional test chambers per concentration may be prepared. A minimum of two fortification spikes (quality control samples) will be prepared and analyzed with each sample set. Additional samples may be collected and analyzed at the discretion of the Study Director.

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 vialed 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:** 25SEP09
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 28Sep09

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64404
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	2	**EFFECTIVE DATE:**	Jan 18, 2010

1. Protocol Section: 13.0 – Report

   A data summary of the range-finding test will be provided to the Sponsor and the results of the range-finding test will be summarized in the final report. A Final report detailing all aspects of the study 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, 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.

Reason: To clarify how the range-finding results will be reported.

Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** 19 JAN 2010
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 19 Jan 2010

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64404
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	3	**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: 10.2.8 – 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 were additional work contracted by the sponsor.

STUDY DIRECTOR:	[redacted]	**DATE:** 20 July 10
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 21 JUL 10

PROTOCOL DEVIATION NOTIFICATION

PROTOCOL TITLE:	Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions
TEST FACILITY:	ABC Laboratories, Inc. **ABC STUDY NO.:** 64404
STUDY SPONSOR:	American Petroleum Institute
DEVIATION NO.:	1 **NOTIFICATION DATE:** July 15, 2010

1. Protocol Section: 10.1 – Range-Finding Test

   The initial range-finding test was performed as a static exposure instead of the static-renewal test design specified in the Protocol.

   Reason: Technical oversight.

   Effect on Study Integrity: None. A second range-finding exposure was performed using a static-renewal test design. The methods and concentrations for the definitive test were established based on the second range-finder.

STUDY DIRECTOR:	[redacted]	**DATE:** 20 July 10
TEST FACILTY MANAGEMENT:	[redacted]	**DATE:** 21 Jul 10

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

1.0 Toxicity test with *Daphnia magna*

1.1 Samples and Methods

In October 2009, two shipments of samples containing the dissolved aqueous fraction (WAFs) of naphthenic acids were received in the Department of Biological Sciences at the University of Alberta, Alberta, Canada.

The first shipment contained six samples of aged-blended freshwater. These were from the *Daphnia magna* test. The concentrations of these were labeled as control (0 mg/L), 5, 10, 20, 40, and 80 mg/L. In this report, these samples are referred to as "*Daphnia* waters". The second shipment contained seven samples of freshwater algal nutrient medium.

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 DCM. The combined DCM extracts were dried under nitrogen to remove the solvent.

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

Two blanks were also prepared and analyzed. One blank consisted of 50 µL of DCM (devoid of naphthenic acids) to which 50 µL of Sigma MTBSTFA derivatizing agent (without 1% *t*-BDMCS) was added and this mixture was heated to 60° C for 20 min. The other blank contained 5 µg of 9-fluorenecarboxylic acid (the surrogate standard used by Young et al. 2008) dissolved in 50 µL of DCM (devoid of naphthenic acids) to which 50 µL of Sigma MTBSTFA derivatizing agent (without 1% *t*-BDMCS) was added and this mixture was heated to 60°C for 20 min. The derivatized samples were analyzed by GC-MS (Young et al. 2008) and the total ion current mass spectra were collected. The data obtained were put into a Microsoft Excel spreadsheet (Holowenko et al. 2002) to prepare tables of the 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. The distributions of ions summarized in each table was used to prepare a three-dimensional plots of the ion abundances for each n and Z value.

1.2 Results and Discussion

The tables of relative abundances of each ion (expressed as percentages) in the six extracted *Daphnia* 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.

Table 1.1

Daphnia water 0 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.9
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.1	1.7	0.0	0.0	0.0	0.0	0.0	2.8
8	0.2	0.7	0.0	0.0	0.0	0.0	0.0	0.9
9	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.6
10	1.2	0.5	0.8	0.0	0.0	0.0	0.0	2.6
11	1.7	0.5	1.6	0.0	0.0	0.0	0.0	3.8
12	9.6	0.5	1.6	0.1	0.0	0.0	0.0	11.8
13	2.3	0.4	0.7	0.4	0.0	0.0	0.0	3.7
14	6.1	0.5	3.7	0.5	0.0	0.0	0.0	10.8
15	1.7	0.9	0.2	0.0	0.0	0.0	0.0	2.8
16	22.6	0.9	0.5	0.1	0.0	0.0	0.0	24.0
17	0.9	0.1	0.0	0.0	0.0	0.0	0.0	1.0
18	20.2	2.9	0.3	0.9	0.0	0.2	0.0	24.5
19	0.4	0.0	0.0	0.0	0.0	0.2	1.3	1.9
20	1.8	0.0	0.0	0.0	0.1	0.9	0.1	2.9
21	0.2	0.0	0.0	0.0	0.0	0.8	0.2	1.1
22	0.0	0.1	0.0	0.0	0.1	1.3	0.0	1.5
23	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.9
24	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5
25	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5
26	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2
27	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2
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	71.3	10.0	9.4	2.0	0.2	5.5	1.6	100.0

Table 1.2

Daphnia water 5 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.4	0.0	0.0	0.0	0.0	0.0	0.0	1.4
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.2	1.2	0.0	0.0	0.0	0.0	0.0	2.4
8	0.6	0.5	0.0	0.0	0.0	0.0	0.0	1.1
9	0.7	0.5	0.0	0.0	0.0	0.0	0.0	1.2
10	1.2	1.0	1.0	0.0	0.0	0.0	0.0	3.2
11	1.4	4.4	4.3	0.0	0.0	0.0	0.0	10.1
12	4.2	7.3	8.7	0.8	0.0	0.0	0.0	21.0
13	2.0	7.2	8.8	1.5	0.0	0.0	0.0	19.6
14	3.0	4.5	6.1	1.5	0.3	0.0	0.0	15.4
15	1.1	2.0	2.7	0.9	0.2	0.0	0.0	7.0
16	6.0	0.9	1.2	0.5	0.1	0.0	0.0	8.8
17	0.4	0.2	0.4	0.2	0.0	0.1	0.0	1.2
18	3.8	0.9	0.2	0.2	0.0	0.1	0.1	5.2
19	0.2	0.0	0.0	0.0	0.0	0.1	0.3	0.5
20	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.3
21	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.2
22	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.4
23	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2
24	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
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	0.1	0.0	0.1
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	27.8	30.8	33.4	5.6	0.7	1.3	0.4	100.0

Table 1.3

Daphnia water 10 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.4	0.0	0.0	0.0	0.0	0.0	0.0	1.4
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.3	1.2	0.0	0.0	0.0	0.0	0.0	2.4
8	0.6	0.5	0.0	0.0	0.0	0.0	0.0	1.1
9	0.5	0.4	0.0	0.0	0.0	0.0	0.0	0.9
10	1.1	0.7	0.7	0.0	0.0	0.0	0.0	2.5
11	1.2	3.3	3.4	0.0	0.0	0.0	0.0	7.9
12	6.1	6.8	8.0	0.8	0.0	0.0	0.0	21.6
13	2.2	7.6	9.1	1.5	0.0	0.0	0.0	20.4
14	3.9	5.2	6.5	1.6	0.4	0.0	0.0	17.6
15	1.5	2.5	3.2	1.1	0.2	0.0	0.0	8.4
16	5.1	1.2	1.4	0.6	0.1	0.0	0.0	8.5
17	0.5	0.2	0.5	0.2	0.0	0.0	0.0	1.5
18	3.2	0.8	0.2	0.2	0.0	0.0	0.0	4.5
19	0.1	0.0	0.0	0.0	0.0	0.0	0.2	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	0.0	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	29.2	30.4	33.1	6.0	0.7	0.3	0.3	100.0

Table 1.4

Daphnia water 20 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.5	0.0	0.0	0.0	0.0	0.0	0.0	1.5
6	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.6
7	1.2	1.1	0.0	0.0	0.0	0.0	0.0	2.3
8	0.9	0.6	0.0	0.0	0.0	0.0	0.0	1.5
9	1.4	0.9	0.0	0.0	0.0	0.0	0.0	2.3
10	1.2	2.5	1.5	0.0	0.0	0.0	0.0	5.2
11	1.3	6.2	5.2	0.0	0.0	0.0	0.0	12.7
12	3.0	8.3	9.2	0.8	0.0	0.0	0.0	21.3
13	1.8	7.6	8.7	1.4	0.0	0.0	0.0	19.6
14	2.1	4.8	5.7	1.4	0.4	0.0	0.0	14.4
15	0.9	2.3	2.8	0.9	0.2	0.0	0.0	7.1
16	2.8	1.1	1.4	0.5	0.1	0.1	0.0	6.0
17	0.2	0.3	0.5	0.2	0.1	0.1	0.0	1.4
18	1.8	0.3	0.2	0.2	0.1	0.1	0.0	2.7
19	0.1	0.0	0.0	0.0	0.0	0.1	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	0.1	0.0	0.1
22	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2
23	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
24	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
25	0.0	0.0	0.0	0.0	0.0	0.0	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	0.0
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	20.9	36.1	35.3	5.7	0.9	0.8	0.2	100.0

Table 1.5

Daphnia water 40 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.5	0.0	0.0	0.0	0.0	0.0	0.0	1.5
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.2	1.0	0.0	0.0	0.0	0.0	0.0	2.2
8	0.8	0.5	0.0	0.0	0.0	0.0	0.0	1.3
9	0.5	0.7	0.0	0.0	0.0	0.0	0.0	1.2
10	0.7	2.1	1.4	0.0	0.0	0.0	0.0	4.2
11	1.0	6.1	5.5	0.0	0.0	0.0	0.0	12.6
12	2.5	9.2	10.3	0.9	0.0	0.0	0.0	22.9
13	1.8	8.9	10.2	1.6	0.0	0.0	0.0	22.5
14	1.8	5.6	6.6	1.6	0.4	0.0	0.0	16.0
15	0.7	2.5	3.1	1.1	0.3	0.0	0.0	7.6
16	1.2	0.9	1.4	0.6	0.2	0.1	0.0	4.4
17	0.1	0.2	0.4	0.2	0.1	0.1	0.0	1.2
18	0.8	0.3	0.1	0.1	0.0	0.0	0.0	1.5
19	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
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	0.1	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	15.0	38.1	39.2	6.2	1.0	0.4	0.1	100.0

Table 1.6

Daphnia water 80 mg/L

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.4	0.0	0.0	0.0	0.0	0.0	0.0	1.4
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.1	1.0	0.0	0.0	0.0	0.0	0.0	2.1
8	0.8	0.6	0.0	0.0	0.0	0.0	0.0	1.4
9	0.4	0.8	0.0	0.0	0.0	0.0	0.0	1.3
10	0.7	2.7	1.4	0.0	0.0	0.0	0.0	4.8
11	1.3	7.5	5.8	0.0	0.0	0.0	0.0	14.7
12	2.3	10.6	10.5	0.9	0.0	0.0	0.0	24.3
13	2.2	9.6	9.9	1.4	0.0	0.0	0.0	23.2
14	1.7	5.6	5.9	1.3	0.4	0.0	0.0	14.9
15	0.6	2.2	2.6	0.8	0.2	0.0	0.0	6.6
16	0.5	0.8	1.1	0.5	0.1	0.1	0.0	3.1
17	0.1	0.2	0.4	0.2	0.1	0.1	0.0	1.0
18	0.3	0.1	0.1	0.1	0.0	0.0	0.0	0.7
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
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	14.0	41.8	37.7	5.2	0.9	0.3	0.1	100.0

The distribu tions of ion s in se lected *Daphnia* water samples were used to prepa re a three-dimensional plots of the ion abundances for each n and Z value. The three-dimensional plots for the *Daphnia* water samples that contained 80 mg WAF/L and 0 mg WAF/L (control) are given in Figure 1.1 and Figure 1.2, respectively. The ion distribution s in the extract of the 80 mg WAF/ L sample is consistent with the distributions found in the neat naphthenic acids.

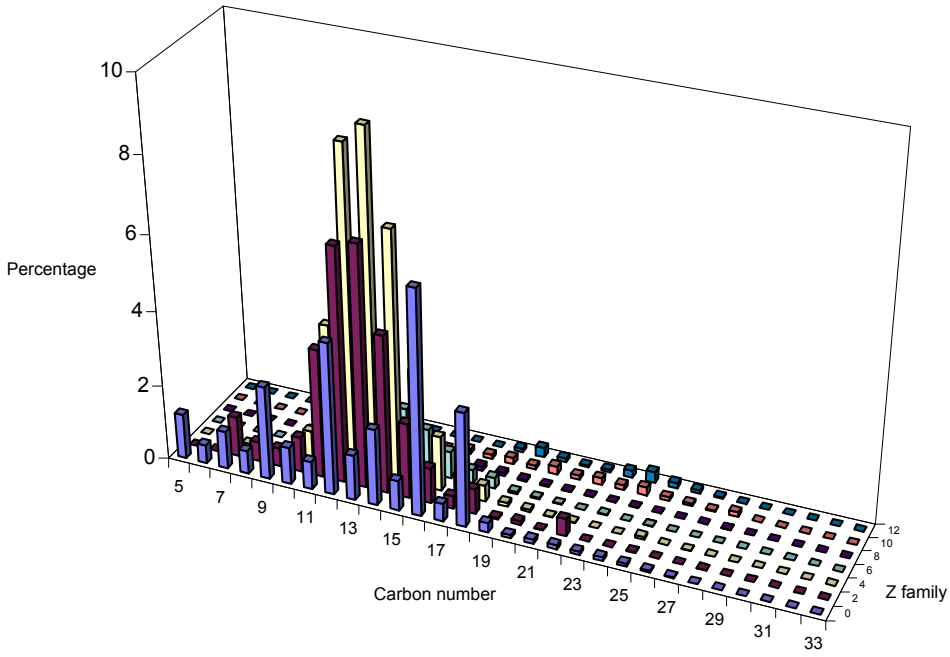


Figure. 1.1. Three-dimensional plot of naphthenic acids in the *Daphnia* water that contained 80 mg WAF/L. The sum of all bars equals 100%.

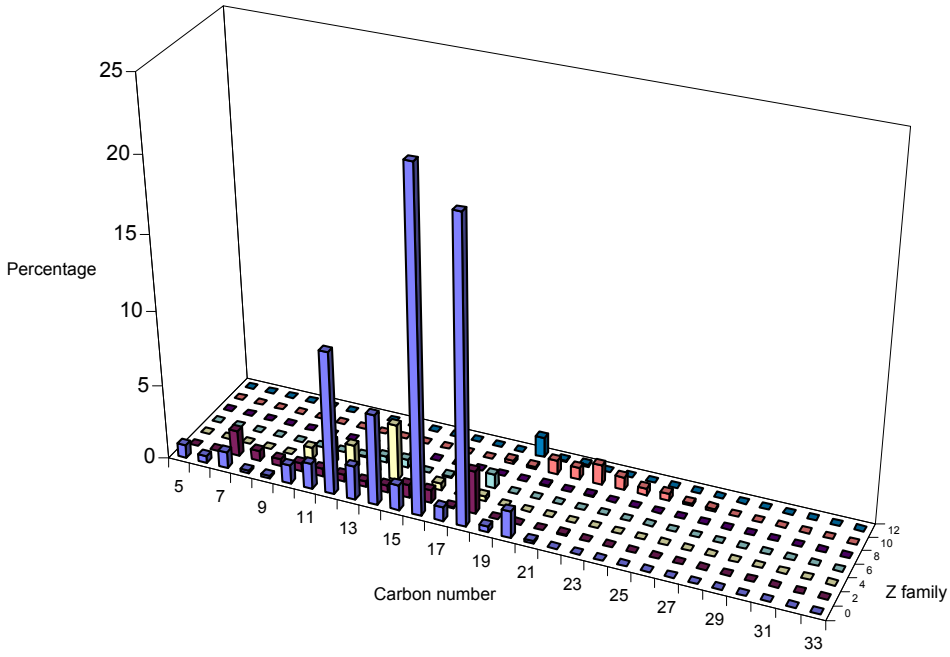


Figure. 1.2. Three-dimensional plot of naphthenic acids in the *Daphnia* water that contained 0 mg WAF/L (control). The sum of all bars equals 100%.

Using the statistical method of Clemente et al. (2003), the data from Figure 1.1 were compared to data from the analyses of samples of neat naphthenic acids previously supplied by API. There were no statistical differences between the naphthenic acids in these two samples.

The three-dimensional plot for the sample that contained 0 mg WAF/L (control) is given in Figure 1.2. This plot is very different from the plot of the naphthenic acids-containing sample (Figure 1.1). The most abundant ions in Figure 1.2 are those corresponding to n = 16, Z = 0 and n = 18, Z = 0. 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 1.2) is not unexpected.

To ensure that these C-16 and C-18 compounds were not artifacts of the GC-MS method, two blanks were prepared and analyzed by GC-MS. One blank consisted of 50 µL of DCM and the derivatizing reagent and the other blank contained DCM, 9-fluorenecarboxylic acid and derivatizing reagent. When analyzed by GC-MS, peaks corresponding to these C-16 and C-18 acids were absent from the chromatograms. Thus, these compounds were not artifacts of the GC-MS method.

The statistical method of Clemente et al. (2003), was also used to determine if there were any differences between the various samples of *Daphnia* water. This method allows two samples to be compared to one another. The 80 mg WAF/L samples were arbitrarily chosen for each set of comparisons. Thus, each *Daphnia* water sample was compared to the 80 mg WAF/L *Daphnia* water sample. The results of these comparisons are summarized in Table 1.7.

Table 1.7. Statistical comparisons of distributions of naphthenic acids in various *Daphnia* waters with the distributions of naphthenic acids in the *Daphnia* water that contained 80 mg WAF/L. "S" indicates a significant difference ($P < 0.05$); "NS" indicates no significant difference ($P > 0.05$)

Sample concentration (mg/L)	Group 1 (C_5 to C_{13})	Group 2 (C_{14} to C_{21})	Group 3 (C_{22} to C_{33})
0	S	NS	S
5	NS	NS	S
10	NS	NS	NS
20	NS	NS	S
40	NS	NS	NS

In this statistical method, the ion distributions from the GC-MS analysis are divided into three groups based on carbon numbers. These are: Group 1 (C_5 to C_{13}), Group 2 (C_{14} to C_{21}), and Group 3 (C_{22} to C_{33}), as shown in Table 1.7. 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-test. 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 80 mg WAF/L *Daphnia* water sample.

Figures. 1.1 and 1.2 show a marked difference between the distribution of ions in the 80 mg WAF/L *Daphnia* water sample and the 0 mg WAF/L *Daphnia* water sample. The statistical comparison of these two samples (Table 1.7) shows that there is a difference between the Group 1 naphthenic acids, and the Group 3 naphthenic acids in these two samples. However, there was no difference detected in the Group 2 naphthenic acids. This result is the same as was observed for the fish water samples and a detailed discussion of the statistical method is given in Section 3.1.2.

The most common differences among samples shown in Table 3.2.7 are in the Group 3 (C_{22} to C_{33}) naphthenic acids. However, commercial naphthenic acids preparations typically lack Group 3 acids (Clemente et al. 2003). The samples of *Daphnia* water were essentially devoid of Group 3 naphthenic acids, and their relative abundances are reported as "0.0" (rounding to the nearest 0.1) in Tables 3.2.2 to 3.2.6. However, the Excel spreadsheet employed for the statistical analyses, uses the actual relative abundance values which are sometimes greater than 0.0, but less than 0.06. Using these very small values, significant differences ($P < 0.05$) are sometimes calculated, but these differences are not meaningful or important.

2.0 References

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APPENDIX E - STATISTICS

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 13OCT09
STUDY NUMBER: 64405 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

PRINTOUT OF RAW DATA
BY EXPOSURE PERIOD

---------- EXP_PERD=24 HOURS ----------

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
CON	0	20	0
L1	5	20	0
L2	10	20	0
L3	20	20	0
L4	40	20	20
L5	80	20	20

---------- EXP_PERD=48 HOURS ----------

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
CON	0	20	1
L1	5	20	0
L2	10	20	0
L3	20	20	5
L4	40	20	20
L5	80	20	20

① Corrected study number. 21July10 MRE

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 13OCT09
STUDY NUMBER: 64405 64 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 24 HOURS
BASED ON NUMBER IMMOBILIZED OF NUMBER EXPOSED

NUMBER OF PROPORTIONS BETWEEN 0 AND 1 = 0

ESTIMATES BASED ON THE PROBIT MODEL CAN NOT BE CALCULATED.

(1) Corrected Study Number, 21JUL10 MRR

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 13OCT09
STUDY NUMBER: 64405 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 24 HOURS
BASED ON NUMBER IMMOBILIZED 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	20	0	0.0000	0.0000
5.0000	20	0	0.0000	0.0000
10.0000	20	0	0.0000	0.0000
20.0000	20	0	0.0000	0.0000
40.0000	20	20	1.0000	1.0000
80.0000	20	20	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

EC50: 28.284 mg/L

95% CONFIDENCE LIMITS CANNOT BE CALCULATED.

THE BEST ESTIMATE FOR THE LOWER LIMIT IS THE
HIGHEST CONCENTRATION WHOSE SMOOTHED
PROPORTION IS ZERO= 20 mg/L

THE BEST ESTIMATE FOR THE UPPER LIMIT IS THE
LOWEST CONCENTRATION WHOSE SMOOTHED
PROPORTION IS ONE= 40 mg/L

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

① CORRECTED STUDY NUMBER - 21OCT09 KMR

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 13OCT09
STUDY NUMBER: 64404 (1) WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 48 HOURS
BASED ON NUMBER IMMOBILIZED OF NUMBER EXPOSED

NUMBER OF PROPORTIONS BETWEEN 0 AND 1 = 1

ESTIMATES BASED ON THE PROBIT MODEL CAN NOT BE CALCULATED.

(1) corrected study number. 21JAN10 MRR

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 13OCT09
STUDY NUMBER: 64405 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 48 HOURS
BASED ON NUMBER IMMOBILIZED 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	20	1	0.0500	0.0000
5.0000	20	0	0.0000	0.0000
10.0000	20	0	0.0000	0.0000
20.0000	20	5	0.2500	0.2373
40.0000	20	20	1.0000	1.0000
80.0000	20	20	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

EC50: 23.995 mg/L
95% LOWER CONFIDENCE LIMIT: 21.030 mg/L
95% UPPER CONFIDENCE LIMIT: 27.377 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 13OCT09
STUDY NUMBER: 64405 64 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

THIS COMPLETE ANALYSIS WAS CONDUCTED

BY: MATTHEW REBSTOCK ON: 13OCT09 MRR 13 Oct 09

THE ANALYSIS WAS REVIEWED

BY: JKJ ON: 22 Oct 09

① corrected study number. 21 Oct 09 MRR

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

PRINTOUT OF RAW DATA
BY EXPOSURE PERIOD

---------- EXP_PERD=24 HOURS ----------

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
CON	0.00	20	0
L1	3.90	20	0
L2	7.68	20	0
L3	17.00	20	0
L4	33.30	20	20
L5	69.00	20	20

---------- EXP_PERD=48 HOURS ----------

TREATMENT GROUP	CONCENTRATION	NUMBER EXPOSED	NUMBER RESPONDING
CON	0.00	20	1
L1	3.90	20	0
L2	7.68	20	0
L3	17.00	20	5
L4	33.30	20	20
L5	69.00	20	20

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 24 HOURS
BASED ON NUMBER IMMOBILIZED OF NUMBER EXPOSED

NUMBER OF PROPORTIONS BETWEEN 0 AND 1 = 0

ESTIMATES BASED ON THE PROBIT MODEL CAN NOT BE CALCULATED.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 24 HOURS
BASED ON NUMBER IMMOBILIZED 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	20	0	0.0000	0.0000
3.9000	20	0	0.0000	0.0000
7.6800	20	0	0.0000	0.0000
17.0000	20	0	0.0000	0.0000
33.3000	20	20	1.0000	1.0000
69.0000	20	20	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

EC50: 23.793 mg/L

95% CONFIDENCE LIMITS CANNOT BE CALCULATED.

THE BEST ESTIMATE FOR THE LOWER LIMIT IS THE
HIGHEST CONCENTRATION WHOSE SMOOTHED
PROPORTION IS ZERO= 17 mg/L

THE BEST ESTIMATE FOR THE UPPER LIMIT IS THE
LOWEST CONCENTRATION WHOSE SMOOTHED
PROPORTION IS ONE= 33.3 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 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 48 HOURS
BASED ON NUMBER IMMOBILIZED OF NUMBER EXPOSED

NUMBER OF PROPORTIONS BETWEEN 0 AND 1 = 1

ESTIMATES BASED ON THE PROBIT MODEL CAN NOT BE CALCULATED.

ABC LABORATORIES, INC.
SAS PROGRAM LC_EC50 (VER 2.3) RUN ON 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

ANALYSIS FOR DAPHNIA MAGNA AT 48 HOURS
BASED ON NUMBER IMMOBILIZED 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	20	1	0.0500	0.0000
3.9000	20	0	0.0000	0.0000
7.6800	20	0	0.0000	0.0000
17.0000	20	5	0.2500	0.2373
33.3000	20	20	1.0000	1.0000
69.0000	20	20	1.0000	1.0000

UNTRIMMED SPEARMAN-KARBER ESTIMATES:

EC50: 19.992 mg/L
95% LOWER CONFIDENCE LIMIT: 17.388 mg/L
95% UPPER CONFIDENCE LIMIT: 22.986 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 22OCT09
STUDY NUMBER: 64404 WITH TEST MATERIAL: NAPHTHENIC ACIDS
DAPHNIA ACUTE DEFINITIVE USING DATA FILE: U:\REBSTOCKM\SAS\64404EC50.PRN

THIS COMPLETE ANALYSIS WAS CONDUCTED

BY: MATTHEW REBSTOCK ON: 22OCT09 MRR 22Oct09

THE ANALYSIS WAS REVIEWED

BY: _____ ON: 22 Oct 09