

ExxonMobil BIOMEDICAL SCIENCES, INC.

EMBSI 2009-104112

***Daphnia magna*, Reproduction Test on Water Accommodated Fractions
of Kerosene**

Final Report

Study Number: 0950146

TEST SUBSTANCE:

**Kerosene
CAS No. 64742-81-0
(MRD-09-501)**

PERFORMED FOR:

**American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070**

PERFORMED AT:

**ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059**

COMPLETION DATE: June 1, 2012

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APPROVAL SIGNATURES



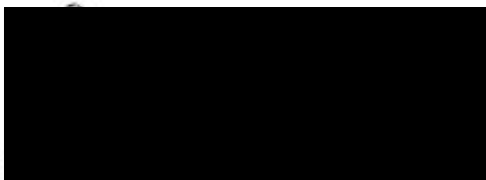
Study Director
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Annandale, NJ 08801-3059

01 JUN 12
Date



Environmental Toxicology & Fate
Laboratory Coordinator

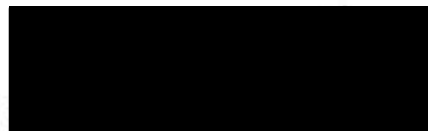
1 Jun 12
Date



Section Head, Environmental Sciences

6-01-2012
Date

The final report was accepted by the Sponsor



Sponsor Representative
American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

30 May 2012
Date

GLP COMPLIANCE STATEMENT

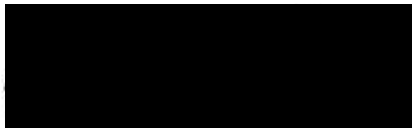
I hereby accept responsibility for the validity of these data and declare that to the best of my knowledge the study contained herein was performed under my supervision in compliance with the OECD Principles of Good Laboratory Practice, C(97) 186/Final, 1997 and the United States Environmental Protection Agency (USEPA) Toxic Substances Control Act, Good Laboratory Practice Standards, 40 CFR Part 792, 1989 with the exceptions listed below.

Contaminant analysis of the water was not performed in a GLP compliant manner. Accutest® laboratory is accredited by the National Environmental Laboratory Accreditation Conference (NELAC). The analyses are performed using standard US EPA methods. Accutest® has been audited by ExxonMobil Biomedical Sciences, Inc. using the ExxonMobil Quality Practices and Guidelines (QP & G v. 5.3).

The sponsor-supplied test substance analyses conducted by Intertek were not performed in a GLP compliant manner. These analyses were not conducted as part of the testing facility's protocol for this study.

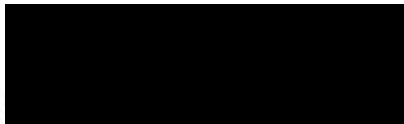
As per protocol, the equilibration and stability trials as well as the range-finding test were not subject to GLP standards.

None of the above exceptions are believed to have an adverse effect on the study results.



Study Director
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Annandale, New Jersey 08801-3059

01 JUN 12
Date



Sponsor Representative
American Petroleum Institute
1220 L Street, NW
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30 May 2012
Date

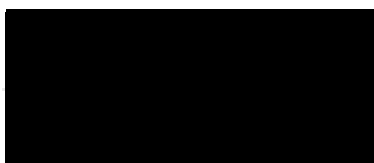
QUALITY ASSURANCE STATEMENT

STUDY NUMBER: 0950146
TEST SUBSTANCE: MRD-09-501
STUDY SPONSOR: American Petroleum Institute

Listed below are the inspections performed by the Quality Assurance Unit of ExxonMobil Biomedical Sciences, Inc., the date(s) of inspection, and the date(s) findings were reported to the Study Director, Principal Investigator and/or Management.

Study Phase Inspected	Date(s) of Inspection	Reported to Study Director and/or Principal Investigator	Reported to Management
Protocol	01 June 2009	01 June 2009	08 July 2009, 15 July 2009
Temperature & Light Measurements	25 September 2009	25 September 2009	29 May 2012 30 May 2012
Test/Control Article Measurements	18 January 2010	19 January 2010	25 January 2010
First review of Final Report & Raw Data (not including analytical chemistry)	09-12 March 2010	15 March 2010	10 February 2011 18 February 2011
First review of Analytical and Characterization section of Final Report & Raw Data	15 March 10	15 March 2010	10 January 2011 14 February 2011
Second review of Analytical and Characterization sections of the Final Report & Raw Data	07 January 2011	07 January 2011	10 February 2011 18 February 2011
Second review of Final Report & Raw Data (not including analytical chemistry)	06, 19 & 28 January 2011 08 February 2011	08 February 2011	10 May 2012

The final report accurately reflects the methods, procedures and observations documented in the raw data.



Quality Assurance Unit Coordinator

30 May 2012
Date

PERSONNEL

Study Director:

Sponsor Representative:

Section Head, Environmental Sciences:
(until July 1, 2011)

Section Head, Environmental Sciences:
(effective July 1, 2011)

Environmental Toxicology & Fate
Laboratory Coordinator:
(until December 31, 2010)

Environmental Toxicology & Fate
Laboratory Coordinator:
(effective January 1, 2011)

Environmental Chemistry Laboratory Coordinator;
Principal Investigator for Characterization &
Analysis of Test Solutions:

Quality Assurance Unit Coordinator:

All personnel involved in the conduct of this study, except the sponsor, are/were located at the testing facility's address. The Sponsor Representative is located at the previously cited address.

SUMMARY

This study was conducted for the Sponsor to assess the effects of the water accommodated fractions (WAFs) of kerosene (CAS No. 64742-81-0) on the reproductive output of *Daphnia magna*. This study was performed as a 21-day semi-static renewal test.

Individual treatments were prepared by adding the appropriate amount of test substance to dilution water in glass aspirator bottles and stirring on magnetic stir plates with a vortex of approximately 9% of the static liquid depth for approximately 24 hours. Approximately one hour after stirring termination, the aqueous portion of each WAF solution was removed for testing. The control and treatment WAFs were prepared every other day at loading rates of 0 (control), 0.08, 0.19, 0.48, 1.2 and 3.0 mg/L.

Ten replicate test chambers were prepared for each test substance loading rate and control. Each replicate test chamber contained one daphnid. Replicate chambers were 130-mL glass bottles containing approximately 130 mL of solution (no headspace) sealed with PTFE-lined screw top caps. Water quality (temperature, pH, dissolved oxygen, and hardness) measurements were measured once or twice a week in each new and old solution for each treatment and the control. Water quality parameters were within acceptable limits throughout the testing period. Adult daphnids were observed daily for immobilization, reproduction, and abnormal behavior/appearance. Any offspring were counted and observed for immobilization at each renewal period and the end of the test.

Concentrations of the test substance hydrocarbon components were quantified against kerosene standards, prepared in acetone, spiked directly into water for automated static headspace gas chromatography with flame ionization detection (HS GC-FID) analysis. The total peak area for eluted hydrocarbon components from WAF headspace analysis was summed for quantification. The distribution and percentage of kerosene components measured in the WAFs differed from the parent kerosene standards owing to the differing solubilities of individual kerosene hydrocarbons. Therefore, measured concentrations do not represent all hydrocarbons constituting the test substance. Due to the complex nature of the test substance, no attempt was made to identify and quantify specific hydrocarbons solubilized in the WAFs. The average concentrations from the measured hydrocarbon analysis during the exposure were ND (Not Detected; control), 0.016, 0.039, 0.092, 0.23, and 0.54 mg/L. All old test solutions ranged from 65 to 120% of the initial measured hydrocarbon concentrations.

Chronic toxicity results are expressed as the Effect Loading 20 and 50 (EL20 and EL50), which are the loading rates of test substance in dilution water calculated to result in a 20% and a 50% reduction in reproductive output, survival, or growth relative to the control group for the test. The No Observed Effect Loading Rate (NOELR) was the highest loading rate that did not exhibit a statistical difference in reproductive output, survival, or growth from the control group. The Lowest Observed Effect Loading Rate (LOELR) was the lowest loading rate that resulted in a statistical difference in reproductive output, survival, or growth from the control group. The Maximum Acceptable Toxicant Loading Rate (MATLR) is the geometric mean of the NOELR and LOELR values. Results expressed as EC, NOEC, LOEC, and MATC values represent the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate. These endpoints are presented below.

SUMMARY (CONT'D)

21-day Endpoints

<u>Response Variable</u>	<u>Loading Rate* (mg/L)</u>	<u>Average Concentration** (mg/L)</u>
Survival	EL20 = 0.41 (could not calculate) EL50 = 0.81 (could not calculate) NOELR = 0.48 LOELR = 1.2 MATLR = 0.76	EC20 = 0.080 (could not calculate) EC50 = 0.15 (could not calculate) NOEC = 0.092 LOEC = 0.23 MATC = 0.15
Reproductive Output ¹	NOELR = 0.48 LOELR ² = 1.2 MATLR = 0.76	NOEC = 0.092 LOEC ² = 0.23 MATC = 0.15
Growth ¹ (Length)	NOELR = 0.48 LOELR ² = 1.2 MATLR = 0.76	NOEC = 0.092 LOEC ² = 0.23 MATC = 0.15

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

** Average concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.

Values in parentheses () are 95% confidence intervals.

¹ Inhibition of reproduction and growth was insufficient to calculate EL20, EL50, EC20 and EC50 values.

² LOELR/LOEC values are based on interpretation of the concentration-response pattern since the levels above the NOELR/NOEC for survival were excluded from statistical analysis for reproduction and growth.

INTRODUCTION

Objective

This study was conducted for the Sponsor to assess the effects of the water-accommodated fractions (WAFs) of kerosene (CAS No. 64742-81-0) on the reproductive output of *Daphnia magna* in a 21-day semi-static (renewal) test.

Sponsor

American Petroleum Institute
1220 L Street, NW
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Testing Facility

ExxonMobil Biomedical Sciences, Inc.
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Initial Characterization

02 June 2009

Study Initiation

24 June 2009

WAF Equilibration and Stability Trial Start (Mixing)

29 June 2009

Experimental Start (Definitive Study)

06 January 2010

Experimental Termination (Definitive Study)

27 January 2010

Experimental Completion

29 July 2010

Compliance

The study was conducted in compliance with OECD¹ and USEPA² Good Laboratory Practice (GLP) standards with the exceptions outlined on page 5. The study was performed in agreement with the OECD³ guidelines with the exception(s) listed on page 21.

MATERIALS and METHODS

Test Substance Identification

EMBSI Identification:	MRD-09-501
Sponsor Identification:	Kerosene
CAS Number:	64742-81-0
Supplier:	EPL Archives, Sterling, Virginia
Date Received:	12 May 2009
Expiration Date:	May 2014

CAS Definition: Kerosene (petroleum), hydrodesulfurized; A complex combination of hydrocarbons obtained from a petroleum stock by treating with hydrogen to convert organic sulfur to hydrogen sulfide which is then removed. It consists of hydrocarbons having carbon numbers predominantly in the range of C9 through C16 and boiling in the range of approximately 150° C to 290° C (302° F to 554° F)⁴.

Additional test substance information supplied by the Sponsor is attached in Appendix I.

Storage Conditions: The neat test substance was stored at room temperature.

Sample Retention

A non-study specific sample of the neat test substance has been retained in the testing facility archives.

Justification of Dosing Route

Potential environmental exposure is by the test substance in water.

Dilution Water

Reconstituted water⁵ (Batch #208A) was prepared with UV-sterilized, deionized well water and reagent grade chemicals (NaHCO₃, CaSO₄, MgSO₄, and KCl), and was aerated prior to use. The reconstituted water contains Ca/Mg and Na/K ratios of 1.2:1 and 12.5:1, respectively. UV-sterilized, deionized well water is distributed throughout the testing facility via PVC and stainless steel pipes. See Appendix E for the dilution water analysis.

MATERIALS and METHODS (CONT'D)

Dilution Water (cont'd)

Contaminants

There were no known contaminants in the feed used for the study, in culturing the organisms or the dilution water believed to be at levels high enough to have interfered with this study. The YTC daphnid feed mixture is analyzed for total solids as well as pesticides and metals by the vendor (Aquatic Biosystems) prior to shipment. The algae is not analyzed. The algae suspension is prepared from the dilution water. The dilution water is prepared from UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless steel pipes. Contaminant analysis of the water is performed by Accutest Laboratories, Inc. The laboratory is accredited by the National Environmental Laboratory Accreditation Conference (NELAC) and has been audited by ExxonMobil Biomedical Sciences using the Quality Practices and Guidelines (QP & G v. 5.3). The analyses are performed using standard US EPA methods, but were not performed in a GLP compliant manner.

Characterization of the Test Substance

The neat test substance was characterized and the stability determined by the testing facility using the following analyses: Ultraviolet/Visible and Infrared Spectrophotometry, density, physical-state, miscibility in water, methanol and/or hexane and a gas chromatography/mass spectrometry (GC/MS) "fingerprint" of the neat test substance. The GC/MS fingerprint is run against an ASTM hydrocarbon standard mixture. The ASTM D3710 standard is applied for higher boiling mixtures with compounds eluting between approximately n-propyl benzene (n-C9) and n-pentadecane (n-C15). Due to the complex nature of the test substance, no reporting of specific hydrocarbon components was made. Instead, an area percent report was generated for both the pre- and post-test analysis to demonstrate stability of the test substance over the testing period. Documentation of characterization and stability assessment is maintained at the testing facility. The test substance was considered stable over the course of the testing period based on the set of analyses presented in Appendix H. The methods of synthesis, fabrication, and/or derivation of the test substance are maintained by the sponsor. The test substance, as received, was considered the "pure" substance for dosing purposes.

Analysis of Test Solutions

Duplicate samples were collected from each new treatment bulk WAF and single samples were collected from the control solution on Day 0, 6, 14 and 18. For the corresponding "old" i.e., used solutions, three individual replicate test chambers were sampled prior to performing the renewal on Day 2, 8, 16, and 20. A different set of replicate chambers were sampled at each interval (starting with replicates 1, 2, and 3 on Day 2). All samples were individually analyzed and not pooled. The samples were taken with no headspace in 40 mL VOA vials and refrigerated pending analysis.

MATERIALS and METHODS (CONT'D)

Analysis of Test Solutions (cont'd)

The method of analysis was automated static headspace gas chromatography with flame ionization detection (HS GC-FID). Analysis was performed on a Perkin Elmer Autosystem XL gas chromatograph. Each concentration measurement represents the concentration of hydrocarbons in mg/L that solubilized from the test substance into each WAF at its respective loading rate.

Concentrations of the test substance hydrocarbon components were quantified against kerosene standards, prepared in acetone, spiked directly into water for HS GC-FID analysis. The total peak area for eluted hydrocarbon components from WAF headspace analysis was summed for quantification. This ensured that the full range of constituent hydrocarbons that could potentially solubilize into the WAF solutions were captured and quantitated. The distribution and percentage of kerosene components measured in the WAFs differed from the parent kerosene standards owing to the differing solubility of individual kerosene hydrocarbons. Due to the complex nature of the test substance, no attempt was made to identify and quantify specific hydrocarbons solubilized in the WAFs. The analytical method is presented in Appendix A.

Test System

Daphnia magna Straus

Justification for Selection of Test System

Daphnia magna has been used in safety evaluations and is a common test species for freshwater toxicity studies.

Supplier

Daphnia magna were cultured at the test facility. Original culture supplied by Aquatic Biosystems, Inc., Fort Collins, Colorado. Starter culture received on 11-Apr-02.

The algae and YTC feed mixtures were supplied by Aquatic Biosystems, Inc., Fort Collins, CO.

Husbandry and Acclimation

Eight to ten daphnids were kept in 1-liter glass culture beakers with approximately 800 mL of reconstituted water (study dilution water). The culture chamber was maintained at $20 \pm 2^\circ\text{C}$ under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux). Day 0 cultures were started at least five days a week. The neonates were less than 24 hours old and came from day 12 to 21 old cultures which experienced less than or equal to 20% adult mortality.

MATERIALS and METHODS (CONT'D)

Test System (cont'd)

Husbandry and Acclimation (cont'd)

Cultures of *Daphnia magna* were fed *Pseudokirchneriella subcapitata* (approximately $3.0 - 4.5 \times 10^5$ cells/mL). They were also fed 1.5 mL of a YTC Daphnid feed mixture. The culture was fed every other day, or more frequently as needed, based on observed algal clearing. Cultures were transferred every other day, with exceptions on holidays or weekends when staff was not present. The brood stock health was evaluated and any mortality, production of males or ephippia was documented as well as any mitigation procedures.

Number and Sex

Number: 60; Sex: female

Age at Initiation of Exposure

Organisms were <24 hours old, taken from 14-day old parents.

Test System Identification

Each replicate, containing one daphnid, was labeled to show study number, loading level, replicate and randomization number.

Feed

Daphnids were fed during renewals by adding between 0.325 mL and 0.447 mL of a 1.3×10^8 cells/mL suspension of *Pseudokirchneriella subcapitata* to provide approximately $3.25 \times 10^5 - 4.47 \times 10^5$ cells/mL. Test organisms were also fed during renewals with between 0.163 mL and 0.244 mL of a YTC Daphnid feed mixture. Feed levels were increased throughout the duration of the study to compensate for daphnid growth and the presence of neonates.

EXPERIMENTAL PROCEDURE

WAF Equilibration and Stability Trials

A WAF equilibration trial was completed prior to testing to determine the most appropriate mixing duration and to verify the analytical method for analyzing dissolved hydrocarbons. Stability of the WAF solutions also was evaluated over a period of 24 and 48 hours. Results of the equilibration trial indicated that a 24-hour mixing period was sufficient to achieve dissolution of the soluble components in the test substance in the WAF solutions. Following analytical sampling at 48 hours, the WAF solutions were determined to be relatively stable over a 48-hour period. Results of the equilibrium and stability trials are presented in Appendix B. These trials were not subject to GLP standards.

Range Finding Test

A non-GLP range-finding trial was performed to determine appropriate loading rates for the definitive trial. The results of the rangefinder are presented in Appendix C.

Definitive Test Design

GROUP	LOADING RATE* (mg/L)	NUMBER OF ORGANISMS
1	0 (Control)	10 (1 per replicate)
2	0.08	10
3	0.19	10
4	0.48	10
5	1.2	10
6	3.0	10

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

Preparation and Administration of Test Substance

Individual treatment WAFs were prepared by adding the appropriate amount of test substance to laboratory dilution water in glass aspirator bottles (capacity of 12.8 L for the 0.08 mg/L loading rate or 4.2 L for all other loading rates). The test substance was added to the aspirator bottles using stainless steel and glass syringes. The vessels were closed with foil-covered rubber stoppers. The control WAF was prepared with 4 L of dilution water in an equivalent sized glass aspirator bottle. The loading rate was determined from the volume of test material added and converted to mass per unit volume (mg/L) based on its density. The mixtures were stirred using a vortex of ~9% (of the static liquid depth) for 24 ± 1 hours with Teflon[®]-coated stir bars on magnetic stir plates. Room temperature during WAF mixing ranged from 23.5 to 25.9°C.

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance (cont'd)

At the end of mixing, the solutions were allowed to settle and cool to test temperature in a waterbath for 1 hour $\pm$ 15 minutes without stirring. The test solutions (aqueous portions of the WAFs) were then removed through the outlet at the bottom of the vessel and into ten replicates. New WAF solutions were prepared every other day during the test for test solution renewals. Renewals were performed by transferring each parent daphnid, via glass pipette, to freshly prepared solutions every 48 hours. At the end of the study, the final renewal was performed on Day 20 and the test terminated on Day 21.

Test Chamber / Organism Loading

The test chambers were 130-mL clear glass containers with screw type caps containing approximately 130 mL of solution (no headspace). The test chambers were sealed with PTFE-lined caps to minimize contamination, evaporation and/or volatilization.

Selection

Organisms were randomly assigned to test chambers using a computer generated randomization scheme using (SAS 9.1)⁶. The test chambers were randomly positioned within the testing location. Printouts of the randomization schedules are included in the raw data.

To ensure that quality organisms were used for the study, neonates were collected from parents that were 14 days old with $\leq 20\%$ adult mortality. Neonates were selected from a pool of organisms larger than that needed for the study. The pool of neonates had $\leq 10\%$ daily mortality on the day of test initiation. The study director determined organism suitability.

Exposure Duration

21 days

Environmental Conditions

An environmental condition study was activated on the laboratory computer system (Watchdog V5 monitoring system), at the start of the study to provide a record of the continuous measurements for temperature and light intensity.

The temperature in the environmental chamber ranged from 19.5 to 19.9°C, continuously monitored by Watchdog in the test area.

Diurnal light: approximately 16 hours light and 8 hours dark. Daylight intensity ranged from approximately 146 – 196 lux during full daylight periods.

EXPERIMENTAL PROCEDURE (CONT'D)

Experimental Evaluation

Observations for immobilization of adult daphnids were performed and recorded at approximately 24-hour intervals following test initiation. Immobilization is defined as the lack of swimming ability or movement within 15 seconds after gentle agitation of the test container. In addition, observations for normal or abnormal adult daphnid behavior or appearance were collected. Observations of test substance insolubility (surface slicks, precipitates and adherence to the test chamber) were noted daily.

The adults were transferred to fresh test solution every 48 hours. Following the appearance of the first brood, neonate presence was noted daily during observations and counted at the time of the renewal. Observations of aborted eggs, neonate immobilization and abnormal appearance were noted when observed. At test termination, all surviving adults were measured for body length (excluding anal spine) to determine growth effects. After completion of the study, the test organisms were discarded and monitoring of the environmental conditions was discontinued.

Water quality measurements (pH, dissolved oxygen, temperature and hardness) were performed at least twice per week during the test in each of the new and old solutions from each treatment and control.

Calculations

Chronic toxicity results are expressed as the Effect Loading 20 and 50 (EL20 and EL50), which are the loading rates of test substance in dilution water calculated to result in a 20% and a 50% reduction in reproductive output, survival or growth relative to the control group for the test. The No Observed Effect Loading Rate (NOELR) was the highest loading rate that did not exhibit a statistical difference in reproductive output, survival or growth from the control group. The Lowest Observed Effect Loading Rate (LOELR) was the lowest loading rate that resulted in a statistical difference in reproductive output, survival or growth from the control group. The Maximum Acceptable Toxicant Loading Rate (MATLR) is the geometric mean of the NOELR and LOELR values. In addition to statistical analyses, interpretation of the concentration-response pattern was used in selecting the NOELR/LOELR values.

Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as EC, NOEC, LOEC, and MATC values represent the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate. The distribution and percentage of kerosene components measured in the WAFs differs from the parent kerosene, owing to the differing solubilities of individual kerosene hydrocarbons.

EXPERIMENTAL PROCEDURE (CONT'D)

Calculations (cont'd)

The EL/EC values and confidence intervals were calculated by using a probit regression calculation based on the methods of Finney⁷, based on the PROC PROBIT procedure and standard data manipulation methods in SAS⁸.

The T-test with Bonferroni adjustment⁹, or Steel's Many-One Rank Test¹⁰ using TOXSTAT¹¹ software were used to determine the LOELR/LOEC and NOELR/NOEC values. Replicates with parent mortality were excluded from the analysis for reproduction and growth. The statistical output is provided in Appendix J.

RESULTS AND DISCUSSION

Three trials of the study were performed. The first two trials did not meet guideline acceptability criteria and were terminated prior to Day 21. A summary of these trials is presented in Appendix D. The third study trial met the acceptability criteria for mortality (not to exceed 20%) and mean number of live offspring produced (≥ 60) in the control group at the end of test. The coefficient of variation around the mean number of living offspring produced per adult in the control was below 25%. The following results are based on the third trial.

The WAF loading rates for this study were 0 (control), 0.08, 0.19, 0.48, 1.2, and 3.0 mg/L. The corresponding average concentrations from the measured hydrocarbon analysis during the exposure were ND (Not Detected; control), 0.016, 0.039, 0.092, 0.23, and 0.54 mg/L. Each concentration measurement represents the concentration of hydrocarbons in mg/L that solubilized from the test substance into each WAF at its respective loading rate. All old test solutions ranged from 65 to 120% of the initial measured hydrocarbon concentrations. The analytical results are presented in Table 1.

At WAF stirring initiation and termination, all treatments appeared clear to slight yellow with test substance visible on the surface of clear test media. Water quality measurements remained consistent throughout the exposure (Table 2). pH measurements were within the 6 to 9 range and did not vary by more than 1.5 units throughout the study. Dissolved oxygen concentrations remained above 3 mg/L throughout the duration of the study. The test water temperatures ranged from 19.8 to 21.8 °C. A complete listing of water quality measurements are provided in Appendix F.

No observation of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) was noted during the time of organism observations. Percent adult immobilization in the control and treatment groups were 10, 20, 0, 0, 90 and 100% in the 0.08, 0.19, 0.48, 1.2 and 3.0 mg/L loading rates, respectively. In the Control group, on Day 18, daphnid #8 was noted as not appearing healthy. In the 0.08 mg/L group on Day 18, daphnid #1 was noted as not appearing healthy. Prior to complete immobilization in the 1.2 mg/L treatment group, observations of abnormality, including small size, off-color and lethargy were noted for some of the daphnids. Neonate immobilization was observed periodically in the control and 0.08, 0.19 and 0.48 treatment groups ranging from Days 8 through 12.

No aborted eggs were observed in any treatment throughout the entire exposure. At test termination, all surviving adults were measured for body length (excluding anal spine) to determine growth effects. Mean survival, neonate production and length data are provided in Table 3. Individual adult daphnid observations, neonate production, survival and length data are provided in Appendix G. The mean cumulative neonate production per adult per loading rate is presented in Figure 1.

There were no statistically significant differences on neonate production and adult daphnid growth (length) below the LOELR for survival. Inhibition of growth (based on length) was insufficient to calculate EL20 or EL50 values. Reliable ELx/ECx values for reproduction could not be calculated within the boundaries of the dataset.

RESULTS AND DISCUSSION (CONT'D)

The NOELR, LOELR, and MATLR values for this study were 0.48, 1.2, and 0.76 mg/L, respectively, based on survival, reproduction and growth. Corresponding NOEC, LOEC, and MATC values were 0.092, 0.23, and 0.15 mg/L, respectively. The EL50 and EL20 values were 0.81 mg/L and 0.41, respectively, based on survival. Corresponding EC50 and EC20 values were 0.15 mg/L and 0.08 mg/L, respectively. A complete listing of the statistical evaluations for individual endpoints is presented below.

<u>21-day Endpoints</u>		
<u>Response Variable</u>	<u>Loading Rate* (mg/L)</u>	<u>Average Concentration** (mg/L)</u>
Survival	EL20 = 0.41 (could not calculate) EL50 = 0.81 (could not calculate) NOELR = 0.48 LOELR = 1.2 MATLR = 0.76	EC20 = 0.080 (could not calculate) EC50 = 0.15 (could not calculate) NOEC = 0.092 LOEC = 0.23 MATC = 0.15
Reproductive Output¹	NOELR = 0.48 LOELR ² = 1.2 MATLR = 0.76	NOEC = 0.092 LOEC ² = 0.23 MATC = 0.15
Growth¹ (Length)	NOELR = 0.48 LOELR ² = 1.2 MATLR = 0.76	NOEC = 0.092 LOEC ² = 0.23 MATC = 0.15

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

**Average concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate.

Values in parentheses () are 95% confidence intervals.

¹ Inhibition of reproduction and growth was insufficient to calculate EL20, EL50, EC20 and EC50 values.

² LOELR/LOEC values are based on interpretation of the concentration-response pattern since the levels above the NOELR/NOEC for survival were excluded from statistical analysis for reproduction and growth.

PROTOCOL DEVIATIONS

Throughout the WAF mixing period, average room temperature ranged from 24 to 25 °C. This exceeded the specified protocol range of $22 \pm 2^{\circ}\text{C}$. WAFs were cooled to test temperature during the settling period (prior to organism exposure).

On Day 0, water temperatures in the treatment group solutions were 21.3 to 21.8 °C. On Day 12, the temperature of the 0.08 mg/L WAF was 21.1 °C. The protocol specified a recommended range of 19 – 21 °C.

Hardness measurements were inadvertently not recorded on Day 0 for each treatment group and the control.

The feed ration in units of mg C/daphnid/day was not determined as specified in the guideline and the protocol. The feed ration used in this study was sufficient for achieving the required number of offspring to meet the test validity criteria.

The equipment used for water quality measurements was required to be reported as per the protocol. This information is available in the raw data but was not added to the report.

The deviations described above are believed to have no impact on the quality or integrity of the data produced through the course of this study.

GUIDELINE EXCEPTIONS

Due to the complex nature and relatively limited solubility of the test substance the following exceptions to the guideline apply for this study:

Consistent with the OECD document on aquatic toxicity testing of complex substances¹², it was deemed more appropriate to prepare individual WAF treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.

RECORDS

All appropriate materials, methods and experimental measurements required in the protocol were recorded and documented in the raw data. Any changes, additions or revisions to the protocol were approved by the Study Director and the Sponsor Representative. These changes were documented in writing, and included the date, the signatures of the Study Director and the Sponsor Representative and the justification for the change.

The protocol, final report, raw data, computer generated listings of raw data, supporting documentation and a non-study specific sample of the neat test substance will be maintained in the archives of the testing facility for 10 years, after which time the records will be offered to the sponsor prior to disposal.

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Table 1. Analytical Results

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration (mg/L)														Mean Measured Concentration (mg/L)	
	1 st renewal			4 th renewal				8 th renewal				10 th renewal				
	Day 0 (new ¹)	Day 2 (old ²)	Day 0-2 Reten. ³	Day 6 (new ¹)	Day 8 (old ²)	Rep	Day 6-8 Reten. ³	Day 14 (new ¹)	Day 16 (old ²)	Rep	Day 14-16 Reten. ³	Day 18 (new ¹)	Day 20 (old ²)	Rep		Day 18-20 Reten. ³
0 (Control)	ND	ND ND ND	--	ND	ND ND ND	4 5 6	--	ND	ND ND ND	7 8 9	--	ND	ND ND ND	10 1 2	--	NA
0.08	0.017 0.016	0.019 0.019 0.017	110%	0.020 0.017	0.015 ⁴ 0.018 0.016	7 5 6	89%	0.018 0.017	0.016 0.013 ⁴ 0.013 ⁴	1 7 9	80%	0.015 ⁴ 0.012 ⁴	0.014 ⁴ 0.018 0.016	2 3 8	120%	0.016
Mean**	0.017	0.018		0.018	0.016			0.018	0.014			0.014	0.016			
0.19	0.040 0.038	0.039 0.038 0.036	97%	0.052 0.048	0.038 0.038 0.031	4 5 6	72%	0.050 0.048	0.029 0.035 0.031	7 8 9	65%	0.040 0.041	0.028 0.035 0.034	1 2 10	80%	0.039
Mean**	0.039	0.038		0.050	0.036			0.049	0.032			0.040	0.032			
0.48	0.090 0.088	0.078 0.088 0.082	93%	0.116 0.113	0.083 0.080 0.090	5 6 7	74%	0.103 0.096	0.081 0.069 0.074	8 9 10	75%	0.112 0.104	0.085 0.075 0.081	1 3 4	74%	0.092
Mean**	0.089	0.083		0.114	0.084			0.100	0.075			0.108	0.080			
1.2	0.247 0.249	0.226 0.210 0.215	88%	0.270 0.257	0.219 ⁵	8	83%	0.247 0.236	0.199 ⁵	8	82%	0.259 0.238	0.186 ⁵	8	75%	0.23
Mean**	0.248	0.217		0.264	0.219			0.242	0.199			0.248	0.186			
3.0	0.550 0.535	0.547 0.542 0.507	98%	-- ⁶	--	--	--	--	--	--	--	--	--	--	--	0.54
Mean**	0.542	0.532														

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

**Means calculated in Excel using unrounded numbers, therefore some rounding differences may be noted.

¹ Analytical samples from the new treatment (duplicate) and control (single) solutions were analyzed.

² Analytical samples (triplicate) from the old treatment and control solutions were analyzed. A different set of replicate chambers were sampled at each renewal interval. Old solution samples were collected from replicates 1, 2, and 3 on Day2; Due to low internal standard recovery for Rep 4, Rep 7 was analyzed on Day 8.

³ Percent retention was determined by dividing the concentration of the old solution to the new solution concentration x 100.

⁴ Hydrocarbons detected but were below PQL (Practical Quantitation Limit). Values were included in mean concentration calculations.

⁵ Only one replicate was available to be sampled, since 90% of the adult daphnids were immobilized by Day 6.

⁶ All daphnids were immobilized by Day 4.

PQL = 0.016 mg/L (lowest analytical standard); NA = Not Applicable; ND = Non Detectable

Table 2. Summary of Water Quality Measurements

Loading Rate* (mg/L)	Dissolved Oxygen (mg/L)		pH		Hardness (mg/L as CaCO ₃)		Temperature (°C)	
	new	old	new	old	new	old	new	old
0 (Control)	7.04 - 7.96	6.08 - 8.40	8.17 - 8.44	8.01 - 8.54	160 - 180	164 - 192	19.8 - 21.0	20.0 - 20.9
0.08	7.29 - 8.17	5.77 - 8.10	8.49 - 8.63	8.12 - 8.68	168 - 184	160 - 172	20.4 - 21.8	20.2 - 21.0
0.19	6.84 - 9.11	5.61 - 7.73	8.53 - 8.68	8.11 - 8.49	164 - 192	164 - 188	20.1 - 21.4	20.4 - 20.9
0.48	6.89 - 7.83	5.73 - 8.33	8.50 - 8.67	8.12 - 8.58	164 - 180	160 - 180	20.1 - 21.3	20.4 - 20.9
1.2	7.05 - 7.93	7.33 - 9.97	8.51 - 8.75	8.28 - 8.81	164 - 180	164 - 180	20.1 - 21.4	20.3 - 20.9
3.0¹	8.03	7.93	8.54	8.64	-- ²	172	21.4	20.7

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ Measurements were collected from Day 0 through Day 2 (100 % mortality).

² Hardness was inadvertently not collected on Day 0.

Table 3. Summary of Observations

Loading Rate* (mg/L)	21-day Survival (%)	21-Day Reproduction Mean offspring/female	Mean Adult Length (mm)
Control	90	109 (10% ¹)	4.9
0.08	80	106	5.0
0.19	100	105	5.0
0.48	100	90	4.8
1.2	10	68 ²	4.3 ²
3.0	0	NA	NA

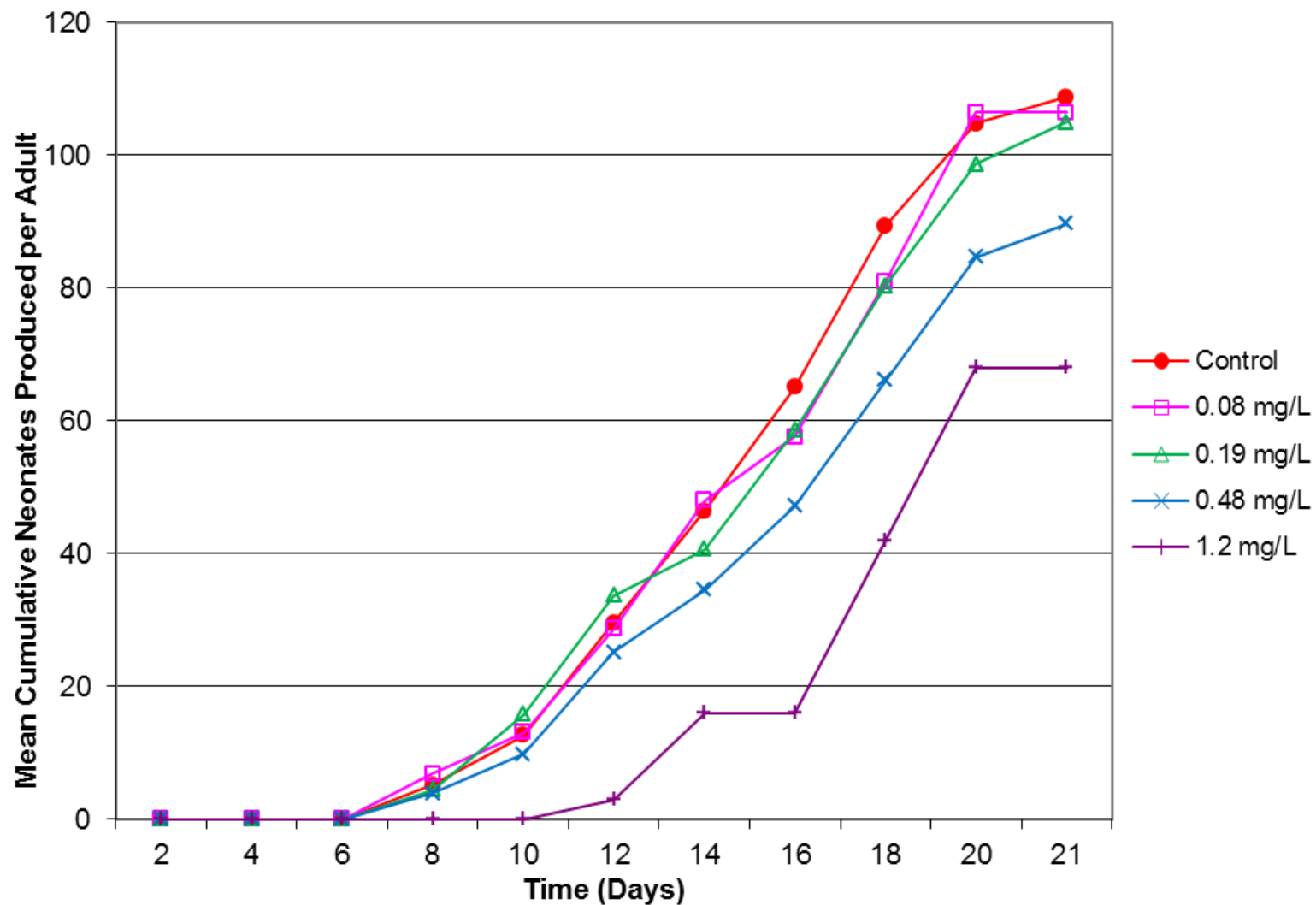
* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹Coefficient of variation should be $\leq 25\%$ for the control group.

²Only one adult daphnid survived until test termination.

NA = Not Applicable.

Figure 1. Mean cumulative neonate production per adult *Daphnid* per Loading Rate



Note: Data for the 1.2 mg/L loading rate are based on a single surviving organism.

APPENDIX A - ANALYTICAL METHOD

Standards and samples of kerosene (CAS No. 64742-81-0) were analyzed by static headspace-trap gas chromatography with flame ionization detection (HS GC-FID). Analysis was performed on a Perkin Elmer Autosystem XL gas chromatograph with a 30 m x 0.53 mm id, 1.5 µm film DB-5 (J&W Scientific) analytical column. The transfer line of a Perkin-Elmer HS-40 Headspace Sampler was connected directly to the analytical column. Samples and standards were equilibrated for 30 minutes at 95°C. The needle and transfer line temperatures were both 140°C, the pressurization time was 3 minutes, and the injection time was 0.3 minutes. The sampler head pressure was 25 psi. The FID was 275°C and the oven temperature was held at 40°C for 4 minutes and then ramped up to 160°C at 40°C/minute. The signal attenuation was -6.

Microliter aliquots of separate kerosene standard and o-xylene internal standard solutions diluted in acetone were spiked directly into the luer lock port of gas tight syringes containing 10 mL reconstituted water. The syringe contents were transferred to headspace (ca. 20 mL) sample vials containing five grams sodium sulfate. The vials were crimp sealed and shaken to solubilize the sodium sulfate prior to being placed on the headspace sampler for analysis. Kerosene standards in water were analyzed at concentrations of 16.3, 40.8, 98.0 and 245 ng/mL with a constant 17.6 ng/mL concentration of the o-xylene internal standard.

WAF samples were similarly prepared for analysis with 10 mL water sample aliquots transferred to gas tight syringes to which a microliter volume of the o-xylene internal standard solution in acetone was added. The syringe contents were transferred to headspace vials containing five grams sodium sulfate. As with the headspace kerosene standards, WAF sample vials were crimp sealed and shaken to solubilize the sodium sulfate prior to analysis. For higher concentration samples, aliquots of five milliliters or less were sampled in appropriate volume gas tight syringes, the internal standard added and the syringe contents transferred to headspace vials containing sodium sulfate and sufficient diluent water to yield a final volume of 10 mL.

Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Standards analysis resulted in a linear response over the standard concentration range. Figure A-1 represents the kerosene standard curve.

APPENDIX A - ANALYTICAL METHOD (CONT'D)

Kerosene (MRD-09-501) eluted as a complex mixture of hydrocarbons between the approximate retention times of 5.2 and 7.6 minutes. Representative kerosene HS GC-FID chromatograms are presented in Figure A-2. The two upper plots display a low and high concentration kerosene standard. The third plot is a control sample; the fourth and fifth chromatograms from the top represent analysis of the 0.48 and 1.2 mg/L loading rates. The total area integrated for the detected hydrocarbons was used for quantification. The o-xylene internal standard eluted at about 4.7 minutes under the analytical conditions utilized. The practical quantitation limit (PQL) was approximately 16 ng/mL (0.016 µg/mL) corresponding to the lowest analyzed standard. All reported concentrations for dissolved hydrocarbons present in kerosene are derived from the use of the standard curve and the internal standard.



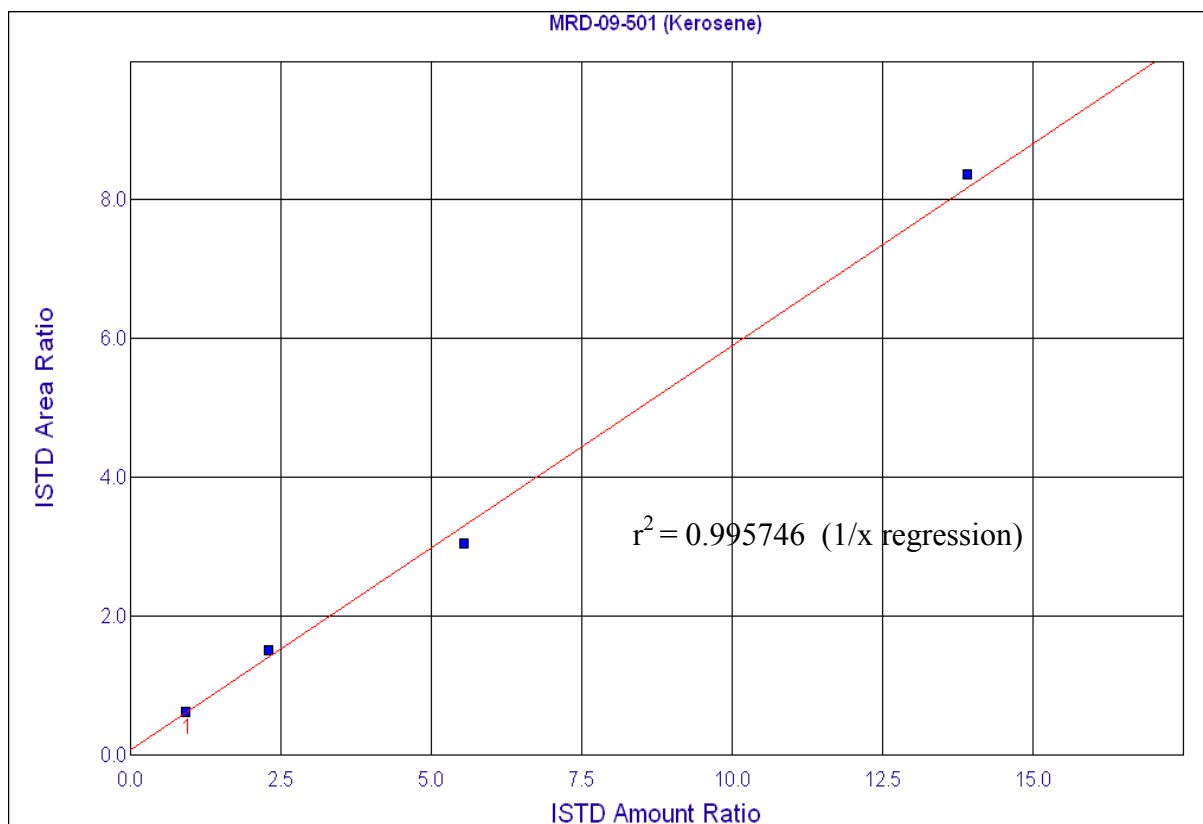
Laboratory Coordinator; Principal Investigator for
Characterization & Analysis of Test Solutions

30 May 2012
Date

APPENDIX A - ANALYTICAL METHOD (CONT'D)

FIGURE A-1

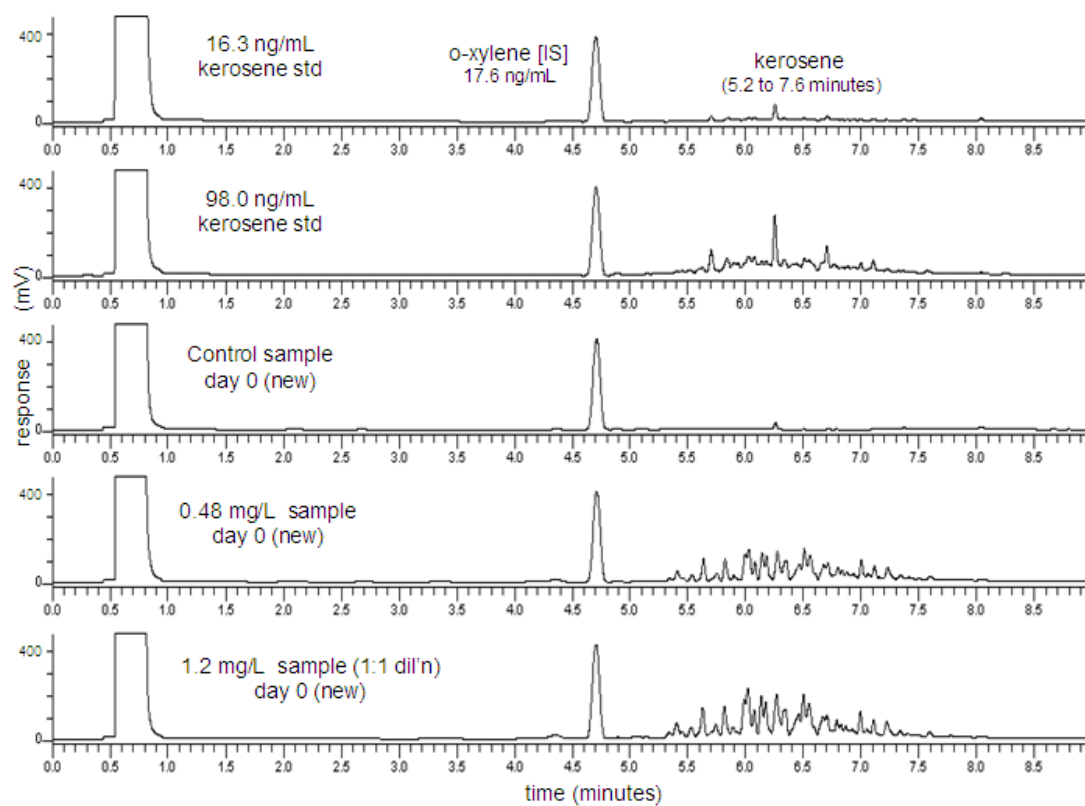
Kerosene Standard Curve



APPENDIX A - ANALYTICAL METHOD (CONT'D)

FIGURE A-2

Kerosene Standard and Sample Chromatograms



APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS

Introduction

A WAF equilibration trial was performed prior to the definitive testing. The purpose of the equilibration trial was to determine the optimum mixing duration to use in WAF preparation. The equilibration trial was also utilized to confirm the analytical method to be used in subsequent testing, and to evaluate the stability of the WAF solutions once they were produced. The stability information was used to establish the renewal interval for the definitive trial.

Mixtures of dilution water and test substance were prepared at loading levels of 0.1, 1.0, and 10 mg/L, in a manner similar to the definitive test. To evaluate equilibration time and WAF stability, WAF samples were collected as described below and analyzed according to the procedures explained in the Analytical Chemistry Methodology section, Appendix A. Sufficient volumes of each WAF were available to assess equilibration time, stability, and any effects of feed (algae) in the WAFs on the stability and chemical analyses.

WAF Equilibration Testing (Assessment of Mixing Duration)

One individual WAF was prepared at each of the three loading levels. At 24, 48 and 72 hours after initiation of mixing, mixing was stopped and the solutions were allowed to settle for one hour. A sample of WAF was removed from each loading level mixture and mixing was resumed at the 24 and 48-hour time points. The concentration of hydrocarbons that had solubilized into the WAF from the test substance was measured following the analytical procedures described in Appendix A. These measurements were used to assess the time required for solubilization of constituent hydrocarbons between the aqueous phase and the un-dissolved fraction of test substance to reach steady-state equilibrium. The equilibration results are shown in Table B1.

Measured concentrations of hydrocarbons in the equilibrated WAFs represent only a portion of the hydrocarbon composition of the test substance due to the very low to negligible aqueous solubility of many of the kerosene components. Evidence of this solubility effect is apparent when comparing measured concentrations of solubilized hydrocarbons to the concentration used to prepare each WAF (i.e., loading). At the lowest WAF loading of 0.1mg/L, measured solubilized hydrocarbon concentrations represent only 26% of the test substance loading rate. This percentage decreases to approximately 20% at the 1mg/L loading and decreases further to approximately 15% as the loading increases to 10 mg/L.

As shown in Figure B1, the analytical results of the WAF Equilibration Testing indicate that in nearly every case, maximum dissolution of kerosene was achieved after mixing for 24 hours. Further mixing time did not result in higher concentrations of solubilized hydrocarbons. It was determined that 24 hours would be a sufficient amount of time to mix for WAF generation. A 24-hour mixing duration is also a logistically convenient period for WAF generation when performing renewals.

APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

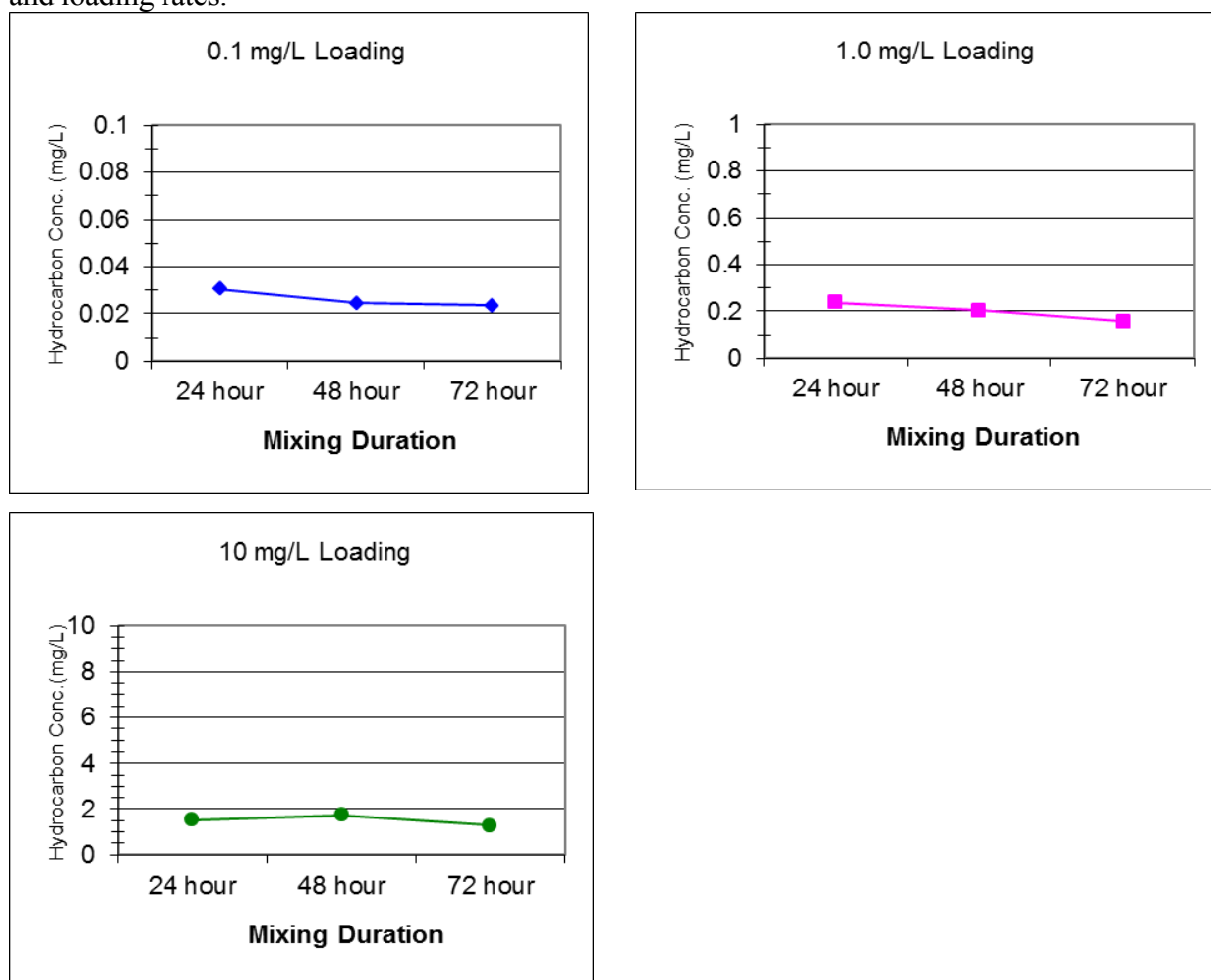
Table B1 - WAF Equilibration Results

Loading Rate*	Measured Hydrocarbon Concentration in WAF (mg/L)					
	24 hour mix	% solubility ¹	48 hour mix	% solubility	72 hour mix	% solubility
0.1 mg/L - 1	0.0306	31%	0.0232	23%	0.0215	22%
0.1 mg/L - 2	-		0.0258	26%	0.0254	25%
mean	0.0306	31%	0.0245	25%	0.0235	23%
1.0 mg/L - 1	0.237	24%	0.206	21%	0.161	16%
1.0 mg/L - 2	-		0.199	20%	0.152	15%
mean	0.237	24%	0.203	20%	0.157	16%
10 mg/L - 1	1.52	15%	1.73	17%	1.33	13%
10 mg/L - 2	-		1.74	17%	1.22	12%
mean	1.52	15%	1.74	17%	1.28	13%

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ Measured solubilized hydrocarbon concentration when compared to the loading rate.

Figure B1. Concentration plots of measured hydrocarbons in WAFs at different mixing times and loading rates.



APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

Assessment of WAF Stability

For the assessment of WAF stability, samples from the WAFs were collected after mixing for 24 and 48 hours. Two samples were collected at each loading level directly into screw-top sealed test chambers (130mL, no headspace) identical to those anticipated for use in the definitive study. To represent test conditions, test feed (0.163mL *Spirulina* and 0.325mL *P. subcapitata*) was added to one sample. No feed was added to the second sample.

All test chambers were set aside under environmental conditions similar to that used for testing. At 24 and 48 hours, test chambers were sampled. Dedicated samples were employed such that no repeated analysis was made on any sample (i.e., samples were destructively analyzed). The stability assessment results are shown below.

Table B2. WAF Stability Assessment Results following a 24 hour WAF mix

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration (mg/L)				
	Initial ¹	without feed		with feed	
		24 hour stability (retention ²)	48 hour stability (retention)	24 hour stability (retention)	48 hour stability (retention)
0.1	0.0306	0.0167	0.0213	0.0141 ³	0.0137 ³
		0.0193	0.0211	0.0155 ³	0.0160
	mean	0.0180 (59%)	0.0212 (69%)	0.0148 (48%)	0.0149 (49%)
1.0	0.237	0.171	0.176	0.149	0.153
		0.144	0.158	0.159	0.146
	mean	0.158 (67%)	0.167 (70%)	0.154 (65%)	0.149 (63%)
10	1.52	1.26	1.02	1.28	0.814
		1.14	1.02	1.23	0.826
	mean	1.20 (79%)	1.02 (67%)	1.26 (83%)	0.820 (54%)

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ 0-hour concentration for stability assessment following a WAF mixed for 24 hours.

² Percent retention was determined by dividing the concentration of the initial solution to the new solution concentration x 100.

³ Test substance detected but was below PQL (Practical Quantitation Limit). Values were included in mean concentration calculations.
PQL = 0.016 mg/L

APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

Table B3. WAF Stability Assessment Results following a 48 hour WAF mix

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration (mg/L)				
	Initial ¹	without feed		with feed	
		24 hour stability (retention ²)	48 hour stability (retention)	24 hour stability (retention)	48 hour stability (retention)
0.1	0.0245	0.0262	0.0106 ³	0.0135 ³	0.0082 ³
		0.0222	0.0113 ³	0.0147 ³	0.0185
	mean	0.0242 (99%)	0.0110 (45%)	0.0141 (58%)	0.0134 (55%)
1.0	0.203	0.173	0.173	0.159	0.135
		0.316 ⁴	0.164	0.140	0.120
	mean	0.173⁴ (85%)	0.169 (83%)	0.150 (74%)	0.128 (63%)
10	1.74	1.23	1.03	1.84	1.01
		1.07	0.907	1.53	0.975
	mean	1.15 (66%)	0.969 (56%)	1.69 (97%)	0.993 (57%)

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ 0-hour concentration for stability assessment following a WAF mixed for 48 hours.

² Percent retention was determined by dividing the concentration of the initial solution to the new solution concentration x 100.

³ Test substance detected but was below PQL (Practical Quantitation Limit). Values were included in mean concentration calculations.

⁴ Only Replicate 1 will be used in calculations as Replicate 2 is a considered an outlier.

PQL = 0.016 mg/L

Assessment of Acute Toxicity

A pre-rangefinder test performed to help establish concentrations to use in a chronic range-finding test. Samples of the 0.1, 1.0, and 10 mg/L WAF solutions created for the equilibration assessment were collected after 24, 48, and 72 hours of mixing, and used in an acute exposure test. The pre-rangefinding test was initiated with the placement five neonate daphnids in a test chamber containing approximately 130 mL of each WAF solution. Chambers were sealed with no headspace, and daphnids were assessed for immobility daily. Daphnids were not fed during the test. The results presented in Table B4 are from the daphnid exposure to the 24-hour mixing period WAFs. Additional observation data that have not been reported are maintained in the raw data.

Table B4. Pre-Rangefinder - Summary of In-life Observations

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration ¹ (mg/L)	% immobilization	
		24 hour	48 hour
0.10	0.0306	0	0
1.0	0.237	0	0
10	1.52	0	20

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ Measured hydrocarbon concentrations are from the 24-hr mixing period of the equilibration trial, for each respective treatment group.

APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS (CONT'D)

Summary

The WAF equilibration trial helped to establish the timing required for mixing during the definitive testing period. It was determined that 24 hours of mixing was sufficient to achieve equilibrium and generate a sufficient WAF. The stability trial showed that the test substance would be stable in the presence of feed. Stability of the dissolved hydrocarbon concentrations between 24 and 48-hours allowed for test solution renewals to be performed every other day. The acute toxicity assessment helped to establish the initial concentrations for the range-finding portion of the method development.

APPENDIX C – RANGE-FINDING TEST

An 11-day static range-finding test was performed for the Sponsor to determine the loading rates of kerosene (MRD-09-501) for the definitive *Daphnia magna* Reproduction Test.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.05, 0.2, 0.6, 1.8 and 5 and 15 mg/L. The measured hydrocarbon concentrations were determined to be 0.012, 0.057, 0.13, 0.35, 0.79 and 1.71 mg/L. Each concentration measurement represents the concentration of hydrocarbons in mg/L that solubilized from the test substance into each WAF at its respective loading rate. A control treatment consisting only of the dilution (hard reconstituted) water also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes; to the dilution water in glass aspirator bottles (mixing vessels) containing Teflon coated stir bars. The mixing vessels were closed with foil covered rubber stoppers and the treatments were stirred using a <10% vortex (of the static liquid depth) at room temperature (approximately 22°C) on magnetic stirplates for 24 hours $\pm$ 1 hour. As stirring initiated and after mixing was stopped, all treatments appeared clear and colorless with clear to slightly yellow test substance evident on the surface. The treatments were allowed to settle and equilibrate to test temperature in an environmental chamber of appropriate temperature to properly reduce the temperature of the WAFs to test temperature for approximately 45 minutes.

Five replicates per treatment were tested. Each replicate contained five daphnids. Replicate chambers were 130 mL glass bottles containing approximately 130 mL of solution (no headspace) closed with aluminum foil lined plastic caps. Water quality (temperature, pH, hardness and dissolved oxygen) measurements were recorded per treatment at the start of the test and at the point of every other renewal thereafter. Observations for immobilization and abnormal behavior or appearance were performed daily.

Samples were collected from the WAFs at the initiation of the test as well as at each renewal for Headspace GC-FID analysis. In addition, composite samples of the "old" solutions were also collected upon each renewal and analyzed.

The immobilization data at 48 hours were used to calculate EL50 and EC50 values. At 48 hours, no immobilization was observed below the 15 mg/l loading level. Based on the Binomial Method¹, the 48-hr EL50 was calculated to be 8.7 mg/L with a 99% confidence interval of 5 to 15 mg/L. The 48-hr EC50 value was 1.2 mg/L with 99% confidence interval of 0.79 to 1.7 mg/L.

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APPENDIX C - RANGE FINDING TEST (CONT'D)

Table C1. Summary of Water Quality Measurements

Loading Rate* (mg/L)	pH	Hardness	Dissolved Oxygen (mg/L)	Temperature (°C)
0 (Control)	7.9	158	7.3	20.7
0.05	8.0	158	7.5	20.7
0.2	8.0	167	7.4	20.8
0.6	8.1	161	7.4	20.8
1.8	8.1	164	7.4	20.9
5.0	8.3	161	8.2	20.9
15	8.3	164	7.9	20.9

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

Table C2. Percent Immobilization by Loading Rate

Loading Rate* (mg/L)	Mean Concentration (mg/L)	% Immobilization	Mean number of Neonates/replicate
0 (Control)	ND	0	61
0.05	0.0116 ¹	0	67
0.2	0.0572	10	58
0.6	0.1273	0	64
1.8	0.3508	16	19
5.0	0.7945	100 ²	NA
15	1.71	100 ³	NA

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹value below the PQL of 0.016 mg/L

²Complete immobilization occurred on Day 3.

³Complete immobilization occurred on Day 2.

ND = Non Detectable. NA = Not Applicable

APPENDIX D – PREVIOUS STUDY TRIALS

An initial trial of the study was performed starting on 07-Sep-2009 and terminating on 27-Sep-2009 (Day 19). Control immobilization exceeded 20% (the guideline acceptability criteria). A conductivity spike exceeding 4000 μ mhos was observed in the Control group on Day 19. It is believed that this is what caused the mortalities. A summary of the in-life observation data for each treatment is shown below.

Summary of Initial Study Trial		
Concentration† (mg/L)	Adult Immobilization (%)	Neonates per Adult◇
0 (Control)	100	85
0.08	0	93
0.19	0	87
0.48	0	78
1.2	60	48
3.0	100	NA

† Loading rate

◇ Average neonates produced per surviving adult at termination.

NA = not applicable

A second trial of the study was performed starting on 13-Oct-2009 and terminating on 28-Oct-2009 (Day 14). Immobilized neonates were noted beginning with the production of the first brood and continuing through the duration of the test. Due to the excessive number of immobilized neonates, the guideline requirement (> 60 neonates/daphnid on average) was unachievable, so the study was terminated. The results are summarized in the table below.

Summary of Second Study Trial		
Concentration† (mg/L)	Adult Immobilization (%)	Neonates per Adult◇
0 (Control)	10	4^
0.08	0	1^
0.19	10	1^
0.48	40	0^
1.2	100	NA
3.0	100	NA

† Loading rate

* Based on one surviving adult when the study was terminated.

◇ Average neonates produced per surviving adult at termination.

^ A large number of neonates were immobilized at the time of observations, number shown is the average number of live neonates.

NA = not applicable

APPENDIX E - DILUTION WATER ANALYSIS

The dilution water was prepared from UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless-steel pipes. Batches of 500 L of this deionized water are reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater*, 20th edition.

The following water quality data are most representative of the dilution water used during the in-life period of the study. Table E1 presents analyses performed on the reconstituted water (RW) on a batch basis. Water quality analyses were performed by Environmental Toxicology laboratory personnel. Total Organic Carbon analysis was performed by the laboratory's Environmental Fate Chemistry group. The quality of the dilution water is monitored annually for priority pollutants, un-ionized ammonia, total suspended solids and bacterial properties. Results of analyses are maintained at the testing facility.

Table E1. RESULTS OF WATER QUALITY ANALYSIS

Sample	Alkalinity as CaCO ₃ (mg/L) ¹	Hardness as CaCO ₃ (mg/L) ²	pH	Temperature (°C)	Dissolved Oxygen (mg/L)	Total Organic Carbon (ppm) ³
Batch 208A	109	176	8.13	20.4	9.44	<0.05

¹ U.S. Environmental Protection Agency. 1979, Revised March 1983. *Methods for Chemical Analysis of Water and Wastes*, EPA-600/4-79-020. Office of Research and Development, Cincinnati, OH. Method 310.1, Alkalinity (Titrimetric, pH 4.5).

² U.S. Environmental Protection Agency. 1979, Revised March 1983. *Methods for Chemical Analysis of Water and Wastes*, EPA-600/4-79-020. Office of Research and Development, Cincinnati, OH. Method 130.2, Hardness (Titrimetric, EDTA).

³ JIS K-0102: "Industrial Waste Water Testing", JIS K-0551: "Total organic carbon (TOC) testing methods for ultra-pure water", U.S. Pharmacopoeia, EPA 415.1 EPA 9060A, ASTM D2575, Standard Methods for Examination of Water and Waste Water 5301B.

APPENDIX F – WATER QUALITY MEASUREMENTS

Day	Variable	Loading Rate* (mg/L)					
		Control	0.08	0.19	0.48	1.2	3.0
0 (new)	D. O. (mg/L)	7.68	8.04	7.70	7.58	7.65	8.03
	pH	8.26	8.49	8.53	8.50	8.51	8.54
	Hardness (mg/L as CaCO ₃)	¹	¹	¹	¹	¹	¹
	Temperature (°C)	21.0	21.8	21.4	21.3	21.4	21.4
2 (old)	D. O. (mg/L)	7.73	7.96	7.64	7.60	7.90	7.93
	pH	8.54	8.52	8.49	8.58	8.56	8.64
	Hardness (mg/L as CaCO ₃)	192	168	164	180	176	172
	Temperature (°C)	20.9	21.0	20.9	20.8	20.4	20.7
4 (new)	D. O. (mg/L)	7.04	7.71	7.34	6.98	7.09	-
	pH	8.44	8.62	8.65	8.64	8.62	-
	Hardness (mg/L as CaCO ₃)	176	180	164	176	180	-
	Temperature (°C)	20.7	20.4	20.1	20.1	20.1	-
6 (old)	D. O. (mg/L)	7.17	7.15	7.13	7.34	9.97	-
	pH	8.29	8.33	8.35	8.27	8.81	-
	Hardness (mg/L as CaCO ₃)	172	160	188	160	172	-
	Temperature (°C)	20.0	20.2	20.4	20.4	20.7	-
8 (new)	D. O. (mg/L)	7.80	7.96	8.62	7.80	7.18	-
	pH	8.39	8.62	8.68	8.64	8.69	-
	Hardness (mg/L as CaCO ₃)	176	184	176	164	168	-
	Temperature (°C)	19.8	20.4	20.4	20.2	20.5	-
10 (old)	D. O. (mg/L)	6.31	7.37	5.97	6.00	7.60	-
	pH	8.13	8.68	8.18	8.27	8.65	-
	Hardness (mg/L as CaCO ₃)	164	164	164	168	176	-
	Temperature (°C)	20.6	20.5	20.5	20.9	20.3	-

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ Hardness measurements were inadvertently not collected on Day 0.

APPENDIX F – WATER QUALITY MEASUREMENTS (CONT'D)

Day	Variable	Loading Rate* (mg/L)					
		Control	0.08	0.19	0.48	1.2	3.0
12 (new)	D. O. (mg/L)	7.96	8.17	9.11	7.83	7.93	-
	pH	8.33	8.63	8.62	8.67	8.75	-
	Hardness (mg/L as CaCO ₃)	180	176	192	180	180	-
	Temperature (°C)	20	21.1	20.3	20.7	20.7	-
14 (old)	D. O. (mg/L)	6.08	5.77	5.61	5.73	7.33	-
	pH	8.01	8.12	8.11	8.14	8.43	-
	Hardness (mg/L as CaCO ₃)	176	172	168	180	180	-
	Temperature (°C)	20.4	20.7	20.5	20.8	20.7	-
16 (new)	D. O. (mg/L)	7.25	7.52	7.22	7.27	7.33	-
	pH	8.17	8.52	8.54	8.57	8.60	-
	Hardness (mg/L as CaCO ₃)	180	168	180	172	168	-
	Temperature (°C)	20.0	20.9	20.2	20.5	20.6	-
18 (old)	D. O. (mg/L)	6.33	6.39	6.14	5.74	8.22	-
	pH	8.09	8.17	8.14	8.12	8.28	-
	Hardness (mg/L as CaCO ₃)	164	172	172	172	164	-
	Temperature (°C)	20.5	20.5	20.6	20.7	20.7	-
20 (new)	D. O. (mg/L)	7.16	7.29	6.84	6.89	7.05	-
	pH	8.34	8.62	8.68	8.67	8.71	-
	Hardness (mg/L as CaCO ₃)	160	180	184	180	164	-
	Temperature (°C)	19.8	20.8	20.4	20.6	20.7	-
21 (old)	D. O. (mg/L)	8.40	8.10	7.73	8.33	8.33	-
	pH	8.08	8.27	8.25	8.38	8.44	-
	Hardness (mg/L as CaCO ₃)	180	172	176	176	164	-
	Temperature (°C)	20.3	20.8	20.9	20.8	20.9	-

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

APPENDIX G – BIOLOGICAL DATA

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.0 mg/L (Control)

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
3	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
4	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
5	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
6	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
7	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
8	0	0	11 [1]	14 [1]	0	7 [2]	0	0	15 [2]	0	0	N: 1-10	100
9	0	P	0	0	P	0	P	0	0	P	0	N: 1-10	100
10	14	14	0	0	9	0	17	0	0	13	0	N: 1-10	100
11	0	0	P	P	0	P	0	P	P	0	0	N: 1-10	100
12	1	16	14	22	16	20	21	29	20	22	0	N: 1-10	100
13	P	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
14	31	0	29	36	0	33	0	33	23	0	0	N: 1-10	100
15	0	P	0	0	P	0	P	0	0	P	0	N: 1-10	100
16	39	31	0	0	38	0	27	0	0	33	0	N: 1-10	100
17	0	0	P	P	0	P	0	P	P	0	0	N: 1-10	100
18	0	35	26	22	41	34	0	22	24	36	0	N: 1-7, 9-10; Z: 8	100
19	P	0	0	0	0	0	P	--	0	0	1	N: 1-7, 9-10; D: 8	90
20	24	0	38	0	0	27	22	--	27	1	1	N: 1-10	90
21	1	15	0	5	1	0	0	--	0	14	1	N: 1-10	90
Total Offspring	110	111	118	99	105	121	87	--	109	119	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted. [] = number of immobilized offspring.

Appearance codes: N = Observed normal. D = Immobilized. Z = daphnid did not appear healthy.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.08 mg/L

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
3	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
4	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
5	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
6	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
7	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
8	9 [2]	10 [1]	12	10 [2]	0	11	0	0	12 [1]	0	0	N: 1-10	100
9	0	0	0	0	P	0	P	P	0	P	0	N: 1-10	100
10	0	0	0	0	12	0	12	3	23	15	0	N: 1-10	100
11	P	P	P	P	0	P	0	0	0	0	0	N: 1-10	100
12	16	25	18	12	12	21	17	17	2	24	0	N: 1-10	100
13	0	0	0	0	0	0	0	0	P	0	0	N: 1-10	100
14	5	32	32	30	0	28	0	0	33	0	0	N: 1-10	100
15	0	0	0	0	P	0	P	P	0	P	0	N: 1-10	100
16	0	0	0	0	25	0	27	25	0	34	1	N: 1-9; D: 10	100
17	0	P	P	P	0	P	0	0	P	--	1	N: 1-9	90
18	0	24	30	33	0	26	23	18	33	--	1	N: 2-9; Z: 1	90
19	--	0	0	0	P	0	0	0	P	--	2	N: 2-9; D: 1	80
20	--	19	42	35	35	36	0	1	35	--	2	N: 2-9	80
21	--	0	0	0	0	0	0	0	0	--	2	N: 2-9	80
Total Offspring	--	110	134	120	84	122	79	64	138	--	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted. [] = number of immobilized offspring.

Appearance codes: N = Observed normal. D = Immobilized. Z = daphnid did not appear healthy.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.19 mg/L

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
3	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
4	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
5	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
6	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
7	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
8	0	0	9 [1]	0	0	12	12 [1]	0	0	12	0	N: 1-10	100
9	P	P	0	P	P	0	0	P	P	0	0	N: 1-10	100
10	14	22	14	17	11	0	0	15	20	0	0	N: 1-10	100
11	0	0	0	0	0	P	P	0	0	P	0	N: 1-10	100
12	23	19	3	22	21	20	14	17	25	15	0	N: 1-10	100
13	0	0	P	0	0	0	0	0	0	0	0	N: 1-10	100
14	0	0	29	0	0	0	16	0	0	25	0	N: 1-10	100
15	P	P	0	P	P	P	0	0	P	0	0	N: 1-10	100
16	28	33	0	27	33	25	1	4	27	0	0	N: 1-10	100
17	0	0	P	0	0	0	P	0	0	P	0	N: 1-10	100
18	4	34	35	41	38	24	18	0	0	24	0	N: 1-10	100
19	P	0	0	0	0	0	0	P	P	0	0	N: 1-10	100
20	32	1	40	0	0	0	27	15	34	34	0	N: 1-10	100
21	0	0	0	24	26	0	13	0	0	0	0	N: 1-10	100
Total Offspring	101	109	130	131	129	81	101	51	106	110	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted. [] = number of immobilized offspring.

Appearance codes: N = Observed normal.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.48 mg/L

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
3	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
4	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
5	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
6	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
7	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
8	10	0	9 [1]	0	0	0	0	12 [1]	0	8 [2]	0	N: 1-10	100
9	0	P	0	P	P	P	0	0	P	0	0	N: 1-10	100
10	0	13	0	10 [1]	13	8 [1]	5 [1]	0	10	0	0	N: 1-10	100
11	P	0	P	0	0	0	0	P	0	P	0	N: 1-10	100
12	23	17	16	13 [2]	23	18	0	17	12	15	0	N: 1-10	100
13	0	0	0	0	0	0	P	0	0	0	0	N: 1-10	100
14	24	0	16	0	0	0	16	19	0	19	0	N: 1-10	100
15	0	P	0	P	P	P	0	0	P	0	0	N: 1-10	100
16	0	19	0	20	26	22	24	0	15	0	0	N: 1-10	100
17	P	0	P	0	0	0	0	P	0	P	0	N: 1-10	100
18	21	0	18	25	31	11	0	23	26	34	0	N: 1-10	100
19	0	P	0	0	0	0	P	0	0	0	0	N: 1-10	100
20	32	28	28	0	0	0	30	30	0	37	0	N: 1-10	100
21	0	0	0	0	28	0	0	0	23	0	0	N: 1-10	100
Total Offspring	110	77	87	68	121	59	75	101	86	113	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted. [] = number of immobilized offspring.

Appearance codes: N = Observed normal.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 1.2 mg/L

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
3	0	0	0	0	--	0 ¹	0	0	0	0	1	N: 1-4, 7-10; L: 6 ² ; D: 5	90
4 ²	0	0	0	0	--	--	0	0	0	0	2	N: 1-4, 7-10; D: 6	80
5	0	--	--	--	--	--	--	0	--	0	7	A: 1, 8, 10 D: 2-4, 7, 9	30
6	--	--	--	--	--	--	--	0	--	--	9	S: 8; D: 1, 10	10
7	--	--	--	--	--	--	--	0	--	--	9	S: 8	10
8	--	--	--	--	--	--	--	0	--	--	9	S: 8	10
9	--	--	--	--	--	--	--	0	--	--	9	S: 8	10
10	--	--	--	--	--	--	--	0	--	--	9	S: 8	10
11	--	--	--	--	--	--	--	0	--	--	9	A: 8	10
12	--	--	--	--	--	--	--	3	--	--	9	N: 8	10
13	--	--	--	--	--	--	--	0	--	--	9	N: 8	10
14	--	--	--	--	--	--	--	13	--	--	9	N: 8	10
15	--	--	--	--	--	--	--	0	--	--	9	N: 8	10
16	--	--	--	--	--	--	--	0	--	--	9	N: 8	10
17	--	--	--	--	--	--	--	P	--	--	9	N: 8	10
18	--	--	--	--	--	--	--	26	--	--	9	N: 8	10
19	--	--	--	--	--	--	--	0	--	--	9	N: 8	10
20	--	--	--	--	--	--	--	26	--	--	9	N: 8	10
21	--	--	--	--	--	--	--	0	--	--	9	N: 8	10
Total Offspring	--	--	--	--	--	--	--	68	--	--	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted. [] = number of immobilized offspring.

Appearance codes: N = Observed normal. S = small. L = Lethargic. D = Immobilized A=Abnormal

¹Daphnid considered very lethargic. ²Daphnids were off-color and lethargic and appeared to be different sizes.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 3.0 mg/L

Test Day	Number of Live Offspring Released per Replicate										Cumulative Daphnid Immobilized	Parent Appearance (Observation: Replicate)	% Survival
	1	2	3	4	5	6	7	8	9	10			
1	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
2	0	--	0	0	--	0	0	0	0	0	2	L: 1, 3-4, 6-10; D: 2, 5	80
3	--	--	0	--	--	0	--	--	--	--	8	C, L: 3, 6; D: 1,4 7-10	20
4	--	--	--	--	--	--	--	--	--	--	10	D: 3, 6	0
Total Offspring	--	--	--	--	--	--	--	--	--	--	--	--	--

* Loading rate is defined by the amount of kerosene per unit volume of dilution water.

P = Neonates present but not counted.

Appearance codes: N = Observed normal. C = Off-color. L = Lethargic D = Immobilized.

APPENDIX G – BIOLOGICAL DATA (CONT'D)

Individual Adult Daphnid Lengths¹ at Test Termination (mm)

Adult	Loading Rate* (mg/L)					
	Control (0)	0.08	0.19	0.48	1.2	3.0
1	5.0	-- ²	5.0	4.8	-- ²	No Surviving Adults
2	4.8	4.9	5.0	4.8	-- ²	
3	5.0	5.0	4.9	4.7	-- ²	
4	4.7	5.0	5.0	4.5	-- ²	
5	5.0	5.0	5.0	4.9	-- ²	
6	4.9	5.1	4.9	4.9	-- ²	
7	4.7	4.7	4.8	4.9	-- ²	
8	-- ²	4.8	4.9	4.9	4.3	
9	5.0	5.1	5.0	4.6	-- ²	
10	5.0	-- ²	5.0	5.0	-- ²	
Mean	4.9	5.0	5.0	4.8	4.3	--

*Loading rate is defined by the amount of kerosene per unit volume of dilution water.

¹ Body length excluding anal spine.

² Daphnid died before test termination.

APPENDIX H - TEST SUBSTANCE CHARACTERIZATION

Kerosene (CAS No. 64742-81-0) was initially characterized on June 2, 2009. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, density, physical state, miscibility and Gas Chromatography-Mass Spectrometry (GC/MS) analysis. Stability of the neat test substance was confirmed by repeating these analyses on February 15, 2010 after the completion of this study.

UV-Vis spectra are presented in Figures H-1 and H-2 representing, the initial and final spectrum at concentrations of 92.0 and 98.8 ppm with hexane, respectively. UV-VIS spectra were acquired on a Hewlett-Packard 8453 diode array UV-VIS spectrophotometer using a 1 cm quartz cell, a scan time of 0.5 seconds, and resolution of 2 nm.

FT-IR spectra of the neat test substance are presented in Figures H-3 and H-4 representing the initial and final spectra. FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of 4 cm⁻¹, gain of 1, and scan number of 32.

The test substance was also characterized by GC/MS using a Varian Saturn 2000 GC/MS system with a Varian 3800 GC. For comparison, the test substance was analyzed under the same instrument conditions against an ASTM D3710 Qualitative Calibration mixture containing a series of known hydrocarbons. Figures H-5 and H-6 represent the initial and final GC/MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components detected between the approximate retention times of 20 and 61 minutes. This corresponds to bracketing by the standard hydrocarbons n-propyl benzene (18.6 minutes) and just after the elution of n-pentadecane (55.6 minutes).

The test substance's density was measured at 20°C using an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 0.8165 g/mL and the final density was 0.8165 g/mL. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water and methanol but miscible in hexane.

Comparison of the initial and final analyses appeared to be substantially similar indicating the neat test substance was stable over the duration of study period.


Principal Investigator for
Characterization (located at the testing facility)

30 May 2012
Date

APPENDIX H - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure H-1 Initial

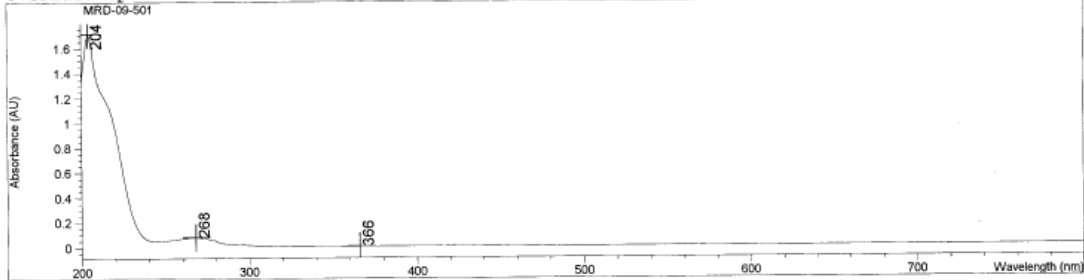
=====

Spectrum/Peak Report Date 06/03/09 Time 17:23:05 Page 1 of 1
Initial characterization MRD-09-501 92 ppm solution with hexane

=====

Method file : CHAR.M (modified) Last update: Date 06/03/09 Time 17:16:35
Information : Default Method
Data File : C:\HPCHEM\1\DATA\09-501I.SD Created : 6/3/09 17:17:16

Overlaid Spectra:



#	Name	Peaks (nm)	Abs (AU)	#	Name	Peaks (nm)	Abs (AU)
1	MRD-09-501	204.0	1.7135	1		366.0	0.007
1		268.0	0.08				

Report generated by : KELLEY

Signature: *P. Kelley*
3 June 09

*** End Spectrum/Peak Report ***

Figure H-2 Final

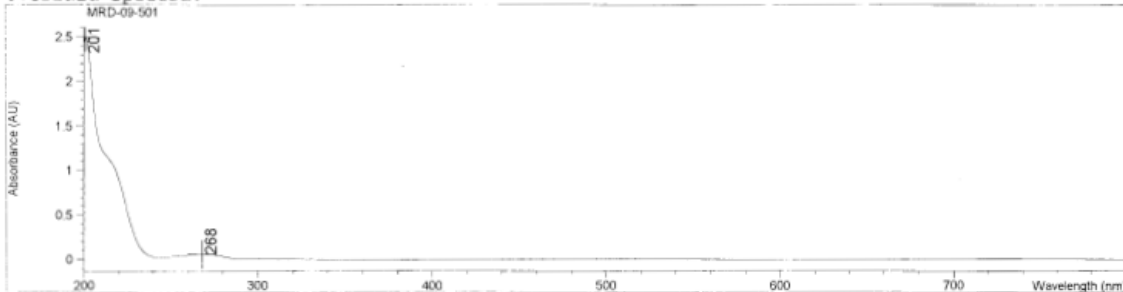
=====

Spectrum/Peak Report Date 02/15/10 Time 13:33:06 Page 1 of 1
Final Characterization MRD-09-501 98.8ppm solution in hexane

=====

Method file : CHAR.M Last update: Date 12/23/05 Time 12:07:09
Information : Default Method
Data File : C:\HPCHEM\UVEXE\09-501F.SD Created : 2/15/10 13:18:32

Overlaid Spectra:



#	Name	Peaks (nm)	Abs (AU)	#	Name	Peaks (nm)	Abs (AU)
1	MRD-09-501	201.0	2.4915	1		268.0	0.058

Report generated by : kelley

Signature: *P. Kelley*
15 Feb 10

*** End Spectrum/Peak Report ***

APPENDIX H - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

IR SPECTRA

Figure H-3 Initial

Initial Characterization MRD-09-501

Collection time: Thu Jun 04 02:58:47 2009

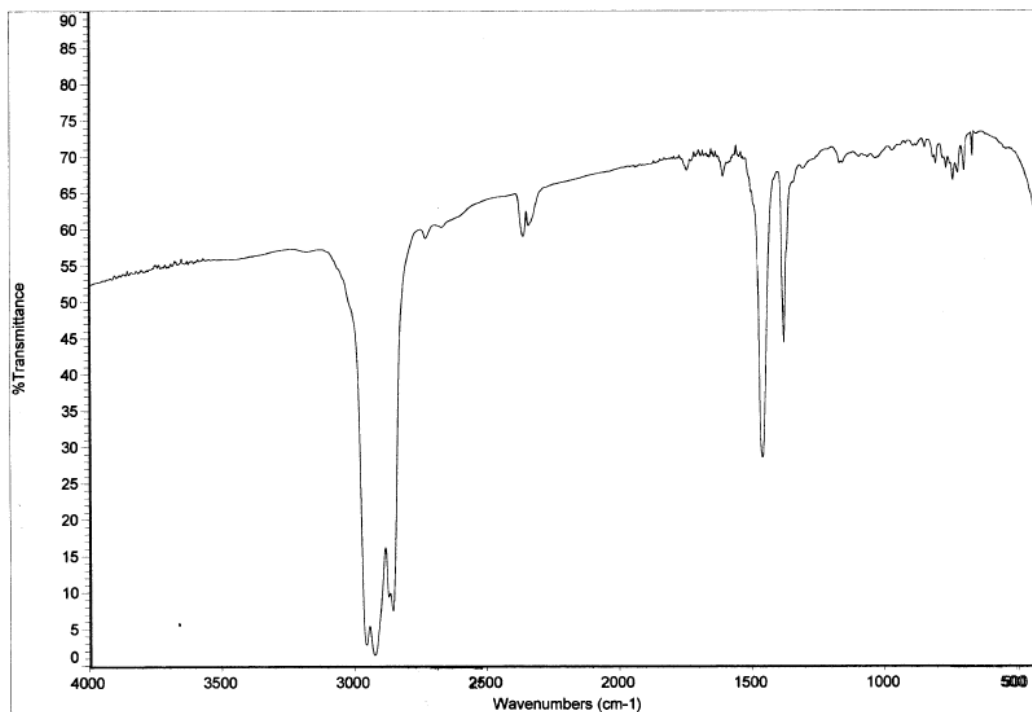
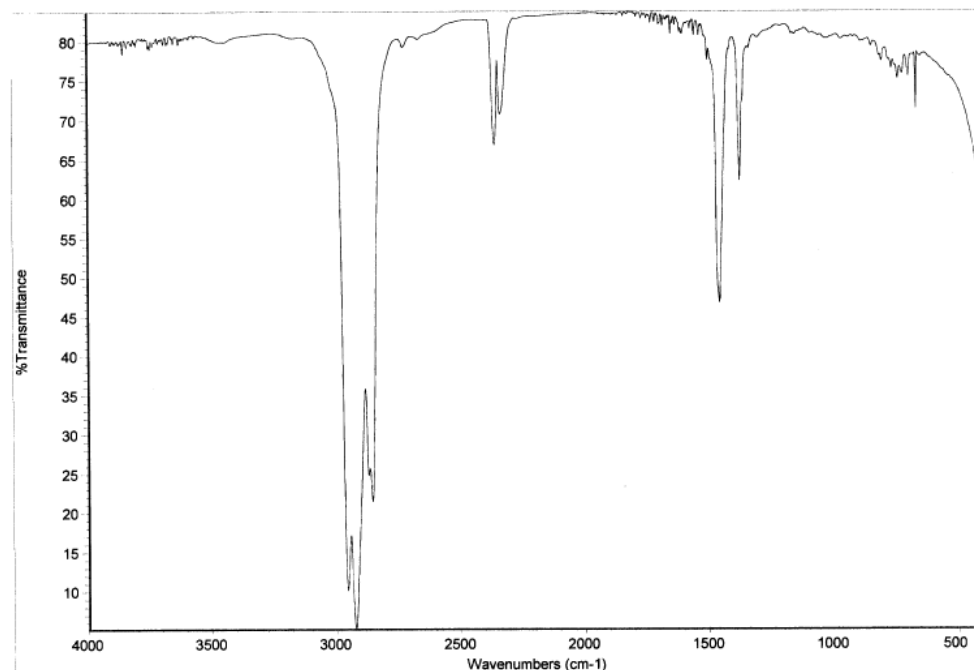


Figure H-4 Final

Final Characterization MRD-09-501

Collection time: Mon Feb 15 09:18:12 2010



APPENDIX H - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure H-5 Initial

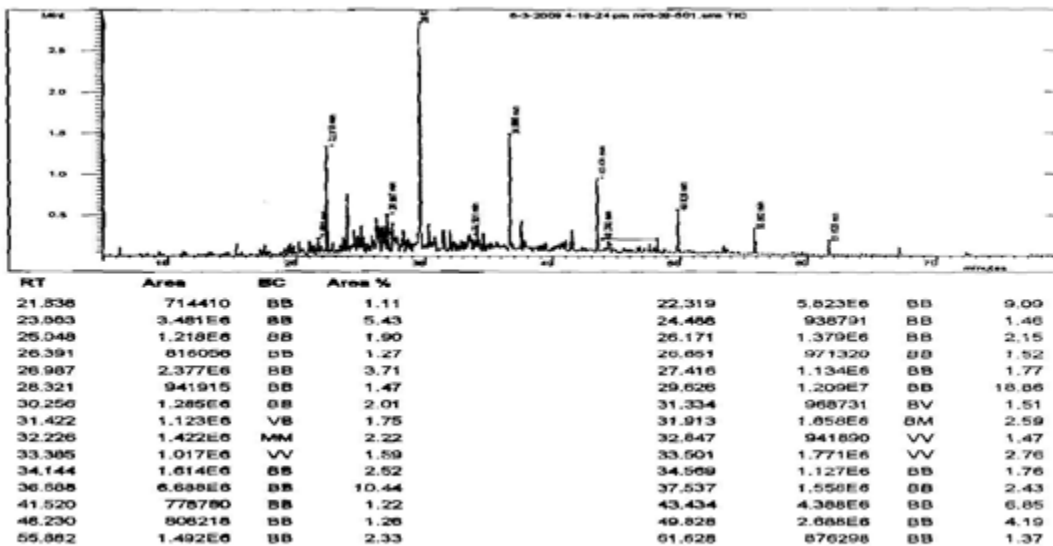
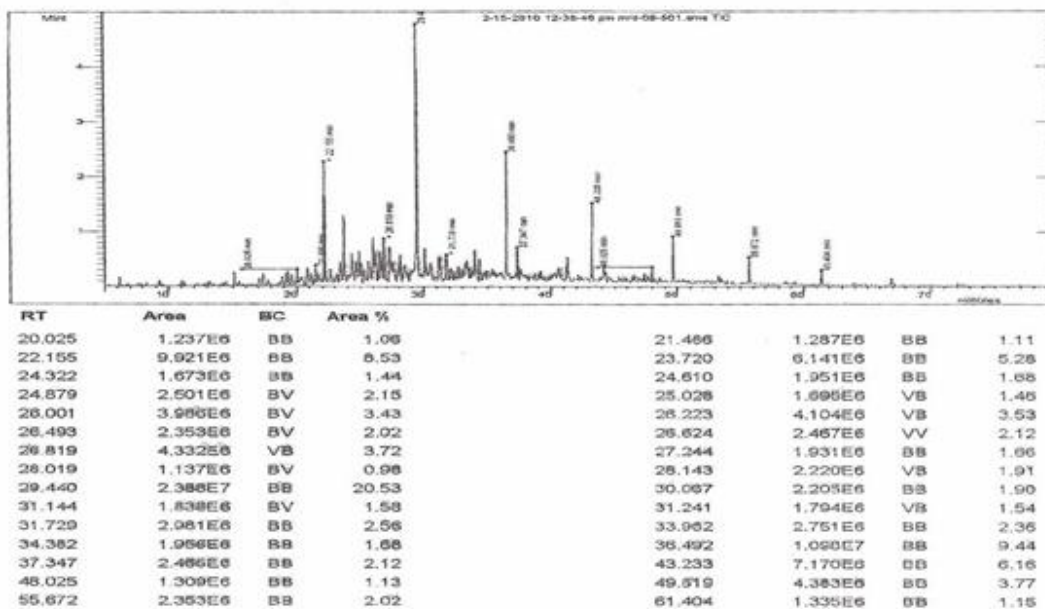


Figure H-6 Final



APPENDIX I – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION



Report of Analysis

INTERIM

[REDACTED] [REDACTED]
[REDACTED] [REDACTED]
[REDACTED] [REDACTED]
[REDACTED] [REDACTED]

[illegible]

████	████	████	████
████	████████████████████		
	████████	████████	
████	████████████████████		
	████	████	████
	████	████	████
	████	████	████

Sample ID: 2009-DRPK-000651-045	Date Taken: 16-January-2009
Drawn By: Client	Date Submitted: 16-January-2009
Sample Designated As: Crude Oil	Date Tested: 26-January-2009
Representing: Site#28 Sx.#7 (As Received)	

Method	Test	Result	Units
ASTM D4052	Density of Liquids by Digital Density Meter		
	Relative Density @ 60/60°F	0.8204	
	API Gravity @ 60°F	41.0	°API
ASTM D2887	Boiling Range Distribution of Petroleum Fractions by GC (Simulated Distillation)		
	Boiling Point Distribution	See Attached Report	
ASTM D1319	Hydrocarbon Types (Aromatics, Olefins, Saturates) by FIA		
	Aromatics	18.8	Vol %
	Olefins	2.9	Vol %
	Saturates	80.4	Vol %

APPENDIX I – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION (CONT'D)



Report of Analysis

INTERIM

Sample ID: 2009-DRPK-000651-045	Date Taken: 16-January-2009
Drawn By: Client	Date Submitted: 16-January-2009
Sample Designated As: Crude Oil	Date Tested: 26-January-2009
Representing: Site#28 Sx.#7 (As Received)	

Method	Test	Result	Units
ASTM D5188	Determination of Aromatic Content and Polynuclear Aromatic Content of Diesel Fuels and Aviation Turbine Fuels by Supercritical Fluid Chromatography		
	Monoaromatics by SFC	22.0	Wt %
	Polynuclear Aromatics by SFC	0.8	Wt %
	Total Aromatics	22.8	Wt %

[illegible]

[illegible]

APPENDIX I – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION (CONT'D)

SAMPLE:	09-0651-46 (Site #28 8 x . #7)					Injection Date:		09/01/16221421-0800	
						Report Date:		1/17/09 1:01	
FILE:	C:\VCP32 Instruments\CD3887 & CD3710\Data\2009\JAN-09\09-0651-46_0002.CDF								
PROCEDURE:	C:\VCP32 Instruments\CD3887 & CD3710\PROCEDURE\22308-CD3887.pro								
EXCEL FILE:	C:\VCP32 Instruments\CD3887 & CD3710\Reports\2009\JAN-09\09-0651-46_0002_CDF.xls								
Boiling Point Distribution Report									
ASTM D2887 Simulated Distillation									
	% Off	BP °F	BP °C	% Off	BP °F	BP °C	% Off	BP °F	BP °C
	IBP	226.1	107.9	40%	392.3	200.1	80%	476.3	246.8
	1%	257.0	125.0	41%	392.8	200.5	81%	480.2	249.0
	2%	287.8	142.1	42%	394.4	201.4	82%	483.3	250.7
	3%	303.5	150.9	43%	396.4	202.5	83%	487.1	252.8
	4%	317.3	158.5	44%	398.2	203.5	84%	490.6	254.8
	5%	325.2	162.9	46%	399.9	204.4	85%	494.1	256.7
	6%	330.5	165.8	48%	401.2	205.1	86%	496.8	258.2
	7%	335.7	168.7	47%	402.4	205.8	87%	501.3	260.7
	8%	339.4	170.8	48%	404.1	206.7	88%	506.7	263.7
	9%	342.1	172.3	48%	405.0	207.8	88%	511.8	266.6
	10%	346.3	174.6	60%	408.1	208.9	89%	516.7	269.3
	11%	349.5	176.4	61%	410.2	210.1	81%	523.4	273.0
	12%	351.0	177.2	62%	412.2	211.2	82%	528.0	275.6
	13%	351.8	177.7	63%	414.0	212.2	83%	536.1	280.1
	14%	353.1	178.4	64%	415.5	213.1	84%	544.6	284.8
	15%	356.3	180.1	66%	417.5	214.1	86%	554.1	290.1
	16%	358.9	181.6	68%	419.5	215.3	88%	564.8	296.0
	17%	360.7	182.6	67%	421.6	216.4	87%	578.0	303.4
	18%	361.9	183.3	68%	423.6	217.6	88%	593.7	312.0
	19%	363.4	184.1	68%	426.1	218.9	89%	620.4	326.9
	20%	364.8	184.9	80%	427.3	219.6	FBP	645.8	341.0
	21%	366.1	185.6	81%	428.3	220.2			
	22%	367.1	186.2	82%	430.0	221.1			
	23%	368.4	186.9	83%	432.2	222.3			
	24%	370.5	188.0	84%	434.0	223.3			
	25%	373.1	189.5	86%	436.6	224.8			
	26%	374.9	190.5	88%	439.0	226.1			
	27%	376.0	191.1	87%	441.3	227.4			
	28%	377.2	191.8	88%	444.3	229.0			
	29%	378.4	192.4	89%	446.8	230.4			
	30%	380.0	193.3	70%	449.2	231.8			
	31%	382.1	194.5	71%	451.8	233.2			
	32%	384.0	195.6	72%	454.3	234.6			
	33%	385.5	196.4	73%	457.3	236.3			
	34%	386.9	197.2	74%	459.7	237.6			
	35%	388.5	198.1	76%	461.9	238.8			
	36%	390.0	198.9	78%	463.3	239.6			
	37%	390.8	199.3	77%	465.9	241.0			
	38%	391.4	199.7	78%	468.9	242.7			
	39%	391.9	199.9	79%	472.8	244.9			
Start Elution Time (min):		0.165		Sample Wt:		0 g			
End Elution Time (min):		12.278		Solvent Wt:		0 g			
				Material Balance:		100.0 W%			
Blank File:		C:\VCP32 Instruments\CD3887 & CD3710\Data\2009\JAN-09\BLANK(1).0005.CDF							
Calib File:		C:\VCP32 Instruments\CD3887 & CD3710\DATA\RTM\060905.0006.CDF							
Resp Factor:		1.000E+00							

APPENDIX J - STATISTICAL OUTPUT

0950146(3) adult immobilization

File: 0950146IM.dat Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	4.020	14.520	22.920	14.520	4.020
OBSERVED	3	0	56	0	1

Calculated Chi-Square goodness of fit test statistic = 79.3113

Table Chi-Square value (alpha = 0.01) = 13.277

Data FAIL normality test. Try another transformation.

Warning - The first three homogeneity tests are sensitive to non-normal data and should not be performed.

0950146(3) adult immobilization

File: 0950146IM.dat Transform: NO TRANSFORMATION

Shapiro - Wilk's test for normality

***** Shapiro - Wilk's Test is aborted *****

This test can not be performed because total number of replicates is greater than 50.

Total number of replicates = 60

0950146(3) adult immobilization

File: 0950146IM.dat Transform: NO TRANSFORMATION

Hartley's test for homogeneity of variance

Bartlett's test for homogeneity of variance

-

These two tests can not be performed because at least one group has zero variance.

Data FAIL to meet homogeneity of variance assumption.

Additional transformations are useless.

TITLE: 0950146(3) adult immobilization

FILE: 0950146IM.dat

TRANSFORM: NO TRANSFORMATION

NUMBER OF GROUPS: 6

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) adult immobilization
File: 0950146IM.dat Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	Control	10	0.000	1.000	0.900
2	0.08mg/L	10	0.000	1.000	0.800
3	0.19mg/L	10	1.000	1.000	1.000
4	0.48mg/L	10	1.000	1.000	1.000
5	1.2mg/L	10	0.000	1.000	0.100
6	3.0mg/L	10	0.000	0.000	0.000

0950146(3) adult immobilization
File: 0950146IM.dat Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	Control	0.100	0.316	0.100	35.14
2	0.08mg/L	0.178	0.422	0.133	52.70
3	0.19mg/L	0.000	0.000	0.000	0.00
4	0.48mg/L	0.000	0.000	0.000	0.00
5	1.2mg/L	0.100	0.316	0.100	316.23
6	3.0mg/L	0.000	0.000	0.000	N/A

0950146(3) adult immobilization
File: 0950146IM.dat Transform: NO TRANSFORMATION

STEEL'S MANY-ONE RANK TEST - Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	RANK SUM	CRIT. VALUE	df	SIG
1	Control	0.900				
2	0.08mg/L	0.800	100.00	75.00	10.00	
3	0.19mg/L	1.000	110.00	75.00	10.00	
4	0.48mg/L	1.000	110.00	75.00	10.00	
5	1.2mg/L	0.100	65.00	75.00	10.00	*
6	3.0mg/L	0.000	60.00	75.00	10.00	*

Critical values use k = 5, are 1 tailed, and alpha = 0.05

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) 21 day EL50 (mg/L) 1
08:57 Tuesday, March 16, 2010

Obs	DOSE	N	MORT
1	0.00	10	1
2	0.08	10	2
3	0.19	10	0
4	0.48	10	0
5	1.20	10	9
6	3.00	10	10

0950146(3) 21 day EL50 (mg/L) 2
08:57 Tuesday, March 16, 2010

Probit Procedure

Iteration History for Parameter Estimates

Iter	Ridge	Loglikelihood	Intercept	DOSE
0	0	-41.588831	0	0
1	0	-21.191365	-1.045776768	0.8624965636
2	0	-18.305206	-1.396131615	1.365859196
3	0	-17.157508	-1.629616477	1.9031887458
4	0	-17.049958	-1.719893628	2.1274358919
5	0	-17.04974	-1.723601084	2.1379227437
6	0	-17.04974	-1.723607922	2.1379454687
7	0	-17.04974	-1.723607922	2.1379454687

Model Information

Data Set	WORK.TOX
Events Variable	MORT
Trials Variable	N
Number of Observations	6
Number of Events	22
Number of Trials	60
Name of Distribution	Normal
Log Likelihood	-17.04973967

Number of Observations Read	6
Number of Observations Used	6
Number of Events	22
Number of Trials	60

Parameter Information

Parameter	Effect
Intercept	Intercept
DOSE	DOSE

0950146(3) 21 day EL50 (mg/L) 3
08:57 Tuesday, March 16, 2010

Probit Procedure

Last Evaluation of the Negative of the Gradient

Intercept	DOSE
-4.21426E-10	-6.20534E-10

Last Evaluation of the Negative of the Hessian

	Intercept	DOSE
Intercept	17.728401126	8.4444500834
DOSE	8.4444500834	7.7102600643

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

Algorithm converged.

Goodness-of-Fit Tests

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	9.1317	4	0.0579
L.R. Chi-Square	11.0881	4	0.0256

Response-Covariate Profile

Response Levels	2
Number of Covariate Values	6

All variances and covariances have been multiplied by the heterogeneity factor H= 2.2829.

Please check to be sure that the large chi-square ($p < 0.0579$) is not caused by systematic departure from the model. A t value of 2.78 will be used in computing fiducial limits.

0950146(3) 21 day EL50 (mg/L) 4
08:57 Tuesday, March 16, 2010

Probit Procedure

Type III Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
DOSE	1	7.3840	0.0066

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits	Chi- Square	Pr > ChiSq
Intercept	1	-1.7236	0.5189	-2.7406 -0.7067	11.04	0.0009
DOSE	1	2.1379	0.7868	0.5959 3.6800	7.38	0.0066

Estimated Covariance Matrix

	Intercept	DOSE
Intercept	0.269218	-0.294853
DOSE	-0.294853	0.619019

Probit Model in Terms of Tolerance Distribution

MU	SIGMA
0.80619826	0.46773878

Estimated Covariance Matrix for Tolerance Parameters

	MU	SIGMA
MU	0.042910	0.020896
SIGMA	0.020896	0.029629

0950146(3) 21 day EL50 (mg/L) 5
08:57 Tuesday, March 16, 2010

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

Probit Procedure

Probit Analysis on DOSE

Probability	DOSE	95% Fiducial Limits	
0.01	-0.28192	.	.
0.02	-0.15442	.	.
0.03	-0.07352	.	.
0.04	-0.01267	.	.
0.05	0.03684	.	.
0.06	0.07897	.	.
0.07	0.11591	.	.
0.08	0.14899	.	.
0.09	0.17908	.	.
0.10	0.20677	.	.
0.15	0.32142	.	.
0.20	0.41254	.	.
0.25	0.49071	.	.
0.30	0.56092	.	.
0.35	0.62597	.	.
0.40	0.68770	.	.
0.45	0.74742	.	.
0.50	0.80620	.	.
0.55	0.86497	.	.
0.60	0.92470	.	.
0.65	0.98643	.	.
0.70	1.05148	.	.
0.75	1.12168	.	.
0.80	1.19986	.	.
0.85	1.29098	.	.
0.90	1.40563	.	.
0.91	1.43332	.	.
0.92	1.46340	.	.
0.93	1.49648	.	.
0.94	1.53343	.	.
0.95	1.57556	.	.
0.96	1.62506	.	.
0.97	1.68592	.	.
0.98	1.76682	.	.
0.99	1.89432	.	.

0950146(3) 21 day EC50 (mg/L) 6
08:57 Tuesday, March 16, 2010

Obs	DOSE	N	MORT
1	0.000	10	1
2	0.016	10	2
3	0.039	10	0
4	0.092	10	0
5	0.230	10	9
6	0.540	10	10

0950146(3) 21 day EC50 (mg/L) 7
08:57 Tuesday, March 16, 2010

Probit Procedure

Iteration History for Parameter Estimates

Iter	Ridge	Loglikelihood	Intercept	DOSE
0	0	-41.588831	0	0
1	0	-20.87998	-1.073240483	4.8354855812
2	0	-18.078356	-1.438376568	7.5843004566
3	0	-17.156617	-1.654301047	10.159780945
4	0	-17.082175	-1.729111687	11.138097907
5	0	-17.082043	-1.73198675	11.180661662
6	0	-17.082043	-1.731991136	11.180736796
7	0	-17.082043	-1.731991136	11.180736796

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

Model Information

Data Set	WORK.TOX
Events Variable	MORT
Trials Variable	N
Number of Observations	6
Number of Events	22
Number of Trials	60
Name of Distribution	Normal
Log Likelihood	-17.0820425

Number of Observations Read	6
Number of Observations Used	6
Number of Events	22
Number of Trials	60

Parameter Information

Parameter	Effect
Intercept	Intercept
DOSE	DOSE

0950146(3) 21 day EC50 (mg/L) 8
08:57 Tuesday, March 16, 2010

Probit Procedure

Last Evaluation of the Negative of the Gradient

Intercept	DOSE
-1.71298E-10	-4.91144E-11

Last Evaluation of the Negative of the Hessian

	Intercept	DOSE
Intercept	17.761571225	1.6308141414
DOSE	1.6308141414	0.2844855759

Algorithm converged.

Goodness-of-Fit Tests

Statistic	Value	DF	Pr > ChiSq
Pearson Chi-Square	9.1931	4	0.0565
L.R. Chi-Square	11.1527	4	0.0249

Response-Covariate Profile

Response Levels	2
Number of Covariate Values	6

All variances and covariances have been multiplied by the heterogeneity factor H= 2.2983.

Please check to be sure that the large chi-square ($p < 0.0565$) is not caused by systematic departure from the model. A t value of 2.78 will be used in computing fiducial limits.

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) 21 day EC50 (mg/L) 9
08:57 Tuesday, March 16, 2010

Probit Procedure

Type III Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
DOSE	1	7.3294	0.0068

Analysis of Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits	Chi- Square	Pr > ChiSq
Intercept	1	-1.7320	0.5227	-2.7564 -0.7076	10.98	0.0009
DOSE	1	11.1807	4.1299	3.0863 19.2751	7.33	0.0068

Estimated Covariance Matrix

	Intercept	DOSE
Intercept	0.273183	-1.566020
DOSE	-1.566020	17.055886

Probit Model in Terms of Tolerance Distribution

MU	SIGMA
0.1549085	0.08943954

Estimated Covariance Matrix for Tolerance Parameters

	MU	SIGMA
MU	0.001578	0.000770
SIGMA	0.000770	0.001091

0950146(3) 21 day EC50 (mg/L) 10
08:57 Tuesday, March 16, 2010

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

Probit Procedure			
Probit Analysis on DOSE			
Probability	DOSE	95% Fiducial Limits	
0.01	-0.05316	.	.
0.02	-0.02878	.	.
0.03	-0.01331	.	.
0.04	-0.00167	.	.
0.05	0.00779	.	.
0.06	0.01585	.	.
0.07	0.02291	.	.
0.08	0.02924	.	.
0.09	0.03499	.	.
0.10	0.04029	.	.
0.15	0.06221	.	.
0.20	0.07963	.	.
0.25	0.09458	.	.
0.30	0.10801	.	.
0.35	0.12045	.	.
0.40	0.13225	.	.
0.45	0.14367	.	.
0.50	0.15491	.	.
0.55	0.16615	.	.
0.60	0.17757	.	.
0.65	0.18937	.	.
0.70	0.20181	.	.
0.75	0.21523	.	.
0.80	0.23018	.	.
0.85	0.24761	.	.
0.90	0.26953	.	.
0.91	0.27483	.	.
0.92	0.28058	.	.
0.93	0.28690	.	.
0.94	0.29397	.	.
0.95	0.30202	.	.
0.96	0.31149	.	.
0.97	0.32313	.	.
0.98	0.33859	.	.
0.99	0.36298	.	.

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

```

0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT      Transform: NO TRANSFORMATION

Shapiro - Wilk's test for normality
-----

D =    54.500

W =     0.908

Critical W (P = 0.05) (n = 37) = 0.936
Critical W (P = 0.01) (n = 37) = 0.914
-----

Data FAIL normality test. Try another transformation.

Warning - The first three homogeneity tests are sensitive to non-normal
          data and should not be performed.


0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT      Transform: NO TRANSFORMATION

-----
Bartlett's test for homogeneity of variance
Calculated B1 statistic =    5.16
-----

Bartlett's test using average degrees of freedom
Calculated B2 statistic =    4.76
    Based on average replicate size of    8.25
-----

Table Chi-square value =   11.34  (alpha = 0.01, df =   3)
Table Chi-square value =    7.81  (alpha = 0.05, df =   3)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.
Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

```

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	Control	9	47.000	50.000	49.000
2	0.08 mg/L	8	47.000	51.000	49.500
3	0.19 mg/L	10	48.000	50.000	49.500
4	0.48 mg/L	10	45.000	50.000	48.000

0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	Control	1.750	1.323	0.441	2.70
2	0.08 mg/L	2.000	1.414	0.500	2.86
3	0.19 mg/L	0.500	0.707	0.224	1.43
4	0.48 mg/L	2.444	1.563	0.494	3.26

0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	3	14.473	4.824	2.921
Within (Error)	33	54.500	1.652	
Total	36	68.973		

Critical F value = 2.92 (0.05,3,30)
Since F > Critical F REJECT Ho: All equal

0950146(3) growth data
File: H:\TOXSTAT\0950146G.DAT Transform: NO TRANSFORMATION

BONFERRONI t-TEST - TABLE 1 OF 2 Ho: Control < Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	Control	49.000	49.000		
2	0.08 mg/L	49.500	49.500	-0.801	
3	0.19 mg/L	49.500	49.500	-0.801	
4	0.48 mg/L	48.000	48.000	1.634	

NOELR = 0.48 mg/L
NOEL = 0.092 mg/L
LOELR = 1.2 mg/L
LOEL = 0.25 mg/L

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

Bonferroni t table value = 2.22 (1 Tailed Value, P=0.05, df=33,3)

0950146(3) growth data

File: H:\TOXSTAT\0950146G.DAT

Transform: NO TRANSFORMATION

BONFERRONI t-TEST		-	TABLE 2 OF 2		Ho:Control<Treatment	
GROUP	IDENTIFICATION		NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	Control		9			
2	0.08 mg/L		8	1.387	2.8	-0.500
3	0.19 mg/L		10	1.311	2.7	-0.500
4	0.48 mg/L		10	1.311	2.7	1.000

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) reproduction data
 File: H:\TOXSTAT\09501463.DAT Transform: NO TRANSFORMATION

Shapiro - Wilk's test for normality

D = 15498.431

W = 0.960

Critical W (P = 0.05) (n = 37) = 0.936

Critical W (P = 0.01) (n = 37) = 0.914

Data PASS normality test at P=0.01 level. Continue analysis.

0950146(3) reproduction data
 File: H:\TOXSTAT\09501463.DAT Transform: NO TRANSFORMATION

Bartlett's test for homogeneity of variance

Calculated B1 statistic = 6.09

Bartlett's test using average degrees of freedom

Calculated B2 statistic = 6.37

Based on average replicate size of 8.25

Table Chi-square value = 11.34 (alpha = 0.01, df = 3)

Table Chi-square value = 7.81 (alpha = 0.05, df = 3)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

Data PASS B2 homogeneity test at 0.01 level. Continue analysis.

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) reproduction data
File: H:\TOXSTAT\09501463.DAT Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	Control	9	87.000	121.000	108.778
2	0.08 mg/L	8	64.000	138.000	106.375
3	0.19 mg/L	10	51.000	131.000	104.900
4	0.48 mg/L	10	59.000	121.000	89.700

0950146(3) reproduction data
File: H:\TOXSTAT\09501463.DAT Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	Control	116.194	10.779	3.593	9.91
2	0.08 mg/L	750.268	27.391	9.684	25.75
3	0.19 mg/L	604.767	24.592	7.777	23.44
4	0.48 mg/L	430.456	20.747	6.561	23.13

0950146(3) reproduction data
File: H:\TOXSTAT\09501463.DAT Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	3	2163.461	721.154	1.536
Within (Error)	33	15498.431	469.649	
Total	36	17661.892		

Critical F value = 2.92 (0.05,3,30)
Since F < Critical F FAIL TO REJECT Ho: All equal

APPENDIX J - STATISTICAL OUTPUT (CONT'D)

0950146(3) reproduction data
File: H:\TOXSTAT\09501463.DAT

Transform: NO TRANSFORMATION

BONFERRONI t-TEST - TABLE 1 OF 2		Ho:Control<Treatment			
GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	Control	108.778	108.778		
2	0.08 mg/L	106.375	106.375	0.228	
3	0.19 mg/L	104.900	104.900	0.389	
4	0.48 mg/L	89.700	89.700	1.916	

Bonferroni t table value = 2.22 (1 Tailed Value, P=0.05, df=33,3)

0950146(3) reproduction data
File: H:\TOXSTAT\09501463.DAT

Transform: NO TRANSFORMATION

BONFERRONI t-TEST - TABLE 2 OF 2		Ho:Control<Treatment			
GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	Control	9			
2	0.08 mg/L	8	23.387	21.5	2.403
3	0.19 mg/L	10	22.114	20.3	3.878
4	0.48 mg/L	10	22.114	20.3	19.078

* NOELR

No observed effect loading rate

The NOEL = 0.092 mg/L

Since the mean measured concentration for the 0.48 mg/L loading was

0.092 mg/L

FOR 16 MAR 10

The LOELR (lowest observed effect loading rate) is 1.2 mg/L which corresponds to the LOEL (lowest observed effect concentration) of 0.23 mg/L based on reproduction, only one replicate survived and only produced 6 neonates, 62% less than the control average.

FOR 20 APR 10

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS

- PROTOCOL -

Contract Number: EMBSI 009-104112
Test Substance: Kerosene
Study Title: *Daphnia magna* Reproduction Test on Kerosene
EMBSI Study Number: 0950146
EMBSI Test Substance Code: MRD-09-501
Date: June 19, 2009
Room Number: LE 337/343

Proposed Key Dates for Completion:

Experimental Start..... 10-Aug-09
Experimental Termination..... 31-Aug-09
Draft Report Completion 18-Oct-09
Final Report Completion 18-Nov-09

Approved By:


Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 Route 22 East, P.O. Box 971
Annandale, New Jersey 08801-0971

24 Jun 09
Date


Sponsor Representative

22 June 2009
Date

SAFETY FIRST

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
0950146; MRD-09-501

PAGE 2

INTRODUCTION

Objective

This study will be conducted for the Sponsor to assess the effects of the water accommodated fractions (WAFs) of Kerosene (MRD-09-501), on the reproductive output of *Daphnia magna* in a 21 day semi-static (renewal) test.

Sponsor

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

Testing Facility

ExxonMobil Biomedical Sciences, Inc.
Laboratory Operations
1545 Route 22 East, P.O. Box 971
Annandale, New Jersey 08801-0971

Compliance

This test will be conducted in general agreement with OECD guidelines¹ and will be conducted in compliance with OECD² and USEPA³ GLP standards except as outlined on pages 5 and 6.

Justification for Selection of Test System

Daphnia magna has been used in safety evaluation and is a common test species for freshwater toxicity studies.

Justification of Dosing Route

Potential environmental exposure is by the test substance in water.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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MATERIALS and METHODS

Test Substance Identification

<u>EMBSI Code</u>	<u>Sponsor's Identification</u>	<u>CAS Number</u>
MRD-09-501	Kerosene	64742-81-0

CAS Definition: 64742-81-0, Kerosene (petroleum), hydrodesulfurized; A complex combination of hydrocarbons obtained from a petroleum stock by treating with hydrogen to convert organic sulfur to hydrogen sulfide which is then removed. It consists of hydrocarbons having carbon numbers predominantly in the range of C9 through C16 and boiling in the range of approximately 150° C to 290° C (302° F to 554° F).

Storage Conditions: The neat test substance will be stored at room temperature.

Characterization of Test Substance

Pre-test and post-test characterization and stability analysis will include the following determinations: FT-IR and UV-Vis spectra, density, physical-state, miscibility in water, methanol and/or hexane and GC-MS "fingerprint" of the neat test substance. The GC-MS fingerprint is run against an ASTM hydrocarbon standard mixture. The ASTM D2887 standard will be applied for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). For more volatile test mixtures, an ASTM D3710 standard is used for compounds eluting between approximately n-heptane (n-C6) and n-pentadecane (n-C15). Due to the complex nature of the test substance, no attempt will be made to identify specific hydrocarbon components. Instead, an area percent report will be generated for both the pre- and post-test analysis to demonstrate stability of the test substance over the testing period. Documentation of characterization and stability assessment will be maintained at the testing facility and the results appended to the final report.

The methods of synthesis, fabrication, and/or derivation of the test substance will be maintained by the sponsor. The test substance, as received, will be considered the "pure" substance.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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MATERIALS and METHODS (CONT'D)

Analysis of Mixtures

Samples will be taken from each "new" treatment WAF and control solution on Day 0 and at three intervals (approximately weekly) during the study, based upon the renewal schedule devised during the Equilibrium study. At least two replicates from each treatment will be analyzed. For the "old", i.e., used solutions three individual replicates will be selected and sampled prior to performing the renewal for at least four intervals (approximately weekly) during the study. Each sample will be individually analyzed (i.e., not pooled.) On the first sampling day, replicates 1, 2, and 3 will be sampled; on the second, replicates 4, 5 and 6, and so on. Specific sampling times as well as the replicates sampled will be documented in the final report. Additional samples may be taken during the course of the study; any taken will be documented and reported. The samples will be taken with no headspace. Gas chromatography with flame ionization detection (GC-FID) or mass spectroscopy (GC-MS) will be used as the analytical technique coupled with static headspace (HS) or solid phase microextraction (SPME) sample introduction. The analytical analysis will quantitate the dissolved (water soluble) fraction of the test substance that is present in the WAFs. The analysis will be standardized against the neat test substance to ensure that the full range is captured and quantitated. A detailed description of the analytical methods used will be documented in the raw data and included in the final report.

Sample Retention

A non-study specific retention sample of the neat test substance will be taken and retained. No retention samples of the mixtures (WAFs) will be taken.

Vehicle/Dilution Water

Reconstituted water⁴ (the vehicle/dilution water) will be prepared from UV-sterilized, deionized well water and reagent grade chemicals (NaHCO₃, CaSO₄, MgSO₄, and KCl). It will be aerated prior to use. The hardness will be >140 mg/L (as CaCO₃).

Test System

Daphnia magna Straus

Supplier

Cultured in the Environmental Toxicology Laboratory, Annandale, New Jersey. The original daphnid culture was received from Aquatic Biosystems, Fort Collins, Colorado.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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MATERIALS and METHODS (CONT'D)

Husbandry and Acclimation

Eight daphnids are kept in 1-liter glass culture beakers with approximately 800 mL of reconstituted water (study vehicle/dilution water). The culture chamber is maintained at $20 \pm 1^\circ\text{C}$ under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux). Day 0 cultures are started daily (at least five days per week) using eight <24 hour old neonates from culture beakers between 12 and 18 days old, exhibiting $\leq 20\%$ adult mortality. Cultures are transferred to fresh reconstituted water on regular intervals to ensure that <24 hour old neonates are available for studies and to start new cultures.

Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately $3.0 - 4.5 \times 10^5$ cells/mL). They are also fed 1.0 mL of a 5.0 g/L *Spirulina* suspension per 800 mL. The culture is fed every other day or as needed based on observed algal clearing. The algae is supplied by Aquatic Biosystems, Inc., Fort Collins, CO. The *Spirulina* is supplied by Salt Creek, Inc., Salt Lake City, UT.

Number and Sex

Number: 60 Sex: female

Age at Initiation of Exposure

< 24 hours (not first brood progeny); age of parents will be noted in the final report.

Test System Identification

Daphnids will not be individually identified. All test chambers will be labeled to show study number, target concentration, replicate and randomization number.

Selection

Organisms will be randomly assigned to intermediate chambers using a computer generated randomization schedule and then transferred to their respective test chambers. The test chambers will be randomly positioned within the test area. A printout of the randomization schedule will be included in the raw data.

To ensure that quality organisms are used for the study, neonates from parents 12-18 days old (with $\leq 20\%$ adult mortality) will be selected. Neonates will be selected from a pool of organisms larger than that needed for the study. The pool of neonates will have $\leq 10\%$ daily mortality on the experimental start day. The study director or his designee will determine organism suitability.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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MATERIALS and METHODS (CONT'D)

Feed

Test organisms will be fed during renewals to achieve a concentration of a 3.0 to 4.5×10^5 cells/mL of *Pseudokirchneriella subcapitata* in the test chambers. They will also be fed during renewals with 0.1 to 0.2 mL of 5.0 g/L *Spirulina* suspension. The feeding ration used is consistent with the recommendations of the OECD 211 guideline.

Contaminants

There are no known contaminants in the feed used for the study, in culturing the organisms or the vehicle/dilution water believed to be at levels high enough to interfere with this study. The algae and *Spirulina* are not analyzed. They are believed to contain no contaminants at levels high enough to affect the daphnids used for studies. The algae suspension is prepared from the vehicle/dilution water. The vehicle/dilution water is prepared from UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless steel pipes. There are no known contaminants in the water believed to be present at levels that may interfere with this study. Contaminant analysis of the water is performed by Accutest Laboratories, Inc. and is not performed in a GLP compliant manner. This is not believed to have an adverse affect on the study results. The laboratory is accredited by the National Environmental Laboratory Accreditation Conference (NELAC) and has been audited by ExxonMobil Biomedical Sciences using the Quality Practices and Guidelines (QP & G v. 5.1). The analyses are performed using standard US EPA methods.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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EXPERIMENTAL PROCEDURE

Equilibrium Test

An equilibrium study will be performed prior to testing to determine the most appropriate mixing duration and to delineate the analytical method, which will be used for the definitive test. Specific analytical procedures, as stated in the analysis of mixtures section, will be used to detect and quantitate the soluble components of the substance. Individual WAFs at 0.1 mg/L, 1 mg/L and 10 mg/L loading rates will be prepared and each WAF will be sampled after 24, 48 and 72 hours of mixing. These loadings were chosen based on a previous GLP daphnid chronic study, run on a "mineral spirits" sample, which appears to have similar properties to kerosene. A sufficient number of samples will be taken after 24, 48, and 72 hours in order to test stability for the renewal as well as equilibrium. The WAFs used in order to measure stability and equilibrium will be documented in the raw data and included in the report. The vortex will be set at $\leq 10\%$ of the static liquid depth. All mixing vessels will be closed using foil covered stoppers during mixing, and minimal headspace will be established given the constraints of the mixing vessel. The equilibrium phase will not extend beyond 72 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. However, if after 72 hours the concentrations are still rising, the mixing time may be extended. Also, if soluble hydrocarbons are not found at the 10 mg/L level, the test will be restarted with WAFs at higher levels. *Daphnia* neonates may be exposed to the WAFs in order to set the loading rates for the range finder. This phase of the study will not be subject to GLP standards.

Range Finding Test

D. magna neonates will be exposed to the WAFs of three to five loading rates plus a control under static renewal conditions (time between renewals will be determined as part of the Equilibrium Test) for long enough to include a reproductive endpoint (generally 8 to 12 days), the loading rates chosen will also aim to capture the 48 hour EC_{50} with analytical measurements as determined during the Equilibrium Test. The WAFs will be prepared by adding the appropriate amount of test substance to vehicle/dilution water in glass vessels. The vessels will be closed using foil covered rubber/neoprene stoppers and will mix on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by equilibrium testing (± 1 hour). The vortex will be set at $\leq 10\%$ of the static liquid depth and minimal headspace will be established given the constraints of the mixing vessel. The treatments will be allowed to settle and equilibrate to test temperature for 1 hour (± 15 mins.) after mixing. At least five replicates at each loading rate will be exposed. The test chambers will be completely filled with the appropriate solution such that zero or minimal headspace exists in the test chambers. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

EXPERIMENTAL PROCEDURE (CONT'D)

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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Definitive Test Design

GROUP	LOADING RATE (µg or mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	10 (1 per replicate)
2	TBD	10
3	TBD	10
4	TBD	10
5	TBD	10
6	TBD	10

TBD = To Be Determined

Preparation and Administration of Test Substance

Individual WAFs will be prepared for each loading rate by adding the appropriate amount of the test substance to the vehicle/dilution water in glass aspirator bottles. The vessels will be sealed with foil covered stoppers. The solutions will be mixed with Teflon® coated stirbars on magnetic stirplates. The vortex will be set at ≤10% of the static liquid depth. The solutions will mix for the appropriate time as determined by equilibrium testing (±1 hour) at room temperature (22°±2°C). At the end of mixing, the solutions will be allowed to settle for 1 hour (±15 minutes). At the end of the settling period the solutions will be removed from the mixing vessels through the outlet at the bottom of the vessels and placed into ten replicate chambers. New WAF solutions will be prepared daily for the renewals.

Test chambers will be completely filled with the appropriate solution such that zero or minimal headspace exists in the test chambers. Treatments will be prepared and renewed based upon the results of the test solution stability established as part of the Equilibrium Test. Renewals will be performed by transferring each parent daphnid, via glass pipette, to fresh solution. The volume of medium transferred will be minimized. If a 48 hour renewal is chosen, then at the end of the study, the final renewal will be performed on Day 20 and the daphnids will only be exposed to those solutions for 24 hours.

Test Chamber and Volume of Solution

The test chambers will be clear glass containers sealed with screw type lids to minimize contamination, evaporation and/or volatilization and will contain no headspace. The volume of solution and test chamber details will be noted in the raw data and reported.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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EXPERIMENTAL PROCEDURE (CONT'D)

Exposure Duration

21 days

Continuous Measurements

Range of acceptable test water temperatures: $20^{\circ} \pm 1^{\circ}\text{C}$.

Diurnal light: 16 hours light, 8 hours dark - light intensity will be documented and reported.

An environmental condition study will be activated on the laboratory computer system (Watchdog V5 monitoring system) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

Experimental Evaluation

Observations for immobilization will be performed and recorded at approximately 24-hour intervals after the beginning of the test. Additional observations may be performed. Immobilization is the lack of swimming ability within 15 seconds after gentle agitation of the test container. Any abnormal behavior or appearance will also be recorded. The adults will be transferred to fresh solution based upon the results of the solution stability established as part of the Equilibrium Test. After the appearance of the first brood, neonate presence will be noted daily during observations, they will be counted at the time of renewal. The presence of aborted eggs or immobilized offspring will also be recorded. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test is assessed.

Observations of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) will be recorded daily at the time of organism observations.

Adult organisms will be measured (body length excluding the anal spine) at termination in order to determine if growth effects occurred. Organisms will be discarded at termination. The monitoring of environmental conditions will be discontinued after completion of the study.

Discrete Measurements

Temperature, dissolved oxygen, hardness and pH will be measured at least twice per week during the test in each "new" and "old" treatment and control. The pH should be within the range of 6-9 and should not vary by more than 1.5 units during the test. Dissolved oxygen levels should be above 3mg/L during the test.

EXPERIMENTAL PROCEDURE (CONT'D)

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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Test Acceptability

The mortality of the control parent animals (female *Daphnia*) should not exceed 20% at the end of the test. The mean number of live offspring produced per parent animal in the control group surviving at the end of the test should be ≥ 60 .

Calculations

Effects will be based on nominal loading rates and measured concentrations. The following study endpoints will be calculated: Effect Loading/Effect Concentration 20 (EL₂₀/EC₂₀), the Effect Loading/Effect Concentration 50 (EL₅₀/EC₅₀), the Lowest Observed Effect Loading Rate/Lowest Observed Effect Concentration (LOELR/LOEC) and No Observed Effect Loading Rate/No Observed Effect Concentration (NOELR/NOEC) based on reproductive output. These endpoints will also be calculated for adult growth if possible.

Examples of the methods used to perform the calculations: analysis of variance (ANOVA) procedures such as Dunnett's⁵ or Wilcoxon Rank Sum⁶ with Bonferroni Adjustment⁷ using TOXSTAT software⁸ may be used to determine the LOELR/LOEC and NOELR/NOEC, the Benchmark Dose (BMD) method⁹ may be used to determine the EL₀₁/EC₀₁ values. If in any of the replicates, the parent animal dies during the test or turns out to be male, then the replicate is excluded from the analysis. The analysis will then be based on a reduced number of replicates. Any statistical procedures employed in analyzing the data will be documented in the final report.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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REPORT

After termination of the study, a final report which includes (but is not limited to) the following information will be submitted:

Test Substance:

- physical nature and relevant physiochemical properties;
- chemical identification data.

Test *Daphnia*:

- scientific name, strain (if applicable), age, supplier, any pretreatment, breeding method (including source, kind and amount of food, feeding frequency, culture conditions).

Test Conditions:

- test procedure used (e.g. semi-static or flow through, volume, loading in number of *Daphnia* per liter);
- photoperiod and light intensity;
- test design (e.g. number of replicates, number of parents per replicate);
- details of culture medium used;
- vehicle/dilution water source and chemical characteristics (pH, temp. dissolved oxygen, TOC, hardness, alkalinity, latest contaminant analysis results);
- method of preparation of the test solutions, frequency of renewals;
- detailed information on feeding, including amount (in mg C/*Daphnia*/day) and schedule, type of food and specific name (species).
- loading rates/concentrations used and any information available on the stability of the concentration of the test substance in solution;
- description of test equipment.

Results:

- results of chemical analysis and methods used, if applicable;
- water quality measurements of the test solutions (pH, temp. dissolved oxygen);
- full record of living offspring by each parent animal (even if parent animal dies during the test);
- survival of parent animals and length, time to production of first brood;
- individual daily observations, including daily and cumulative immobilization, survival and behavior;
- the coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive at the end of the test);
- LOELR/LOEC, NOELR/NOEC, EL_{xx}/EC_{xx} values (reproduction and growth, if possible) with confidence limits, if possible;
- statistical procedures followed and statistical software output;
- graph of the loading rate/concentration reproduction-response curve at the end of the test.

REPORT (CONT'D)

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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Study Conduct:

- compliance statement;
- quality assurance statement;
- protocol with amendments appended to the report;
- evidence that the quality criteria have been fulfilled;
- incidents in the course of the test which may have influenced the results.
- deviations from experimental design

RECORDS

All appropriate materials, methods and experimental measurements required in this protocol will be recorded and documented in the raw data. Any changes, additions or revisions of this protocol must be approved by the Study Director and the Sponsor Representative. These changes will be documented in writing, including the date the justification for the change and the signatures of the Study Director and Sponsor Representative.

The protocol, final report, raw data or computer generated listings of raw data, supporting documentation, and a non-study specific sample of the test substance will be maintained in the Archives of the testing facility for 10 years, after which time the records will be offered to the sponsor prior to disposal.

QUALITY ASSURANCE

The Quality Assurance Unit of ExxonMobil Biomedical Sciences, Inc. will audit the protocol, conduct study based phase inspection(s), and audit the draft final report (before sponsor review) to assure that they are in conformance with company SOPs, the appropriate guidelines, and Good Laboratory Practice regulations.

GUIDELINE EXCEPTIONS

Due to the complex nature of the test substance the following exceptions to the guideline will apply for this study:

The concentration of the test substance in solutions will not be determined prior to use. Due to the limited solubility of the test substance, it may not be possible for analytical analysis to demonstrate that the initial concentration of the test substance will be maintained at 80% throughout the test.

It is deemed more appropriate to prepare individual treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
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REFERENCES

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APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Daphnia magna Reproduction Test on Kerosene:
0950146; MRD-09-501

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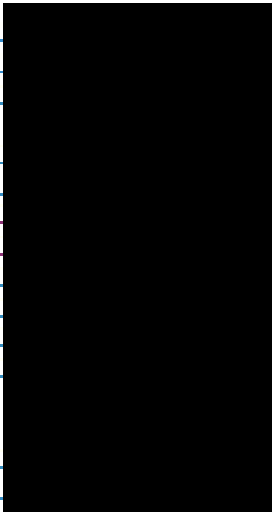
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APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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Study Number: 0950146

Revision Number: 1

Date: 31-Aug-09

Pg. 1 / Proposed Key Dates

Previous Statement:

Experimental Start	10-Aug-09
Experimental Completion.....	31-Aug-09
Draft Report Completion.....	18-Oct-09
Final Report Completion	18-Nov-09

Revised Statement:

Experimental Start	08-Sep-09
Experimental Completion.....	29-Sep-09
Draft Report Completion.....	12-Nov-09
Final Report Completion	12-Dec-09

Pg. 4 / Analysis of Mixtures

Previous Statement:

Gas chromatography with flame ionization detection (GC-FID) or mass spectroscopy (GC-MS) will be used as the analytical technique coupled with static headspace (HS) or solid phase microextraction (SPME) sample introduction.

Revised Statement:

Gas chromatography with flame ionization detection (GC-FID) will be used as the analytical technique coupled with static headspace (HS).

Justification:

Equilibrium study determined the use of Gas chromatography with flame ionization detection (GC-FID) coupled with static headspace (HS) was an adequate analytical technique for the analysis of water samples in this test.

Pg. 7 / Equilibrium Test

Previous Statement:

An equilibrium study will be performed prior to testing to determine the most appropriate mixing duration and to delineate the analytical method, which will be used for the definitive test. Specific analytical procedures, as stated in the analysis of mixtures section, will be used to detect and quantitate the soluble components of the substance. Individual WAFs at 0.1 mg/L, 1 mg/L and 10 mg/L loading rates will be prepared and each WAF will be sampled after 24, 48 and 72 hours of mixing. These loadings were chosen based on a previous GLP daphnid chronic study, run on a "mineral spirits" sample, which appears to have similar properties to kerosene. A sufficient number of samples will be taken after 24, 48, and 72 hours in order to test stability for the renewal as well as equilibrium.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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Study Number: 0950146

Revision Number: 1

Date: 31-Aug-09

The WAFs used in order to measure stability and equilibrium will be documented in the raw data and included in the report. The vortex will be set at $\leq 10\%$ of the static liquid depth. All mixing vessels will be closed using foil covered stoppers during mixing, and minimal headspace will be established given the constraints of the mixing vessel. The equilibrium phase will not extend beyond 72 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. However, if after 72 hours the concentrations are still rising, the mixing time may be extended. Also, if soluble hydrocarbons are not found at the 10 mg/L level, the test will be restarted with WAFs at higher levels. Daphnia neonates may be exposed to the WAFs in order to set the loading rates for the range finder. This phase of the study will not be subject to GLP standards.

Revised Statement:

An equilibrium study was performed to determine the most appropriate mixing duration and to delineate the analytical method, which will be used for the definitive test. Specific analytical procedures, as stated in the analysis of mixtures section, were used to detect and quantitate the soluble components of the substance. Individual WAFs at 0.1 mg/L, 1 mg/L and 10 mg/L loading rates were prepared and each WAF were sampled after 24, 48 and 72 hours of mixing. These loadings were chosen based on a previous GLP daphnid chronic study, run on a "mineral spirits" sample, which appears to have similar properties to kerosene. A sufficient number of samples were taken after 24, 48, and 72 hours in order to test stability for the renewal as well as equilibrium. All three loading levels were utilized to determine stability and equilibrium. The vortex was set at $\leq 10\%$ of the static liquid depth. All mixing vessels were closed using foil covered stoppers during mixing, and minimal headspace was established given the constraints of the mixing vessel. The equilibrium phase did not extend beyond 72 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test.

It was determined through the analysis of the concentration verification samples that a 24 hour mixture will suffice for the test. An increase in concentration after 48 hours of mixing was only seen at the 10 mg/L level and as the test concentrations will not exceed 5.0 mg/L, a 24 hour mix will be adequate.

Stability samples were analyzed with and without the appropriate algae concentrations. Through these analyses, it was determined that a 48 hour renewal will be acceptable for the test as the concentration did not experience a significant drop between 24 and 48 hour stability sampling. A significant drop was seen between the Day 0 and 24 hour stability concentrations.

This phase of the study was not subject to GLP standards.

Justification:

Completion of Equilibrium Study as per protocol.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

Page 3 of 6

Study Number: 0950146

Revision Number: 1

Date: 31-Aug-09

Pg. 7 / Range Finding Test

Previous Statement:

D. magna neonates will be exposed to the WAFs of three to five loading rates plus a control under static renewal conditions (time between renewals will be determined as part of the Equilibrium Test) for long enough to include a reproductive endpoint (generally 8 to 12 days), the loading rates chosen will also aim to capture the 48 hour EC₅₀ with analytical measurements as determined during the Equilibrium Test. The WAFs will be prepared by adding the appropriate amount of test substance to vehicle/dilution water in glass vessels. The vessels will be closed using foil covered rubber/neoprene stoppers and will mix on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by equilibrium testing (± 1 hour). The vortex will be set at $\leq 10\%$ of the static liquid depth and minimal headspace will be established given the constraints of the mixing vessel. The treatments will be allowed to settle and equilibrate to test temperature for 1 hour (± 15 mins.) after mixing. At least five replicates at each loading rate will be exposed. The test chambers will be completely filled with the appropriate solution such that zero or minimal headspace exists in the test chambers. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

Revised Statement:

D. magna neonates were exposed to WAFs of six loading rates plus a control under 48 hour static renewal conditions for eleven days in order to reach a reproductive endpoint. The loading rates chosen captured an EC₅₀ of 1.18 mg/L and an EL₅₀ of 8.7 mg/L were calculated.

The WAFs were prepared by adding the appropriate amount of test substance to vehicle/dilution water in glass vessels. The vessels were closed using foil covered rubber stoppers and were mixed on magnetic stirplates with Teflon® coated stirbars for the 24 hours (± 1 hour). The vortex was set at $\leq 10\%$ of the static liquid depth and minimal headspace was established given the constraints of the mixing vessel. The treatments were allowed to settle and equilibrate to test temperature for 1 hour (± 15 mins.) after mixing. Five replicates at each loading rate were exposed. The test chambers were completely filled with the appropriate solution such that zero headspace existed in the test chambers. This phase of the study was not be subject to GLP standards.

Justification:

Completion of Range Finding Study as per protocol.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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Study Number: 0950146

Revision Number: 1

Date: 31-Aug-09

Pg. 8 / Definitive Study Design

Previous Statement:

GROUP	LOADING RATE (μg or mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	10 (1 per replicate)
2	TBD	10
3	TBD	10
4	TBD	10
5	TBD	10
6	TBD	10

TBD = To Be Determined

Revised Statement:

GROUP	LOADING RATE (μg or mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	10 (1 per replicate)
2	3.0	10
3	1.2	10
4	0.48	10
5	0.19	10
6	0.08	10

Justification:

Completion of Range Finding Study as per protocol.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

Page 5 of 6

Study Number: 0950146

Revision Number: 1

Date: 31-Aug-09

Pg. 8 / Preparation and Administration of Test Substance

Previous Statement:

The solutions will mix for the appropriate time as determined by equilibrium testing (± 1 hour) at room temperature ($22^{\circ}\pm 2^{\circ}\text{C}$).

Revised Statement:

The solutions will mix for 24 hours (± 1 hour) at room temperature ($22^{\circ}\pm 2^{\circ}\text{C}$).

Justification: Results of Equilibrium Study

Pg. 8 / Preparation and Administration of Test Substance

Previous Statement:

Treatments will be prepared and renewed based upon the results of the test solution stability established as part of the Equilibrium Test. Renewals will be performed by transferring each parent daphnid, via glass pipette, to fresh solution. The volume of medium transferred will be minimized. If a 48 hour renewal is chosen, then at the end of the study, the final renewal will be performed on Day 20 and the daphnids will only be exposed to those solutions for 24 hours.

Revised Statement:

Treatments will be prepared 24 hours prior to each 48 hour renewal. Renewals will be performed by transferring each parent daphnid, via glass pipette, to fresh solution. The volume of medium transferred will be minimized. At the end of the study, the final renewal will be performed on Day 20 and the daphnids will only be exposed to those solutions for 24 hours.

Justification: Results of Equilibrium Study

Pg. 9 / Experimental Evaluation

Previous Statement:

The adults will be transferred to fresh solution based upon the results of the solution stability established as part of the Equilibrium Test.

Revised Statement:

The adults will be transferred to fresh solutions every 48 hours.

Justification: Results of Equilibrium Study

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

Page 6 of 6

Revision Number: 1

Date: 31-Aug-09

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04 Sep 09
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APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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Study Number: 0950146

Revision Number: 2

Date: 05-Jan-10

Pg. 1 / Proposed Key Dates

Previous Statement:

Experimental Start	08-Sep-09
Experimental Completion.....	29-Sep-09
Draft Report Completion	12-Nov-09
Final Report Completion	12-Dec-09

Revised Statement:

Experimental Starting Date (OECD)	10-Aug-09
Current Experimental Start (EPA).....	06-Jan-10
Experimental Completion.....	27-Jan-10
Draft Report Completion	15-Mar-10
Final Report Completion	15-Apr-10

Justification:

Dates revised to reflect the initiation of trial 3 and also to designate OECD and EPA start dates.

Pg. 5 / Husbandry and Acclimation

Previous Statement:

Day 0 cultures are started daily (at least five days per week) using eight <24 hour old neonates from culture beakers between 12 and 18 days old, exhibiting ≤20% adult mortality.

They are also fed 1.0 mL of a 5.0 g/L *Spirulina* suspension per 800 mL. The culture is fed every other day or as needed based on observed algal clearing. The algae is supplied by Aquatic Biosystems, Inc., Fort Collins, CO. The *Spirulina* is supplied by Salt Creek, Inc., Salt Lake City, UT.

Revised Statement:

Day 0 cultures are started daily (at least five days per week) using eight <24 hour old neonates from culture beakers between 12 and 21 days old, exhibiting ≤20% adult mortality.

They are also fed 1.5 mL of a YTC Daphnid feed mixture per 800 mL. The culture is fed every other day or as needed based on observed algal clearing. The algae and YTC Daphnid feed mixtures are supplied by Aquatic Biosystems, Inc., Fort Collins, CO.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

Page 2 of 4

Study Number: 0950146

Revision Number: 2

Date: 05-Jan-10

Justification:

Cultured organisms now maintained to 21 days.

Spirulina replaced as secondary feed with YTC Daphnid feed mixture due to inconsistency in *Spirulina* rotifer content.

Pg. 5 / Selection

Previous Statement:

To ensure that quality organisms are used for the study, neonates from parents 12-18 days old (with $\leq 20\%$ adult mortality) will be selected.

Revised Statement:

To ensure that quality organisms are used for the study, neonates from parents 12-21 days old (with $\leq 20\%$ adult mortality) will be selected.

Justification:

Cultured organisms now maintained to 21 days.

Pg. 6 / Feed

Previous Statement:

Test organisms will be fed during renewals to achieve a concentration of a 3.0 to 4.5×10^5 cells/mL of *Pseudokirchneriella subcapitata* in the test chambers. They will also be fed during renewals with 0.1 to 0.2 mL of 5.0 g/L *Spirulina* suspension. The feeding ration used is consistent with the recommendations of the OECD 211 guideline.

Revised Statement:

Test organisms will be fed during renewals to achieve a concentration of a 3.0 to 4.5×10^5 cells/mL of *Pseudokirchneriella subcapitata* in the test chambers. They will also be fed during renewals with 0.2 to 0.3 mL of YTC Daphnid feed mixture. The feeding ration used is consistent with the recommendations of the OECD 211 guideline.

Justification:

Spirulina replaced as secondary feed with YTC Daphnid feed mixture

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

Page 3 of 4

Study Number: 0950146

Revision Number: ① 2

Date: 05-Jan-10

Pg. 6 / Contaminants

Previous Statement:

There are no known contaminants in the feed used for the study, in culturing the organisms or the vehicle/dilution water believed to be at levels high enough to interfere with this study. The algae and *Spirulina* are not analyzed.

Revised Statement:

There are no known contaminants in the feed used for the study, in culturing the organisms or the vehicle/dilution water believed to be at levels high enough to interfere with this study. The algae are not analyzed. The YTC Daphnid feed mixture is analyzed for total solids as well as pesticides and metals by the vendor (Aquatic Biosystems) prior to shipment.

Justification:

Spirulina replaced as secondary feed with YTC Daphnid feed mixture.

OK 03 May 12

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

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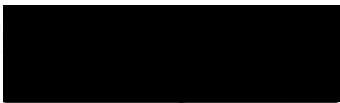
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06/20/2010
Date



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06/20/10
Date

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

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Study Numbers: 0950146

Revision Number: 3

Date: 04Apr12

Page 1 /

Previous Statement: Sponsor Representative – [REDACTED]

Revised Statement: Sponsor Representative – [REDACTED]

Justification: [REDACTED] has retired from American Petroleum Institute and has been replaced with [REDACTED]

Page 10 / CALCULATIONS:

Previous Statement: These endpoints will also be calculated for adult growth if possible.

Revised Statement: These endpoints will also be calculated for adult growth and survival if possible.

Additional Statement: A maximum acceptable toxicant concentration (MATC) will also be calculated.

Justification: Additional statistical endpoint calculations per Sponsor request.

Page 11 / REPORT:

Previous Statement: *vehicle/dilution water source and chemical characteristics (pH, temp. dissolved oxygen, TOC, hardness, alkalinity, latest contaminant analysis results);

Revised Statement: *dilution water source and chemical characteristics (pH, temp. dissolved oxygen, TOC, hardness, alkalinity);

Justification: Contaminant analysis results are maintained at the testing facility.

Page 14 / PERSONNEL:

Previous Statement: Environmental Toxicology and Fate Coordinator [REDACTED]

Revised Statement: Environmental Toxicology and Fate Coordinator – [REDACTED]

Justification: [REDACTED] has been replaced with [REDACTED] as Lab Coordinator effective January 1, 2011.

APPENDIX K – PROTOCOL and PROTOCOL REVISIONS (CONT'D)

PROTOCOL CHANGE RECORD

Page 2 of 2

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Study Numbers: 0950146

Revision Number: 3

Date: 04Apr12

Previous Statement: Environmental Sciences, Section Head - [REDACTED]

Revised Statement: Environmental Sciences, Section Head - [REDACTED]

Justification: [REDACTED] has been replaced with [REDACTED] as Section Head effective July 1, 2011

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[REDACTED]

Study Director

10Apr12

Date