BIOMEDICAL SCIENCES, INC.

EMBSI 2010-104821

Daphnia magna, Reproduction Test on Water Accommodated Fractions of a Light Hydrocracked Gas Oil

Final Report

Study Number: 1057746

TEST SUBSTANCE:

Light Hydrocracked Gas Oil
CAS No. 64741-77-1
(MRD-10-577)

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: March 28, 2012

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APPENDICES:

APPROVAL SIGNATURES

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Date

The final report was accepted by the Sponsor

Russell White, Ph.D.
Sponsor Representative
American Petroleum Institute
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21 March 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 vehicle dilution/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.

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

T.M. Knarr-Cousineau, M.S.
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28Mar12
Date

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21 March 2012
Date

QUALITY ASSURANCE STATEMENT

STUDY NUMBER: 1057746

TEST SUBSTANCE: MRD-10-577

STUDY SPONSOR: API

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 and Management.

Study Phase Inspected	Date(s) of Inspection	Reported to Study Director	Reported to Management
Protocol	15, 16 MAY 2011	20 FEB 2012	24 FEB 2012 27 FEB 2012
Day 21 Observations, Individual Replicate Sampling and Compositing of "old" replicates for Water Quality Measurements	12 JUL 2011	27 JUL 2011	24 FEB 2012 27 FEB 2012
Review of Draft Report & Raw Data: Analytical Chemistry	12, 16 JAN 2012	16 JAN 2012	24 FEB 2012 27 FEB 2012
Review of Draft Report & Raw Data: Environmental Toxicology	07, 09 FEB 2012	09 FEB 2012	24 FEB 2012 27 FEB 2012

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

Robert Pristas — 21 MAR '12

Robert Pristas, M.S.
Quality Assurance Coordinator

Date

PERSONNEL

Study Director:	T. M. Knarr-Cousineau, M.S.
Sponsor Representative:	Russell White, Ph.D.
Section Head, Environmental Sciences: (until July 1, 2011)	T. F. Parkerton, Ph.D.
Section Head, Environmental Sciences: (effective July 1, 2011)	R. A. Barter, Ph.D.
Environmental Toxicology & Fate Laboratory Coordinator:	G. E. Bragin, M.S
Environmental Chemistry Laboratory Coordinator; Principal Investigator for Characterization & Analysis of Test Solutions:	D. J. Letinski, M.S.
Quality Assurance Unit Coordinator:	R. Pristas, M.S.

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 evaluate the effects of the water accommodated fractions (WAFs) of light hydrocracked gas oil (CAS No. 64741-77-1) 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 10% 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), 0.04, 0.08, 0.16, 0.32 and 0.64 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) closed 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, with exception of temperature exceeding protocol specifications on several occasions. 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 gas oil 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 gas oil components measured in the WAFs differed from the parent gas oil standards owing to the differing solubilities of individual gas oil 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 time-weighted average concentrations from the measured hydrocarbon analysis during the exposure were ND (Not Detected; control), 0.013, 0.021, 0.037, 0.072 and 0.13 mg/L. All old test solutions ranged from 74 to 101% 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 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 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 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 were calculated for adult growth and survival where possible, and are presented below.

SUMMARY (CONT'D)

21-day Endpoints

Response Variable	Loading Rate* (mg/L)	Time-Weighted Average Concentration** (mg/L)
Survival	EL20 = not calculable EL50 = not calculable NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	EC20 = not calculable EC50 = not calculable NOEC = 0.13 LOEC = > 0.13 MATC = not calculable
Reproductive Output	EL20 = not calculable EL50 = not calculable NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	EC20 = not calculable EC50 = not calculable NOEC = 0.13 LOEC = > 0.13 MATC = not calculable
Growth (Length)	NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	NOEC = 0.13 LOEC = > 0.13 MATC = not calculable

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.

**Time-weighted average concentration represents the concentration of hydrocarbons that solubilized from the test substance into each WAF at its respective loading rate. See calculations section for explanation of time-weighted average equation.

INTRODUCTION

Objective

This study was conducted for the Sponsor to assess the effects of the water-accommodated fractions (WAFs) of light hydrocracked gas oil (CAS No. 64741-77-1) 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.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

Initial Characterization

12 July 2010

Study Initiation

25 May 2011

WAF Equilibration and Stability Trial Start (Mixing)

13 September 2010

Experimental Start (Definitive Study)

21 June 2011

Experimental Termination (Definitive Study)

12 July 2011

Final Characterization

26 July 2011

Compliance

The study was conducted in compliance with OECD[1] and USEPA[2] Good Laboratory Practice (GLP) standards with the exceptions outlined on page 5. The study was performed in agreement with the OECD[3] and USEPA[4] guidelines with the exceptions listed on page 21.

MATERIALS and METHODS

Test Substance Identification

EMBSI Identification:	MRD-10-577
Sponsor Identification:	Light hydrocracked gas oil Distillates (Petroleum)
CAS Number	64741-77-1
Supplier:	EPL Archives, Sterling. VA
Date Received:	24 June 2010
Expiration Date:	June 2015

CAS Definition: Distillates (petroleum) light hydrocracked. A complex combination of hydrocarbons produced by the distillation of products from a hydrocracking process. It consists predominantly of saturated hydrocarbons having carbon numbers predominantly in the range of C10 through C18, and boiling in the range of approximately 160 to 320 °C (320 to 608 °F)[5].

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

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 water[6] (Batches #228A and #230A) was prepared with UV-sterilized deionized well water and reagent grade chemicals ($NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl) with Ca/Mg and Na/K ratios of 1.2:1 and 12.5:1, respectively. The dilution water was aerated prior to use. UV-sterilized, deionized well water is distributed throughout the testing facility via PVC and stainless steel pipes. See Appendix C for the dilution water analysis.

MATERIALS and METHODS (CONT'D)

Dilution Water (cont'd)

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 Vita-Chem were not analyzed. 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 was performed by Accutest Laboratories, Inc and was not conducted in a GLP compliant manner. 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.

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 Fourtier Transform Infrared Spectrophotometry (FT-IR), density, physical–state, miscibility in water, methanol and /or hexane and a GC-MS "fingerprint" of the neat test substance. The GC-MS fingerprint is run against an ASTM hydrocarbon standard mixture. The ASTM D2887 standard is applied for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). 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 F. 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 control solution on Day 0, 6, 14, and 20. For the corresponding “old” i.e., used solutions, three individual replicate test chambers were sampled prior to performing the renewal. Old solution samples were collected from replicates 1, 2, 3 (Day2); 4, 5, 6 (Day 8); 7, 8, 9 (Day 16) and 1, 2, 10 (Day 21). On Day 21, solution from replicate 10 for the 0.04 mg/L treatment group was inadvertently not sampled. A composite sample from this treatment group was collected and was analyzed in place of replicate 10. 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 gas oil 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 gas oil components measured in the WAFs differed from the parent gas oil standards owing to the differing solubilities of individual gas oil 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 feed was supplied by Aquatic Biosystems, Inc., Fort Collins, CO. The Vita chem was manufactured by Boyd Enterprises, Inc. and supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin.

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 ± 2°C under a 16 hour light 8 hour dark photoperiod (10 - 20 foot/candles, 108 - 215 Lux). Two sets of Day 0 cultures were started at least five days a week. The neonates were less than 24 hours old and came from a day 12-18 culture which experienced less than an estimated 10% neonate mortality and 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 4.5 - 6.0 x 10^5 cells/mL). They were also fed 25µL/L of Vita chem Fresh formula mixed on a magnetic stir plate with the reconstituted water prior to feeding with algae. 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 13-day old parents.

Test System Identification

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

Feed

Daphnids were fed daily during the study. Following WAF settling, two liters of each WAF was removed and 50 µL of Vita-chem fresh water formula was added to the two liters to provide a concentration of 25 µL/L. The Vita-chem feed was added to the treatment WAF, rather than to individual test chambers, to provide a consistent concentration between replicates. Additionally, daphnids were fed at the initiation of the test and during renewals by adding 0.350 mL of a 1.3 x 10^8 cells/mL suspension of *Pseudokirchneriella subcapitata* to provide approximately 3.3 x 10^5 cells/mL, which is equivalent to 0.13 mgC/daphnid/day. Beginning on Day 7, they were fed an additional 0.200 mL 1.3 x 10^8 cells/mL suspension of *Pseudokirchneriella subcapitata* on non-renewal days to provide an additional 1.9 x 10^5 cells/mL (approximate) of algae, which is equivalent to 0.07 mgC/daphnid/day.

EXPERIMENTAL PROCEDURE

WAF Equilibration and Stability Trial

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 24 and 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.

Range Finding Test

A range-finding trial was not performed for this study. However, the loading rates selected for this study were based on results from a 48-hour acute immobilization study (Study number 1057742)[7] with *Daphnia magna*.

Definitive Test Design

GROUP	LOADING RATE* (mg/L)	NUMBER OF ORGANISMS
1	0 (Control)	10 (1 per replicate)
2	0.04	10
3	0.08	10
4	0.16	10
5	0.32	10
6	0.64	10

* Loading rate is defined by the amount of light hydrocracked gas oil 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 20 L of laboratory dilution water in equivalent sized glass aspirator bottles. The control WAF was prepared at 2 L of dilution water in equivalent sized glass aspirator bottle. The test substance was added to the aspirator bottles using stainless steel syringes. 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 mixing vessels were sealed with foil-covered rubber stoppers. The mixtures were stirred using a vortex ≤ 10% (of the static liquid depth) for 24 hours ± 2 hours on magnetic stir plates with Teflon®-coated stir bars. The temperature in the environmental chamber used for WAF mixing and settling ranged from 20.0 to 20.8°C.

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance (cont'd)

At the end of mixing, the solutions were allowed to settle for 1 hour ± 30 minutes before removing the test solutions (aqueous portions of the WAFs) through the outlet at the bottom of the vessel. Two liters of each WAF was removed and 50 µL of Vita chem freshwater formula was added to the 2 L to provide a concentration of 25 µL/L. Ten replicates for each treatment group were prepared by completely filling the test chambers with the 2 L WAF solution (no headspace). Ten replicates of the control were prepared in the same manner using only laboratory dilution water plus feed. New WAF solutions were prepared every other day during the test for test solution renewals.

Renewals were performed by transferring each parent daphnid 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 PTFE-lined screw type caps to minimize contamination, evaporation and/or volatilization. All test chambers were completely filled with test solution (no headspace).

Selection

Organisms were randomly assigned to intermediate chambers using a computer generated randomization scheme using (SAS 9.2)[7]. Following randomization, the organisms were transferred to their respective test chambers. 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 13 days old with ≤20% adult mortality. Neonates were selected from a pool of organisms larger than that needed for the study. The pool of neonates had ≤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. Light intensity was measured twice daily using a LI-COR light meter with photometric sensor.

EXPERIMENTAL PROCEDURE (CONT'D)

Environmental Conditions (cont'd)

The temperature in the environmental chamber ranged from 20.0°C to 20.8°C, continuously monitored by computer in the test area.

Diurnal light: approximately 16 hours light and 8 hours dark. Daylight intensity ranged from approximately 176 - 205 lux during full daylight periods.

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 and at the end of the study. 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 with two exceptions. Only one "old" water quality interval was measured during the first week (Day 0 – 7) of the study. On the final week of the study (Days 15 - 21) only one "new" interval was collected.

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 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 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 from the control group. The Maximum Acceptable Toxicant Loading Rate (MATLR) is the geometric mean of the NOELR and LOELR values. These endpoints were calculated for adult growth and survival where possible.

EXPERIMENTAL PROCEDURE (CONT'D)

Calculations (cont'd)

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 gas oil components measured in the WAFs differs from the parent gas oil, owing to the differing solubilities of individual gas oil hydrocarbons. Endpoints based on concentration were determined using the 21-day time-weighted mean concentration of solubilized hydrocarbons determined at each loading rate. Calculation of time-weighted mean is explained below.

The EL/EC values and confidence intervals for survival and reproductive output could not be calculated. The T-test with Bonferroni adjustment[8], Wilcoxon Rank Sum with Bonferroni adjustment[9] and Fisher's Exact Test[10,11] using TOXSTAT[12] software were used to determine the LOELR/LOEC and NOELR/NOEC values. The statistical output is provided in Appendix H.

The time-weighted mean (OECD Guideline 211) concentration of solubilized hydrocarbons for each loading rate was calculated so that the area under the time-weighted mean equaled the area under the concentration curve. The area under the exponential curve for each renewal period was calculated by:

$$\text{Area} = \frac{\mathit{Conc\ 0} - \mathit{Conc\ 1}}{\mathit{Ln(Conc\ 0)} - \mathit{Ln(Conc\ 1)}} \ \mathit{x\ Days}$$

Where:
Days is the number of days in the renewal period
Conc 0 is the measured concentration of solubilized hydrocarbons at the start of each renewal period
Conc 1 is the measured concentration of solubilized hydrocarbons at the end of each renewal period
Ln(Conc 0) is the natural logarithm of Conc 0
Ln(Conc 1) is the natural logarithm of Conc 1

The areas calculated for each renewal period were summed, and the time-weighted mean *(TW Mean)* equaled the *Total Area* divided by the *Total Days.*

RESULTS AND DISCUSSION

This study 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 WAF loading rates for this study were 0 (control), 0.04, 0.08, 0.16, 0.32, and 0.64 mg/L. The corresponding time-weighted average concentrations from the measured hydrocarbon analysis during the exposure were ND (Not Detected; control), 0.013, 0.021, 0.037, 0.072 and 0.13 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 74 to 101% of the initial measured hydrocarbon concentrations. The analytical results are presented in Table 1.

At WAF stirring initiation and termination, all treatments appeared clear with test substance visible on the surface. Water quality measurements remained consistent throughout the exposure (Table 2). pH measurements were within the 6 to 9 range throughout the study. Dissolved oxygen concentrations remained above 3 mg/L throughout the duration of the study. The test water temperatures ranged from 20.7 to 22.2 °C. A complete listing of water quality measurements are provided in Appendix D.

No observation of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) was noted during the time of organism observations. No immobilization or abnormal appearance was observed in the control group or any treatment group throughout the entire exposure, with the exception of one adult daphnid in the 0.64 mg/L treatment group. This adult daphnid appeared small and/or off-color from day 3 through day 14 and then was considered normal until test termination. Neonate immobilization was observed once in the 0.08 mg/L treatment group.

No aborted eggs were observed in the control group or any treatment group throughout the entire exposure. 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 E. The mean cumulative neonate production per adult per loading rate is presented in Figure 1.

There were no statistically significant differences on adult daphnid growth (length) and neonate production for all the treatment groups when compared to the control. Inhibition of growth (based on length) was insufficient to calculate EL20 or EL50 values.

The NOELR and LOELR values for this study were 0.64 and > 0.64, respectively, based on reproduction and growth. Corresponding NOEC and LOEC values were 0.13 and > 0.13, respectively. MATLR and MATC values could not be calculated, since the NOELR and NOEC values were determined to be the highest treatment group tested. Based on survival and reproductive output, EL/EC (20 and 50) values could not be determined. A complete listing of the statistical evaluations for individual endpoints is presented below.

RESULTS AND DISCUSSION (CONT'D)

21-day Endpoints

Response Variable	Loading Rate* (mg/L)	Time-Weighted Average Concentration** (mg/L)
Survival	EL20 = not calculable EL50 = not calculable NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	EC20 = not calculable EC50 = not calculable NOEC = 0.13 LOEC = > 0.13 MATC = not calculable
Reproductive Output	EL20 = not calculable EL50 = not calculable NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	EC20 = not calculable EC50 = not calculable NOEC = 0.13 LOEC = > 0.13 MATC = not calculable
Growth (Length)	NOELR = 0.64 LOELR = > 0.64 MATLR = not calculable	NOEC = 0.13 LOEC = > 0.13 MATC = not calculable

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.

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

PROTOCOL DEVIATIONS

On several occasions at the time of water quality measurements, test water temperatures from the control and the treatment groups were above the recommended protocol temperature range of 19 – 21 ºC. Measurements varied from 0.1 to 1.2 ºC above the specified range. At no time did the temperature within the test area (environmental chamber) exceed 21 ºC.

The temperature of the new 0.64 mg/L test solution was inadvertently not recorded on Day 6 "new" (27 Jun 2011).

On Day 21, only two individual replicates were analytically sampled for the 0.04 treatment group instead of the protocol specified three individual replicates. A composite sample was collected as a back-up and analyzed.

Neonate presence was inadvertently not recorded on Day 7 for the 0.32 and 0.64 mg/L treatment groups.

The protocol specified 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. During the first week (Day 0 – 7) of the study, measurements were only collected from one "old" interval on Day 2. On the last week of the study (Day 15 - 21), measurements were collected on only one "new" interval (Day 20).

None of the deviations described above are believed to have any 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 substances the following exceptions to the guideline apply for this study:

> Consistent with the OECD document on aquatic toxicity testing of complex substances[13], 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)												Time-Weighted Average Concentration** (mg/L)
	1st renewal			4th renewal			8th renewal			11th renewal			
	Day 0 (new[1])	Day 2 (old[2])	Day 0-2 Retention[3]	Day 6 (new[1])	Day 8 (old[2])	Day 6-8 Retention[3]	Day 14 (new[1])	Day 16 (old[2])	Day 14-16 Retention[3]	Day 20 (new[1])	Day 21 (old[2])	Day 20-21 Retention[3]	
0 (Control)	ND ND	ND ND ND	--	ND ND	ND ND ND	--	ND ND	ND ND ND	--	ND ND	ND ND ND	--	NA
0.04	0.0134 0.0115[4]	0.0104[4] 0.0127[4] 0.00953[4]	87%	0.0145 0.0155	0.0133 0.0116[4] 0.0114[4]	81%	0.0133 0.0141	0.0109[4] 0.0113[4] 0.0106[4]	80%	0.0223 0.0213	0.0163 0.0182 0.0153	76%	0.013
mean	**0.0125**	**0.0109**		**0.0150**	**0.0121**		**0.0137**	**0.0109**		**0.0218**	**0.0166**		
0.08	0.0185 0.0189	0.0168 0.0194 0.0203	101%	0.0243 0.0270	0.0188 0.0228 0.0188	78%	0.0235 0.0228	0.0188 0.0191 0.0184	81%	0.0206 0.0201	0.0174 0.0181 0.0183	88%	0.021
mean	**0.0187**	**0.0188**		**0.0257**	**0.0201**		**0.0232**	**0.0188**		**0.0204**	**0.0179**		
0.16	0.0409 0.0377	0.0305 0.0348 0.0373	87%	0.0413 0.0440	0.0305 0.0288 0.0361	74%	0.0410 0.0416	0.0346 0.0345 0.0335	83%	0.0422 0.0431	0.0321 0.0323 0.0355	78%	0.037
mean	**0.0393**	**0.0342**		**0.0427**	**0.0318**		**0.0413**	**0.0342**		**0.0427**	**0.0333**		
0.32	0.0652 0.0694	0.0657 0.0609 0.0638	94%	0.0709 0.0721	0.0629 0.0624 0.0598	86%	0.0899 0.0907	0.0694 0.0706 0.0718	78%	0.0854 0.0823	0.0761 0.0704 0.0736	87%	0.072
mean	**0.0673**	**0.0635**		**0.0715**	**0.0617**		**0.0903**	**0.0706**		**0.0839**	**0.0734**		
0.64	0.118 0.115	0.113 0.106 0.110	94%	0.150 0.147	0.125 0.126 0.127	84%	0.142 0.147	0.123 0.112 0.121	82%	0.133 0.130	0.120 0.113 0.119	89%	0.13
mean	**0.117**	**0.110**		**0.149**	**0.126**		**0.145**	**0.119**		**0.132**	**0.0117**		

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
** See calculations section for explanation of time-weighted average equation.
[1] Analytical samples (duplicate) from the new treatment and control solutions were analyzed.
[2] Analytical samples (triplicate) from the old treatment and control solutions were analyzed. Old solution samples were collected from replicates 1,2,3 (Day2); 4,5,6 (Day 8); 7,8,9 (Day 16) and 1,2,10 (Day 21), except for the 0.04 mg/L treatment where replicate 10 was inadvertently not collected. A composite sample for the 0.04 treatment group was collected and analyzed in its place.
[3] Percent retention was determined by dividing the concentration of the old solution to the new solution concentration x 100.
[4] Test substance detected but below PQL (Practical Quantitation Limit). Values were included in mean concentration calculations.
ND = Non Detectable.
NA = Not Applicable.
PQL (Practical Quantitation Limit) = 0.013 mg/L (lowest analytical standard).

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)	8.53 - 9.02	6.42 - 8.81	8.14 - 8.33	7.51 - 8.18	162 - 172	160 - 174	20.7 - 21.9	21.2 - 22.2
0.04	8.79 - 9.07	6.89 - 8.44	8.19 - 8.38	7.84 - 8.36	160 - 170	164 - 170	20.8 - 21.8	21.1 - 22.2
0.08	8.46 - 9.07	6.15 - 8.32	8.17 - 8.40	7.79 - 8.32	166 - 168	164 - 168	20.7 - 21.7	21.1 - 22.1
0.16	8.48 - 9.37	6.32 - 8.45	8.32 - 8.47	7.97 - 8.38	160 - 170	162 - 168	20.7 - 21.3	21.1 - 22.2
0.32	8.61 - 9.08	6.81 - 8.37	8.35 - 8.41	7.93 - 8.36	160 - 170	162 - 170	20.7 - 21.3	21.0 - 22.1
0.64	8.62 - 9.47	6.56 - 8.36	8.29 - 8.49	7.99 - 8.38	164 - 168	160 - 170	20.8 - 21.7	21.1 – 22.1

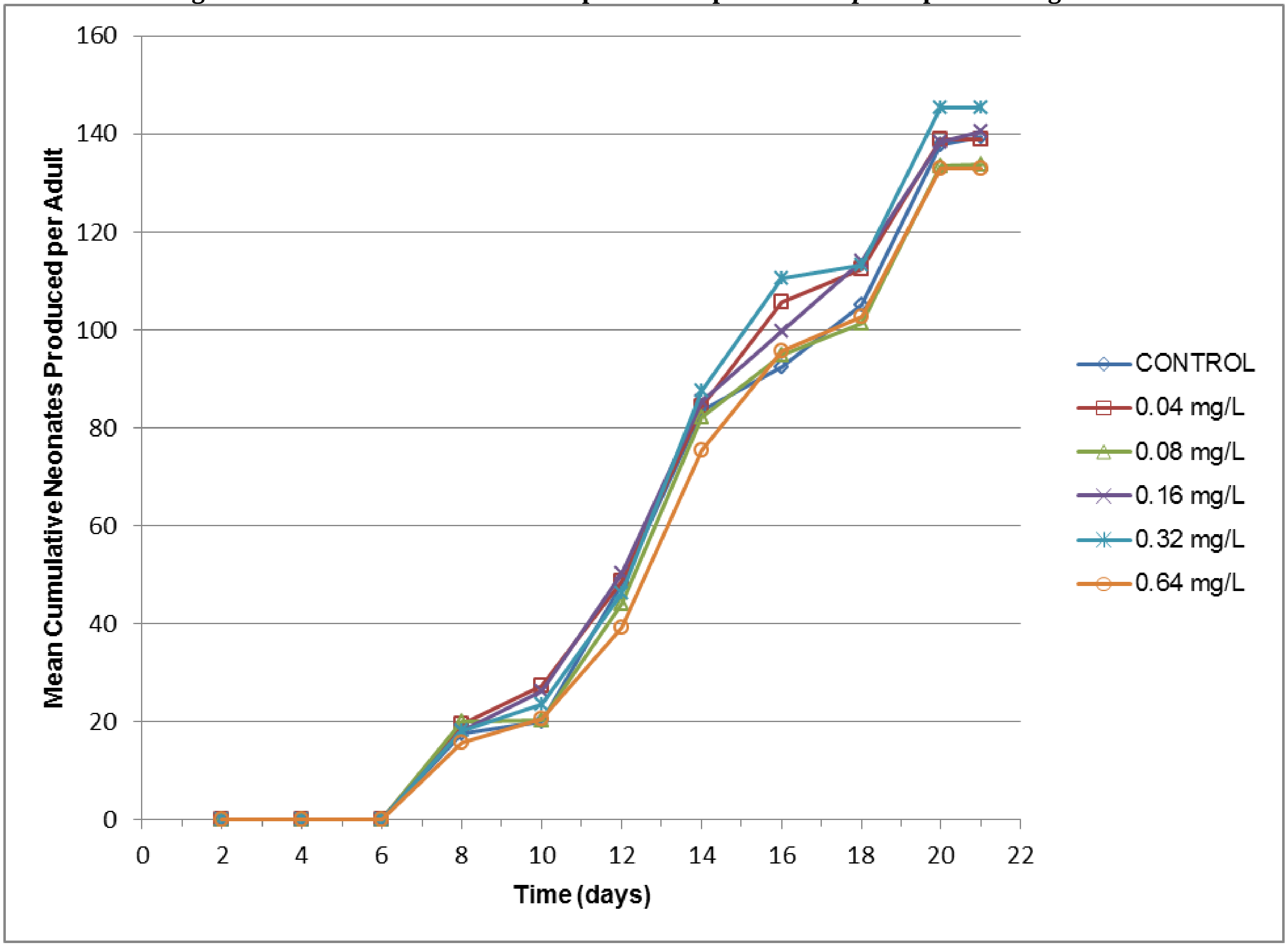
***** Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.

Table 3. Summary of Observations

Loading Rate* (mg/L)	21-day Survival (%)	21-Day Reproduction Mean offspring/female	Mean Adult Length (mm)
Control	100	139 (4.8%[1])	4.9
0.04	100	139	4.9
0.08	100	134	5.0
0.16	100	140	4.9
0.32	100	145	4.9
0.64	100	133	4.9

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1]Coefficient of variation should be ≤25% for the control group.

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



APPENDIX A - ANALYTICAL METHOD

Standards and samples of light hydrocracked gas oil (CAS No. 64741-77-1) were analyzed by static headspace-trap gas chromatography with flame ionization detection (HS-Trap 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 TurboMatrix 40 Trap Headspace Sampler was connected directly to the analytical column. Samples and standards were equilibrated for 45 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 0.15 minutes. The sampler head pressure was 28 psi. The HS trap packing was 1:1 Carbopack C / Tenax. The low trap temperature was 35^{o}C and the desorb temperature was 290^{o}C. The trap hold time was 7 minutes with a 5 minute dry purge and 0.5 minute desorption time. Both the headspace vial shaker and outlet split were on. The FID was 275°C and the oven temperature was held at 50°C for 3 minutes and then ramped up to 300°C at 45°C/minute. The signal attenuation was -5.

Microliter aliquots of separate gas oil 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. Gas oil standards in water were analyzed at concentrations of 12.6, 31.5, 98.5 and 197 ng/mL with a constant 27.0 ng/mL concentration of the 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 gas oil standards, WAF sample vials were crimp sealed and shaken to solubilize the sodium sulfate prior to analysis.

Data were acquired and processed using Perkin Elmer TotalChrom Workstation software (version 6.3.1). Results are presented in Table A1. Standards analyses resulted in a linear response over the standard concentration range. Figure A-1 represents the gas oil standard curve.

APPENDIX A - ANALYTICAL METHOD (CONT'D)

Light hydrocracked gas oil (MRD-10-577) eluted as a complex mixture of hydrocarbons between the approximate retention times of 3.5 and 6.3 minutes. Representative gas oil HS GC-FID chromatograms are presented in Figure A-2. The two upper plots display a low and high concentration gas oil standard. The third plot is a control sample with the fourth and fifth chromatograms from the top representing analysis of low (0.08 mg/L) and high (0.64 mg/L) sample loadings. The total area integrated for the detected hydrocarbons was used for quantification. The o-xylene internal standard eluted at about 3.0 minutes under the analytical conditions utilized. The practical quantitation limit (PQL) was approximately 13 ng/mL (0.013 mg/L) corresponding to the lowest analyzed standard. All reported concentrations for dissolved hydrocarbons are derived from the use of the standard curve and the internal standard.

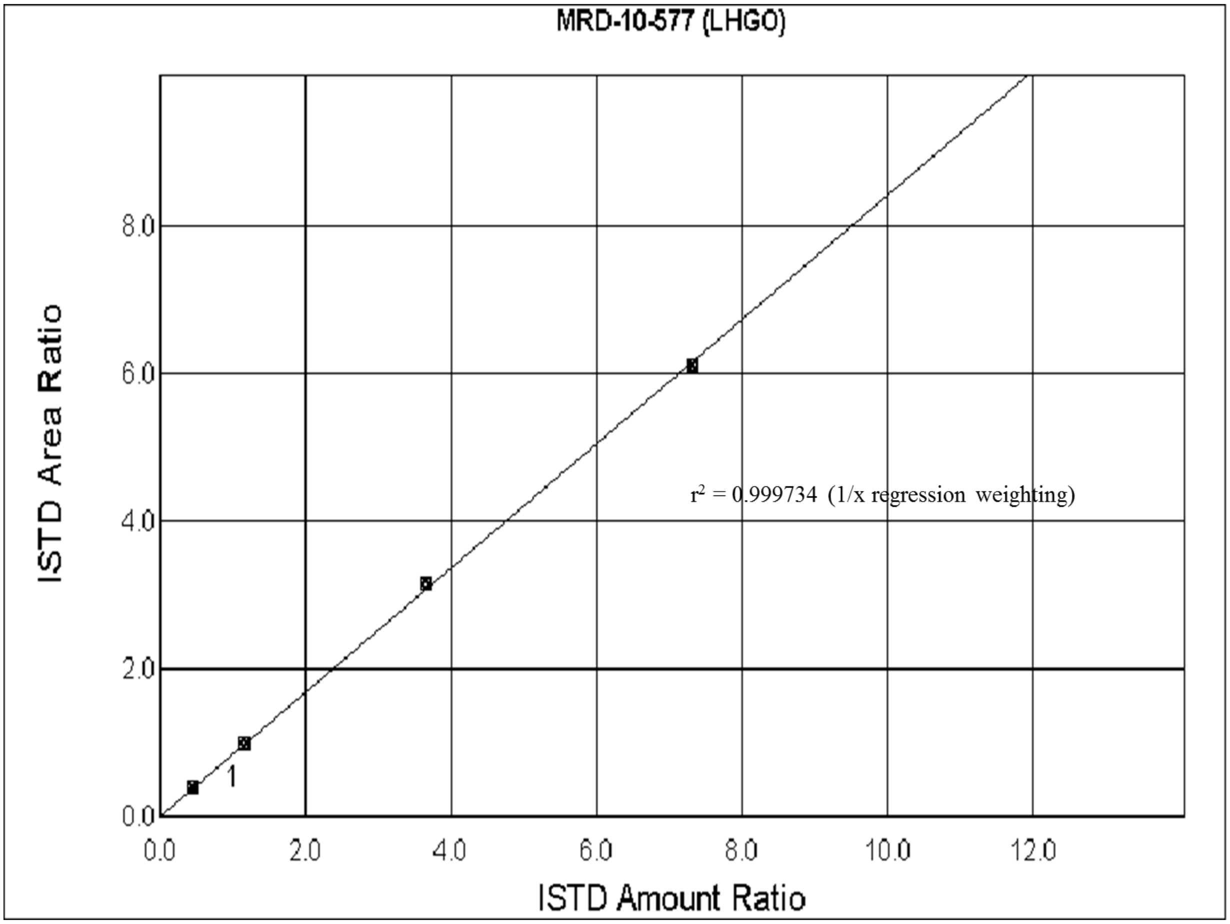
D. J. Letinski, M.S., Environmental Chemistry Laboratory Coordinator; Principal Investigator for Characterization & Analysis of Test Solutions

21 March 2012
Date

APPENDIX A - ANALYTICAL METHOD (CONT'D)

FIGURE A-1

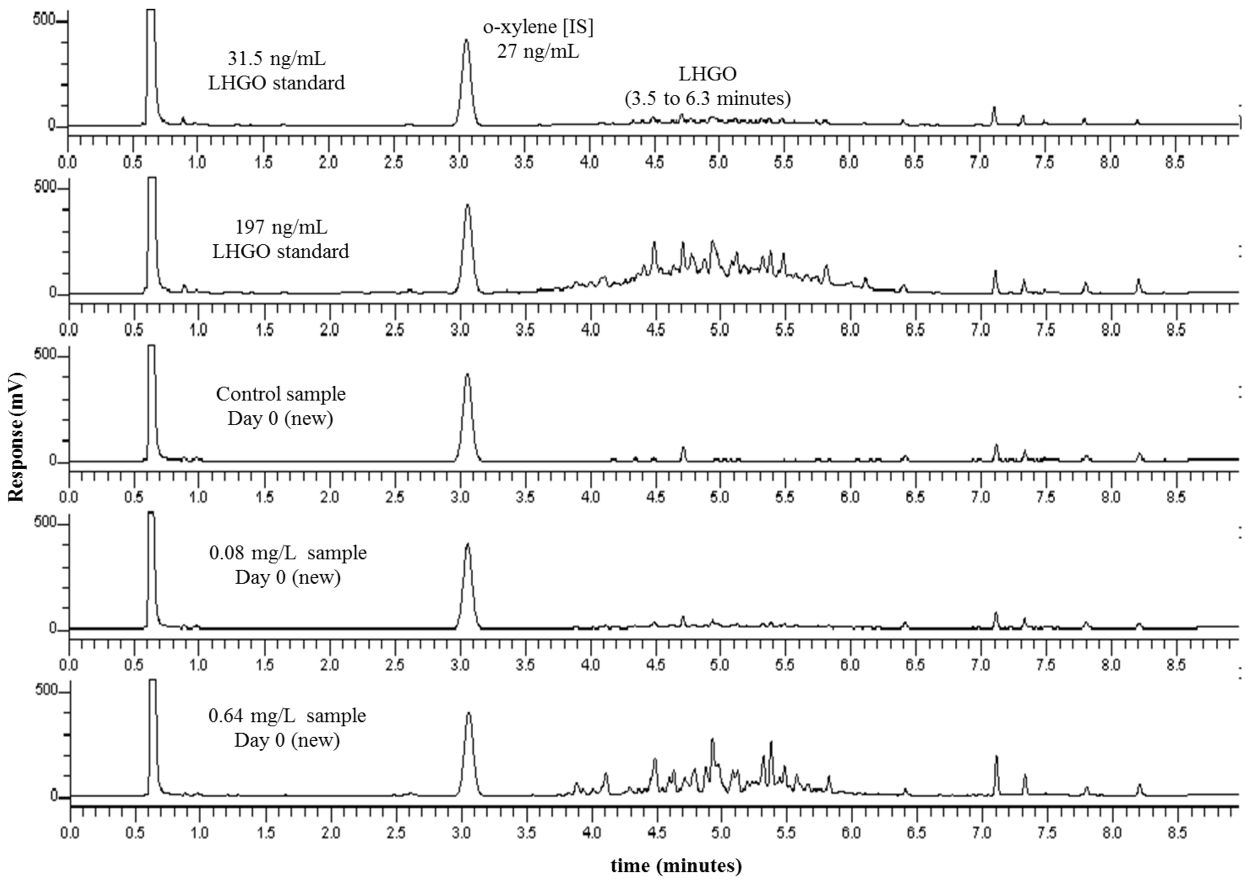
Gas Oil Standard Curve



APPENDIX A - ANALYTICAL METHOD (CONT'D)

FIGURE A-2

Gas Oil Standard and Sample Chromatograms



APPENDIX B - WAF EQUILIBRATION AND STABILITY TRIALS

Introduction

A WAF equilibration trial was performed prior to the range-finding and 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 that would be used in subsequent testing, and to evaluate the stability of the WAF solutions once they were produced. The stability information would be used to establish the renewal interval for a 21-day chronic study, and to determine whether or not a renewal would be needed for the definitive acute toxicity test.

Mixtures of dilution water and test substance were prepared at loading levels of 0.1, 0.5 and 5.0 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 gas oil 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 WAF loadings of 0.1 and 0.5 mg/L, measured concentrations represent about 28 to 33% of the test substance loading. This percentage decreases to 18% at the 5 mg/L loading.

As shown in Figure B1, the analytical results of the WAF Equilibration Testing indicate that in nearly every case, maximum dissolution of the gas oil 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 (mg/L)					
	24 hour mix	% solubility[1]	48 hour mix	% solubility	72 hour mix	% solubility
0.1 mg/L - 1	0.028	28%	0.027	27%	0.022	22%
0.1 mg/L - 2	0.028	28%	0.027	27%	0.023	23%
mean	0.028	28%	0.027	27%	0.023	23%
0.5 mg/L - 1	0.167	33%	0.156	31%	0.123	25%
0.5 mg/L - 2	0.163	33%	0.159	32%	0.125	25%
mean	0.165	33%	0.158	32%	0.124	25%
5 mg/L - 1	0.895	18%	0.923	18%	0.822	16%
5 mg/L - 2	0.894	18%	0.918	18%	0.771	15%
mean	0.895	18%	0.921	18%	0.797	16%

*Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1] Measured solubilized hydrocarbon concentration when compared to the loading rate.

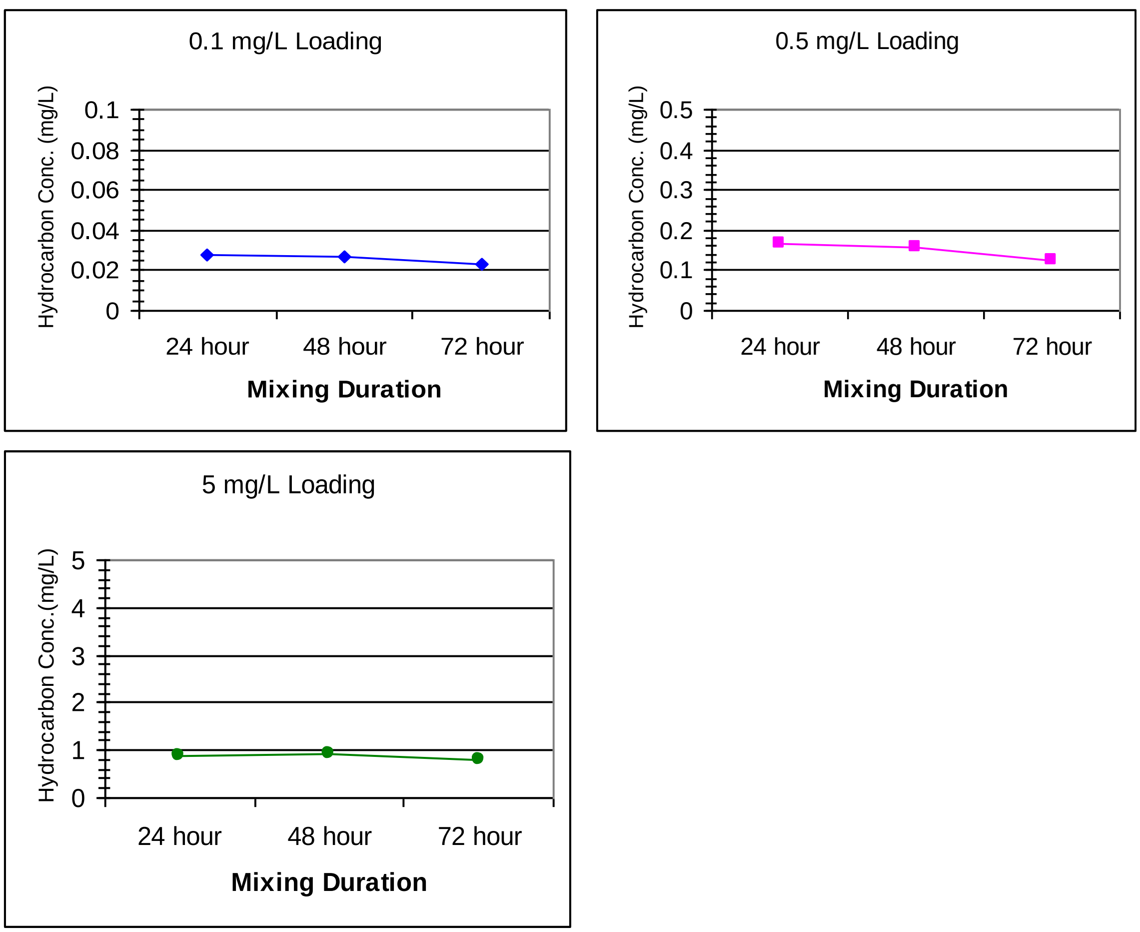


Figure B1. Concentration plots of measured hydrocarbons in WAFs at different mixing times and loading rates.

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 48 hours. For WAF stability related to an acute exposure, 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 acute study.

For WAF stability related to a 21-day chronic exposure, 2 L of the 0.1 and 0.5 mg/L WAF was placed into 2 L volumetric flasks. *Daphnia* chronic test feed (25ul/L Vita chem vitamin solution and 5 mL/L *P. subcapitata*) was added to the volumetric flasks. Following approximately 15 minutes of mixing, samples were taken for 24 hour and 48 hour stability assessments. The samples were placed in screw-top sealed test chambers (no headspace) identical to those anticipated for use in the definitive life cycle study.

All test chambers were set aside under environmental conditions similar to that used for testing. At 24 and again at 48 hours, test chambers were sampled and held under refrigeration pending analysis.

Dedicated samples were employed such that no repeated analysis was made on any sample (i.e., samples were destructively analyzed). The equilibration phase demonstrated good reproducibility between replicate samples; therefore, single samples were used for the stability assessment. The stability assessment results are shown below.

Table B2. WAF Stability Assessment Results

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	0.027	0.022 (82%)	0.025 (93%)	0.024 (89%)	0.023 (83%)
0.5	0.158	0.144 (91%)	0.157 (100%)	0.123 (78%)	0.114 (72%)
5.0	0.921	0.838 (91%)	0.913 (99%)	Not analyzed[3]	

*Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1] 0-hour concentration for stability assessment from 48 hour mix.
[2] Percent retention was determined by dividing the concentration of the initial solution to the new solution concentration x 100.
[3] Stability determinations with feed are applicable at lower concentrations related to chronic testing.

Based on the analytical results of the WAF Stability Testing, the sponsor determined that a renewal was not necessary for the 48-hour daphnid acute testing (Study Number 1057742) and that a 48-hour renewal period will suffice for the chronic testing.

APPENDIX C - DILUTION WATER ANALYSIS

The dilution water was prepared from UV-sterilized, deionized well water that was treated and distributed throughout the testing facility via PVC and stainless-steel pipes. Batches of 500 L of this deionized water were reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater*, 21st edition.

The following water quality data are most representative of the dilution water used during the in-life period of the study. Table C1 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 was monitored annually for priority pollutants, un-ionized ammonia, total suspended solids, and annually for bacterial properties. Results of analyses are maintained at the testing facility.

Table C1. 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 228A	110	164	8.05	21.7	9.09	0.12
Batch 230A	120	182	8.49	20.6	8.92	0.090

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. Pharmacopoceia, EPA 415.1 EPA 9060A, ASTM D2575, Standard Methods for Examination of Water and Waste Water 5301B.

APPENDIX D – WATER QUALITY MEASUREMENTS

Day	Variable	Loading Rate* (mg/L)					
		Control	0.04	0.08	0.16	0.32	0.64
0 (new)	D. O. (mg/L)	9.02	8.79	8.68	8.67	8.73	8.71
	pH	8.22	8.37	8.39	8.38	8.37	8.36
	Hardness (mg/L as CaCO3)	164	164	166	164	164	164
	Temperature (ºC)	20.8	20.8	20.8	20.8	20.8	20.9
2 (old)	D. O. (mg/L)	8.38	8.44	8.32	8.45	8.37	8.36
	pH	8.18	8.36	8.32	8.38	8.36	8.38
	Hardness (mg/L as CaCO3)	160	170	164	162	162	160
	Temperature (ºC)	21.5	21.5	21.7	21.6	21.6	21.5
6 (new)	D. O. (mg/L)	8.77	9.07	9.07	9.37	9.08	9.47
	pH	8.14	8.21	8.17	8.37	8.35	8.29
	Hardness (mg/L as CaCO3)	162	160	166	160	160	164
	Temperature (ºC)	21.1	21.0	20.7	20.7	20.7	--[1]
8 (old)	D. O. (mg/L)	7.17	7.51	8.08	7.66	7.36	7.44
	pH	7.66	8.02	8.11	8.03	8.05	8.03
	Hardness (mg/L as CaCO3)	160	164	164	164	164	170
	Temperature (ºC)	21.2	21.1	21.2	21.1	21.0	21.1
10 (new)	D. O. (mg/L)	8.53	8.80	8.77	8.68	8.81	8.64
	pH	8.33	8.33	8.40	8.47	8.41	8.49
	Hardness (mg/L as CaCO3)	166	166	166	170	170	168
	Temperature (ºC)	21.1	21.0	20.9	21.0	21.0	21.1

***** Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1] Data inadvertently not recorded.

APPENDIX D – WATER QUALITY MEASUREMENTS (CONT'D)

Day	Variable	Loading Rate* (mg/L)					
		Control	0.04	0.08	0.16	0.32	0.64
12 (old)	D. O. (mg/L)	6.42	6.89	6.15	6.32	7.04	6.65
	pH	7.51	7.84	7.79	7.97	8.09	8.13
	Hardness (mg/L as CaCO3)	170	166	168	166	166	168
	Temperature (ºC)	21.5	21.5	21.5	21.5	21.4	21.4
14 (new)	D. O. (mg/L)	8.60	8.82	8.46	8.48	8.61	8.62
	pH	8.18	8.19	8.25	8.32	8.35	8.39
	Hardness (mg/L as CaCO3)	164	164	168	168	166	168
	Temperature (ºC)	20.7	20.8	20.8	20.8	20.7	20.8
16 (old)	D. O. (mg/L)	7.78	7.05	7.61	6.62	6.81	6.56
	pH	8.02	8.01	8.00	8.00	7.93	7.99
	Hardness (mg/L as CaCO3)	174	170	168	168	170	168
	Temperature (ºC)	21.2	21.1	21.1	21.1	21.0	21.1
20 (new)	D. O. (mg/L)	8.69	8.81	8.83	8.73	8.71	8.69
	pH	8.33	8.38	8.31	8.37	8.41	8.45
	Hardness (mg/L as CaCO3)	172	170	168	168	168	168
	Temperature (ºC)	21.9	21.8	21.7	21.3	21.3	21.7
21 (old)	D. O. (mg/L)	8.81	7.78	7.73	7.60	7.45	7.55
	pH	8.02	8.09	8.17	8.12	8.11	8.12
	Hardness (mg/L as CaCO3)	172	168	166	168	166	166
	Temperature (ºC)	22.2	22.2	22.1	22.2	22.1	22.1

***** Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.

APPENDIX E – 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	3	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	16	15	0	23	23	21	20	20	16	20	0	N: 1-10	100
9	0	0	P	0	0	0	0	0	0	0	0	N: 1-10	100
10	0	0	24	0	0	0	0	0	0	0	0	N: 1-10	100
11	P	P	0	P	P	P	P	P	P	P	0	N: 1-10	100
12	27	32	31	27	27	25	29	32	24	26	0	N: 1-10	100
13	0	P	0	P	0	P	0	P	0	P	0	N: 1-10	100
14	33	39	2	38	40	42	39	44	36	41	0	N: 1-10	100
15	0	0	P	0	0	0	0	0	0	0	0	N: 1-10	100
16	0	18	21	0	0	17	0	16	0	16	0	N: 1-10	100
17	P	0	P	P	P	0	P	0	P	0	0	N: 1-10	100
18	22	0	31	15	18	0	18	0	23	0	0	N: 1-10	100
19	P	P	0	P	P	P	0	P	P	P	0	N: 1-10	100
20	42	36	0	33	32	30	39	35	44	37	0	N: 1-10	100
21	0	3	12	0	0	0	0	1	0	0	0	N: 1-10	100
Total Offspring	140	143	124	136	140	135	145	148	143	140	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted.
Appearance codes: N = Observed normal.

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.04 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	P	0	0	0	0	N: 1-10	100
8	21	25	18	23	16	22	10	23	15	22	0	N: 1-10	100
9	0	0	0	0	0	0	P	0	0	0	0	N: 1-10	100
10	27	0	24	0	0	1	24	0	1	2	0	N: 1-10	100
11	0	P	0	P	P	P	P	P	P	P	0	N: 1-10	100
12	0	28	0	24	22	25	41	20	25	28	0	N: 1-10	100
13	P	P	P	0	P	P	0	P	P	0	0	N: 1-10	100
14	43	45	35	36	41	41	0	36	36	42	0	N: 1-10	100
15	0	0	0	0	0	0	P	0	0	0	0	N: 1-10	100
16	50	18	37	0	4	18	28	20	19	21	0	N: 1-10	100
17	0	0	0	P	P	0	P	0	0	0	0	N: 1-10	100
18	0	0	0	16	16	0	34	1	0	0	0	N: 1-10	100
19	P	P	P	P	P	P	0	P	P	P	0	N: 1-10	100
20	20	35	24	27	30	39	2	27	23	37	0	N: 1-10	100
21	0	0	0	0	0	0	0	1	0	0	0	N: 1-10	100
Total Offspring	161	151	138	126	129	146	139	128	119	152	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted.
Appearance codes: N = Observed normal.

APPENDIX E – 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	16	21	25	18	21	18	19	22	20	21	0	N: 1-10	100
9	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
10	1	0	0	0 [2]	0	1	0	0	0	0	0	N: 1-10	100
11	P	P	P	P	P	P	P	P	P	P	0	N: 1-10	100
12	29	23	21	12	32	23	23	24	24	27	0	N: 1-10	100
13	P	0	P	P	P	0	P	P	0	P	0	N: 1-10	100
14	41	37	36	38	40	39	37	41	35	37	0	N: 1-10	100
15	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
16	2	0	18	36	16	0	19	16	0	19	0	N: 1-10	100
17	P	P	0	0	0	P	0	0	P	0	0	N: 1-10	100
18	18	17	0	0	0	19	0	0	12	0	0	N: 1-10	100
19	P	0	P	P	P	0	P	P	0	P	0	N: 1-10	100
20	36	23	29	27	37	38	33	30	29	39	0	N: 1-10	100
21	0	1	0	0	1	0	0	0	0	0	0	N: 1-10	100
Total Offspring	143	122	129	131	147	138	131	133	120	143	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted. [I] = number of immobilized offspring.
Appearance codes: N = Observed normal.

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.16 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	11	0	0	0	0	0	0	0	0	N: 1-10	100
7	P	0	P	0	0	0	0	0	0	0	0	N: 1-10	100
8	13	18	2	19	15	21	22	20	21	20	0	N: 1-10	100
9	P	0	P	0	0	0	0	0	0	0	0	N: 1-10	100
10	25	32	23	0	0	0	0	0	0	0	0	N: 1-10	100
11	0	0	0	P	P	P	P	P	P	P	0	N: 1-10	100
12	35	0	37	26	26	26	25	16	26	25	0	N: 1-10	100
13	0	P	0	0	P	P	0	0	0	P	0	N: 1-10	100
14	4	40	36	37	42	43	39	28	38	42	0	N: 1-10	100
15	P	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
16	40	45	0	0	26	5	4	0	0	24	0	N: 1-10	100
17	0	0	P	P	P	P	P	P	P	0	0	N: 1-10	100
18	28	0	34	14	0	14	15	19	17	1	0	N: 1-10	100
19	0	P	0	0	P	0	P	0	0	P	0	N: 1-10	100
20	0	7	33	35	26	35	34	16	32	27	0	N: 1-10	100
21	15	0	0	1	0	1	0	2	0	1	0	N: 1-10	100
Total Offspring	160	142	176	132	135	145	139	101	134	140	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted.
Appearance codes: N = Observed normal.

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.32 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[1]	-	-	-	-	-	-	-	-	-	-	0	N: 1-10	100
8	20	16	10	22	18	18	21	24	15	19	0	N: 1-10	100
9	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
10	0	0	27	0	0	1	25	0	1	0	0	N: 1-10	100
11	P	P	0	P	P	P	0	P	P	P	0	N: 1-10	100
12	27	28	0	31	29	28	1	24	25	34	0	N: 1-10	100
13	0	P	P	P	0	0	P	P	P	P	0	N: 1-10	100
14	41	41	41	45	44	36	44	38	44	39	0	N: 1-10	100
15	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
16	30	15	45	16	19	0	43	20	26	15	0	N: 1-10	100
17	0	0	0	0	0	P	0	0	0	0	0	N: 1-10	100
18	2	0	0	0	3	20	0	2	0	0	0	N: 1-10	100
19	P	P	P	P	P	0	P	P	P	P	0	N: 1-10	100
20	35	35	20	34	41	30	19	32	34	39	0	N: 1-10	100
21	1	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
Total Offspring	156	135	143	148	154	133	153	140	145	146	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted.
Appearance codes: N = Observed normal.
[1]Neonate presence data was inadvertently not recorded.

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Survival and Reproduction of Adult daphnids

Loading Rate*: 0.64 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: 1-8,10; 9[1]	100
4	0	0	0	0	0	0	0	0	0	0	0	N: 1-8,10; S,C: 9	100
5	0	0	0	0	0	0	0	0	0	0	0	N: 1-8,10; S,C: 9	100
6	0	0	0	0	0	0	0	0	0	0	0	N: 1-8,10; S,C: 9	100
7[2]	-	-	-	-	-	-	-	-	-	-	0	N: 1-8,10; S,C: 9	100
8	18	20	19	18	16	17	15	15	0	19	0	N: 1-8,10; S,C: 9	100
9	0	0	0	0	0	0	0	0	0	0	0	N: 1-8,10; S,C: 9	100
10	0	0	0	25	0	0	1	0	0	24	0	N: 1-8,10; S,C: 9	100
11	P	P	P	0	P	P	P	P	0	P	0	N: 1-8,10; S,C: 9	100
12	25	21	27	0	28	27	28	26	3	1	0	N: 1-8,10; S,C: 9	100
13	0	P	P	P	P	P	0	P	0	P	0	N: 1-8,10; S[3]: 9	100
14	35	36	37	47	37	38	37	35	21	39	0	N: 1-8,10; S: 9	100
15	0	0	0	0	0	0	0	0	0	0	0	N: 1-10	100
16	0	23	23	43	20	26	0	21	0	46	0	N: 1-10	100
17	P	0	0	0	0	0	P	0	P	0	0	N: 1-10	100
18	20	0	0	0	0	0	19	0	30	0	0	N: 1-10	100
19	P	P	P	P	P	P	P	P	0	P	0	N: 1-10	100
20	32	23	33	23	32	37	31	30	43	18	0	N: 1-10	100
21	0	0	1	0	0	0	0	0	0	1	0	N: 1-10	100
Total Offspring	130	123	140	156	133	145	131	127	97	148	--	--	--

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
P = Neonates present but not counted.
Appearance codes: N = Observed normal. S = Small. C = Off-color. [3]Color has improved.
[1] Daphnid was observed stuck in some type of fiber or piece of molt. [2]Neonate presence data was inadvertently not recorded.

APPENDIX E – BIOLOGICAL DATA (CONT'D)

Individual Adult Daphnid Lengths[1] at Test Termination (mm)

Adult	Loading Rate* (mg/L)					
	Control (0)	0.04	0.08	0.16	0.32	0.64
1	4.8	5.0	4.9	4.8	5.0	4.9
2	4.9	5.0	5.0	5.0	4.9	4.9
3	4.9	4.8	5.0	5.0	5.0	5.0
4	5.0	4.9	5.0	4.8	4.9	5.0
5	5.0	4.8	4.8	4.9	4.9	4.8
6	4.8	4.9	5.0	5.0	4.9	5.0
7	4.9	4.8	5.0	5.0	5.0	5.0
8	5.0	5.0	4.9	4.9	4.9	4.9
9	5.0	4.8	5.0	5.0	4.9	4.7
10	5.0	4.9	5.0	4.8	5.0	5.0
Mean	4.9	4.9	5.0	4.9	4.9	4.9

*Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1]Body length excluding anal spine.

APPENDIX F – TEST SUBSTANCE CHARACTERIZATION

TEST SUBSTANCE CHARACTERIZATION

The light hydrocracked gas oil (CAS No. 64741-77-1) was initially characterized on July 12, 2010. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and Gas chromatography-mass spectrometry (GC-MS) analysis. Stability of the neat test substance was confirmed by repeating these same analyses on July 26, 2011 after completion of this study.

UV-VIS spectra are presented in Figures UV-VIS-1 and UV-VIS-2 representing, the initial and final spectrum at concentrations of 908 ppm and 760 ppm, 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 FTIR-1 and FTIR-2 representing the initial and final spectra. Initial and final FT-IR spectra were acquired on a FTIR 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 Hewlett-Packard HP5890 Series II gas chromatograph with 5972 mass selective detector. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, MRD-10-577 was analyzed against an ASTM D2887 calibration mixture. Figures Total Ion Chromatogram-1 and Total Ion Chromatogram-2 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components between retention times of approximately 9 and 23 minutes. This corresponds to bracketing by standard hydrocarbons n-nonane (n-C9) and n-heptadecane (n-C17) under the analytical conditions employed.

The test substance's initial and final density was measured at 20°C with an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter, the initial density was measured as 0.8207 g/mL@20°C and final density was measured as 0.8207 g/mL@20°C. 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 the study period.

D. J. Letinski, M.S.; Principal Investigator for Characterization (located at the testing facility)

8 Nov 2011
Date

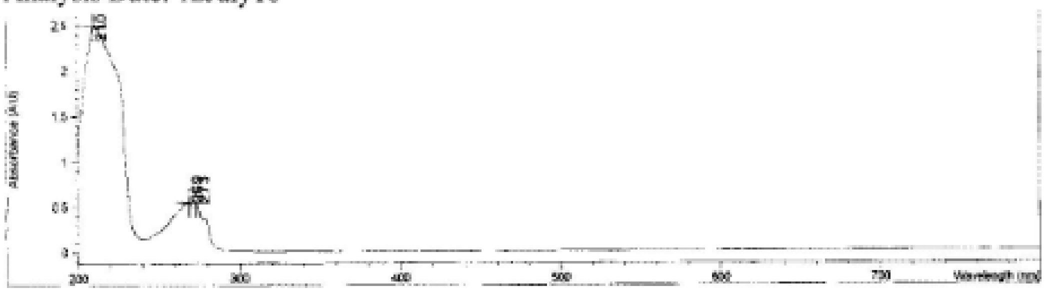
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure: UV-VIS-1
Initial

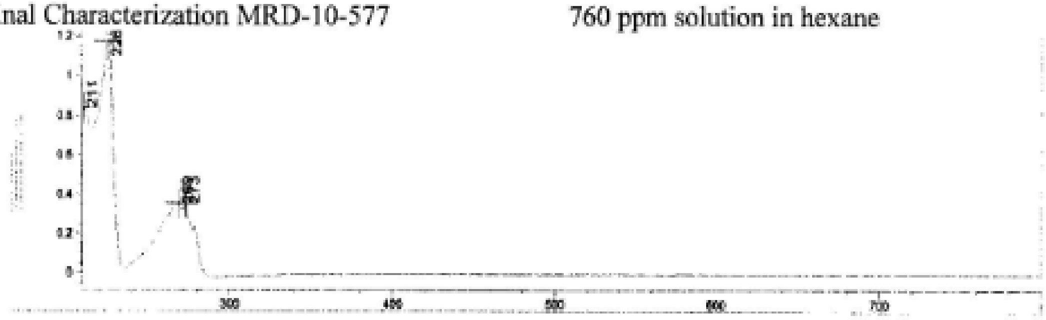
Initial Characterization MRD-10-577 908 ppm solution in hexane
Analysis Date: 12July10



Peak 210nm Absorbance = 2.5019
Peak 269nm Absorbance = 0.5468
Peak 273nm Absorbance = 0.5451

Figure UV-VIS-2
Final

Final Characterization MRD-10-577 760 ppm solution in hexane



Analysis Date: 26Jul11

Peak 226nm Absorbance = 1.17330
Peak 211nm Absorbance = 0.83978
Peak 269nm Absorbance = 0.35515
Peak 273nm Absorbance = 0.35396

APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FT-IR SPECTRA

Figure: FTIR-1
Initial

Initial Characterization MRD-10-577 Analysis Date: 12Jul10

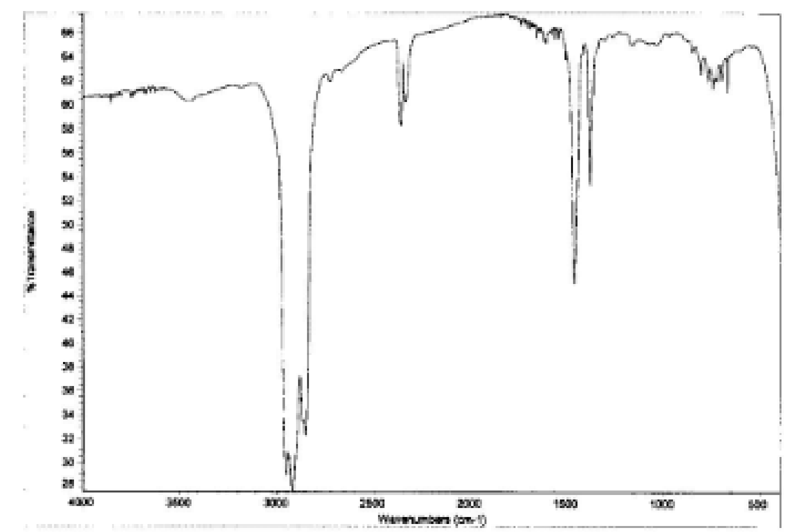
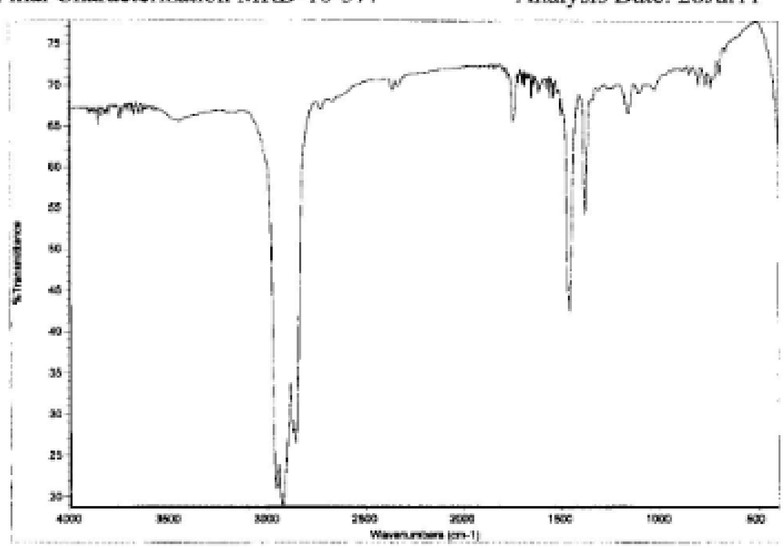


Figure: FTIR-2
Final

Final Characterization MRD-10-577 Analysis Date: 26Jul11



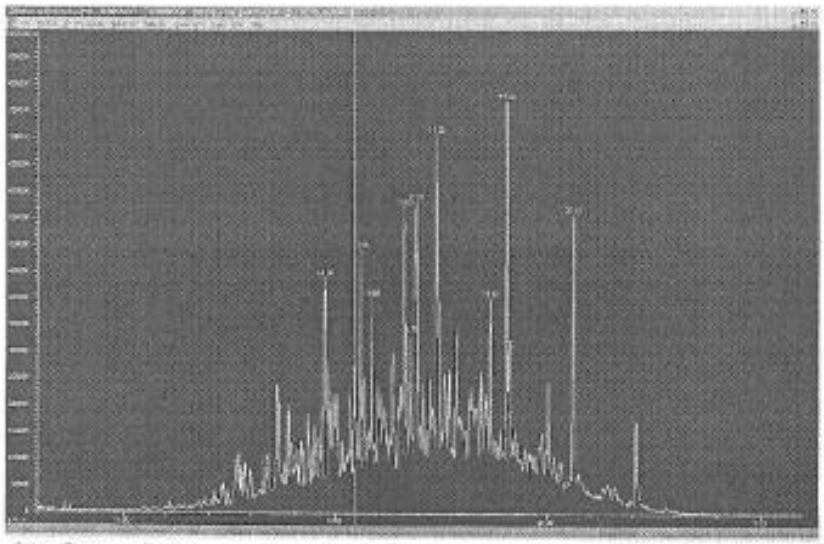
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure: Total Ion Chromatogram-1

INITIAL



Area Percent Report

```
Data File : C:\HPCHEM\1\DATA\CHAR2010\12JUL03A.D          Vial: 14
Acq On    : 13 Jul 2010   3:23                          Operator:
Sample    : MRD-10-577 (initial characterization) 20 Inst     : GC/MS Ins
Misc      : distillates (petroleum) light hydrocrack Multiplr: 1.00
                                                  Sample Amount: 0.00
MS Integration Params: MRD10577.E

Method    : C:\HPCHEM\1\METHODS\CHAR2010.M (Chemstation Integrator)
Title     :

Signal    : TIC
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	14.739	1491	1497	1504	VV 4	367477	13365157	57.65%	11.848%
2	15.589	1597	1603	1608	VV 3	396816	10073761	50.99%	8.930%
3	15.845	1631	1636	1643	VV	300934	6897096	34.91%	6.115%
4	16.630	1729	1733	1743	VV 3	456526	12644174	64.00%	11.209%
5	16.751	1743	1748	1756	VV 3	220665	6532093	33.06%	5.791%
6	16.911	1758	1768	1777	VV 6	462840	19757075	100.00%	17.515%
7	17.392	1821	1828	1841	VV 2	566662	18492440	93.60%	16.394%
8	18.673	1983	1988	1998	VB	282352	5562197	28.15%	4.931%
9	19.066	2026	2037	2040	BV	592190	8962945	45.37%	7.946%
10	20.628	2205	2232	2242	PB	502906	10515100	53.22%	9.322%

Sum of corrected areas: 112802828

12JUL03A.D CHAR2010.M Wed Jul 14 08:50:53 2010

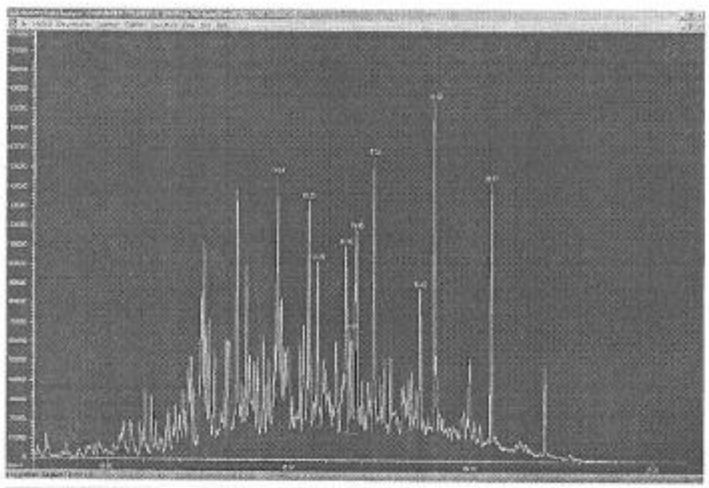
APPENDIX F – TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

TOTAL ION CHROMATOGRAM

Figure: Total Ion Chromatogram-2

FINAL



Area Percent Report

```
Data File : C:\HPCHEM\1\DATA\CHAR2010\26JUL03.D                Vial: 3
Acq On    : 26 Jul 2011  10:53                              Operator:
Sample    : MRD-10-577(final characterization)20%v/v Inst     : GC/MS Ins
Misc      : distillates (petroleum) light hydrocrack Multiplr: 1.00
                                                    Sample Amount: 0.00
MS Integration Params: 577.2

Method    : C:\HPCHEM\1\METHODS\CHAR2010.M (Chemstation Integrator)
Title     :

Signal    : TIC
```

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	14.681	2243	2253	2263	VV 5	1083640	31865216	73.62%	11.691%
2	15.534	2399	2414	2421	BV 2	1139110	25500505	58.92%	9.356%
3	15.788	2455	2462	2472	VV	851466	17649089	40.79%	6.475%
4	16.566	2603	2609	2614	VV 2	957642	20500568	47.37%	7.521%
5	16.686	2624	2632	2646	VVA5	526058	16422931	37.95%	6.026%
6	16.958	2646	2664	2682	VV 7	1050447	43281045	100.00%	15.879%
7	17.340	2764	2755	2772	VV 3	1376680	38921803	89.93%	14.279%
8	18.616	2989	2996	3011	VV 2	718106	13237543	30.59%	4.857%
9	19.029	3060	3074	3092	BV 2	1652915	41674367	96.29%	15.289%
10	20.575	3357	3366	3376	VB	1310136	23517748	54.34%	8.628%

Sum of corrected areas: 272571833

26JUL03.D CHAR2010.M   Tue Jul 26 14:28:51 2011

APPENDIX G – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION

Intertek

Report of Analysis

INTERIM

[redacted]

[redacted]

[redacted]

[redacted]

Sample ID:	2009-DRPK-003707-009	**Date Taken:**	01-April-2009
Drawn By:	Client	**Date Submitted:**	01-April-2009
Sample Designated As:	Various	**Date Tested:**	16-April-2009
Representing:	Site #7, Sx. #3 (As Received)		

Method	Test	Result	Units
ASTM D4052	Density of Liquids by Digital Density Meter		
	Density @ 15°C/59°F	0.8244	g/mL
	Relative Density @ 60/60°F	0.8248	
	API Gravity @ 60°F	40.1	°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	16.7	Vol %
	Olefins	1.5	Vol %
	Saturates	81.8	Vol %

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Information not relevant to the test sample has been blacked out.

1114 Seaco Avenue, Deer Park, Texas 77536 USA
Tel.: (713) 844-3200

20-Apr-2009 12:19
US785-0017157

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

Intertek **Report of Analysis** **INTERIM**

Sample ID: 2009-DRPK-003707-009
Drawn By: Client
Sample Designated As: Various
Representing: Site #7, Sx. #3 (As Received)

Date Taken: 01-April-2009
Date Submitted: 01-April-2009
Date Tested: 16-April-2009

Method	Test	Result	Units
ASTM D5186	Determination of Aromatic Content and Polynuclear Aromatic Content of Diesel Fuels and Aviation Turbine Fuels by Supercritical Fluid Chromatography		
	Monoaromatics by SFC	20.8	Wt %
	Polynuclear Aromatics by SFC	< 0.5	Wt %
	Total Aromatics	21.0	Wt %

[redacted]

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86999

Information not relevant to the test sample has been blacked out.

1114 Seaco Avenue, Deer Park, Texas 77536 USA
Tel.: (713) 844-3200

20-Apr-2009 12:19
US785-0017157

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

SAMPLE:	**09-3707-9 (Site #7 Sx. #3)**	Injection Date:	)090402005303-0600
		Report Date:	4/8/09 9:38
FILE:	C:\CP32 Instruments\D2887 & D3710\Data\2009\APR-09\09-3707-9.0004.CDF		
PROCEDURE:	C:\CP32 Instruments\D2887 & D3710\PROCEDURES\D2887-031709.prc		
EXCEL FILE:	C:\CP32 Instruments\D2887 & D3710\Reports\2009\APR-09\09-3707-9_0004_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	273.8	134.4	**40%**	395.6	202.0	**80%**	467.3	241.8
1%	283.7	139.8	**41%**	396.6	202.6	**81%**	470.7	243.7
2%	293.1	145.0	**42%**	397.8	203.2	**82%**	473.3	245.2
3%	309.0	153.9	**43%**	399.2	204.0	**83%**	476.6	247.0
4%	315.9	157.7	**44%**	401.1	205.0	**84%**	479.7	248.7
5%	320.6	160.3	**45%**	403.1	206.2	**85%**	482.2	250.1
6%	326.2	163.4	**46%**	405.0	207.2	**86%**	486.1	252.3
7%	329.8	165.4	**47%**	406.5	208.0	**87%**	488.7	253.7
8%	332.2	166.8	**48%**	407.9	208.9	**88%**	490.2	254.5
9%	336.7	169.3	**49%**	409.7	209.8	**89%**	492.9	256.0
10%	341.9	172.2	**50%**	411.8	211.0	**90%**	496.7	258.2
11%	346.1	174.5	**51%**	413.5	211.9	**91%**	501.1	260.6
12%	350.6	177.0	**52%**	415.3	212.9	**92%**	505.7	263.2
13%	352.6	178.1	**53%**	417.4	214.1	**93%**	509.2	265.1
14%	354.1	178.9	**54%**	419.7	215.4	**94%**	513.8	267.7
15%	355.7	179.8	**55%**	421.3	216.3	**95%**	518.9	270.5
16%	356.9	180.5	**56%**	422.7	217.0	**96%**	521.3	271.8
17%	357.9	181.1	**57%**	424.4	218.0	**97%**	528.6	275.9
18%	358.9	181.6	**58%**	425.8	218.8	**98%**	537.7	280.9
19%	360.3	182.4	**59%**	426.9	219.4	**99%**	548.5	287.0
20%	362.9	183.8	**60%**	428.3	220.2	**FBP**	554.6	290.4
21%	365.5	185.3	**61%**	429.5	220.8			
22%	367.3	186.3	**62%**	430.4	221.4			
23%	368.8	187.1	**63%**	431.7	222.0			
24%	370.7	188.1	**64%**	433.5	223.0			
25%	372.6	189.2	**65%**	435.3	224.1			
26%	374.0	190.0	**66%**	437.1	225.0			
27%	375.5	190.8	**67%**	438.7	225.9			
28%	377.3	191.8	**68%**	440.4	226.9			
29%	379.2	192.9	**69%**	442.2	227.9			
30%	380.7	193.7	**70%**	444.1	229.0			
31%	382.4	194.6	**71%**	445.9	229.9			
32%	384.1	195.6	**72%**	447.7	231.0			
33%	385.9	196.6	**73%**	450.0	232.2			
34%	387.7	197.6	**74%**	451.6	233.1			
35%	389.6	198.7	**75%**	452.9	233.9			
36%	391.1	199.5	**76%**	454.9	234.9			
37%	392.4	200.2	**77%**	457.4	236.4			
38%	393.6	200.9	**78%**	460.4	238.0			
39%	394.5	201.4	**79%**	463.6	239.8			

Start Elution Time (mins):	0.094	**Sample Wt:**	0 **g**
End Elution Time (mins):	8.752	**Solvent Wt:**	0 **g**
		Material Balance:	100.0 **Wt%**
Blank File:	C:\CP32 Instruments\D2887 & D3710\Data\2009\APR-09\BLANK2-040109.0002.CDF		
Calib File:	D:\CP32 Instruments\D2887 & D3710\DATA\RTMIX-060905.0006.CDF		
Resp Factor:	1.000E+00		

APPENDIX H - STATISTICAL OUTPUT

```
577Load reproduction data
File: 577R.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      5           11                25             17           2
--------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   2.7196
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

577Load reproduction data
File: 577R.dat       Transform: NO TRANSFORMATION

--------------------------------------------------------------------------
Bartlett's test for homogeneity of variance
Calculated B1 statistic =   14.97
--------------------------------------------------------------------------
Table Chi-square value =   15.09  (alpha = 0.01, df =   5)
Table Chi-square value =   11.07  (alpha = 0.05, df =   5)

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

577Load reproduction data
File: 577R.dat        Transform: NO TRANSFORMATION
```

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 1 of 2

GRP	IDENTIFICATION	N	MIN	MAX	MEAN
1	Control	10	124.000	148.000	139.400
2	0.04 mg/L	10	119.000	161.000	138.900
3	0.08 mg/L	10	120.000	147.000	133.700
4	0.16 mg/L	10	101.000	176.000	140.400
5	0.32 mg/L	10	133.000	156.000	145.300
6	0.64 mg/L	10	97.000	156.000	133.000

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	Control	44.489	6.670	2.109	4.78
2	0.04 mg/L	181.878	13.486	4.265	9.71
3	0.08 mg/L	81.122	9.007	2.848	6.74
4	0.16 mg/L	374.489	19.352	6.120	13.78
5	0.32 mg/L	60.900	7.804	2.468	5.37
6	0.64 mg/L	265.778	16.303	5.155	12.26

APPENDIX H - STATISTICAL OUTPUT (CONT'D)

577Load reproduction data
File: 577R.dat Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	1040.950	208.190	1.238
Within (Error)	54	9077.900	168.109	
Total	59	10118.850		

Critical F value = 2.45 (0.05,5,40)
Since F < Critical F FAIL TO REJECT Ho: All equal

577Load reproduction data
File: 577R.dat Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	Control	139.400	139.400		
2	0.04 mg/L	138.900	138.900	0.086	
3	0.08 mg/L	133.700	133.700	0.983	
4	0.16 mg/L	140.400	140.400	-0.172	
5	0.32 mg/L	145.300	145.300	-1.018	
6	0.64 mg/L	133.000	133.000	1.104	

Dunnett table value = 2.31 (1 Tailed Value, P=0.05, df=40,5)

DUNNETT'S 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	10			
2	0.04 mg/L	10	13.394	9.6	0.500
3	0.08 mg/L	10	13.394	9.6	5.700
4	0.16 mg/L	10	13.394	9.6	-1.000
5	0.32 mg/L	10	13.394	9.6	-5.900
6	0.64 mg/L	10	13.394	9.6	6.400

APPENDIX H - STATISTICAL OUTPUT (CONT'D)

```
577Load length data
File: 577L.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   4          16                11             29          0

-------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =  24.8103
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.

577Load length data
File: 577L.dat        Transform: NO TRANSFORMATION
-------------------------------------------------------------------------
Bartlett's test for homogeneity of variance
Calculated B1 statistic =    4.56
-------------------------------------------------------------------------
Table Chi-square value =   15.09  (alpha = 0.01, df =   5)
Table Chi-square value =   11.07  (alpha = 0.05, df =   5)

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

577Load length data
File: 577L.dat         Transform: NO TRANSFORMATION

           SUMMARY STATISTICS ON TRANSFORMED DATA  TABLE 1 of 2
-------------------------------------------------------------------------

GRP  IDENTIFICATION    N      MIN        MAX        MEAN
--- ---------------- ---- ---------- ---------- ----------
 1          Control   10      4.800      5.000      4.930
 2        0.04 mg/L   10      4.800      5.000      4.890
 3        0.08 mg/L   10      4.800      5.000      4.960
 4        0.16 mg/L   10      4.800      5.000      4.920
 5        0.32 mg/L   10      4.900      5.000      4.940
 6        0.64 mg/L   10      4.700      5.000      4.920
-------------------------------------------------------------------------
```

APPENDIX H - STATISTICAL OUTPUT (CONT'D)

577Load length data
File: 577L.dat Transform: NO TRANSFORMATION

SUMMARY STATISTICS ON TRANSFORMED DATA TABLE 2 of 2

GRP	IDENTIFICATION	VARIANCE	SD	SEM	C.V. %
1	Control	0.007	0.082	0.026	1.67
2	0.04 mg/L	0.008	0.088	0.028	1.79
3	0.08 mg/L	0.005	0.070	0.022	1.41
4	0.16 mg/L	0.008	0.092	0.029	1.87
5	0.32 mg/L	0.003	0.052	0.016	1.05
6	0.64 mg/L	0.011	0.103	0.033	2.10

577Load length data
File: 577L.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	4.930				
2	0.04 mg/L	4.890	92.00	75.00	10.00	
3	0.08 mg/L	4.960	115.50	75.00	10.00	
4	0.16 mg/L	4.920	102.50	75.00	10.00	
5	0.32 mg/L	4.940	106.00	75.00	10.00	
6	0.64 mg/L	4.920	104.00	75.00	10.00	

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

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

- PROTOCOL -

Contract Number:	EMBSI 2010-104821
Test Substance:	Gas oil; CAS RN 64741-77-1, Distillates (petroleum), light hydrocracked
Study Title:	*Daphnia magna* Reproduction Test on Water Accommodated Fractions of a Light Hydrocracked Gas Oil
EMBSI Study Number:	1057746
EMBSI Test Substance Code:	MRD-10-577
Date:	May 20, 2011
Room Number:	LE 337/343

Proposed Key Dates for Completion:

Initial Characterization.......................12-Jul-10
WAF Equilibration and Stability Trial Start 13-Sep-10
Experimental Start 21-Jun-11
Experimental Termination.......................12-Jul-11
Draft Report Completion 12-Aug-11
Final Report Completion 30-Sep-11

Approved By:

Tricia M. Kharr-Cousineau, M.S.E.H. — 25May11 Date
Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-3059

RDW for P. Podhasky — 25 May 2011 Date
Paula Podhasky
Sponsor Representative

SAFETY FIRST

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

INTRODUCTION

Objective

This study will be conducted for the Sponsor to assess the effects of the water accommodated fractions (WAFs) of a light Hydrocracked gas oil (CAS RN 64741-77-1), 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 US Highway 22 East
Annandale, New Jersey 08801-3059

Compliance

This test will be conducted in general agreement with the OECD[1] and EPA[2] guidelines, and will be conducted in compliance with OECD[3] and USEPA[4] GLP standards.

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 I – PROTOCOL AND PROTOCOL REVISIONS

MATERIALS and METHODS

Test Substance Identification

EMBSI code	Test Substance
MRD-10-577	CAS 64741-77-1

CAS Definition: Distillates (petroleum) light hydrocracked. A complex combination of hydrocarbons produced by the distillation of products from a hydrocracking process. It consists predominantly of saturated hydrocarbons having carbon numbers predominantly in the range of C10 through C18, and boiling in the range of approximately 160 to 320 °C (302 to 608 °F)[5].

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 was applied as it is used for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). 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 I – PROTOCOL AND PROTOCOL REVISIONS

MATERIALS and METHODS (CONT'D)

Analysis of Test Solutions

Samples will be taken from each "new" treatment WAF and control solution on Day 0 and at least three additional intervals during the study (approximately weekly). At least two replicates from each treatment will be analyzed. For the corresponding "old", i.e., used solutions, three individual replicates will be selected and sampled prior to performing the renewal. Each treatment 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. Samples will be analyzed using static headspace gas chromatography with flame ionization detection (HS GC-FID). The analysis will quantitate the concentration of hydrocarbons present in the WAFs. The analysis will be standardized against the neat test substance to ensure that the full range of constituent hydrocarbons that could potentially solubilize into the WAF solutions is captured and quantitated. Based on the levels at which the test will be conducted, if the concentration of hydrocarbons in any "new" solution is found to be unable to be quantified, the corresponding "old" solution will not be analyzed. However, as long as the concentration of hydrocarbons in any "new" solution is quantifiable, the corresponding "old" solution will be analyzed. A detailed description of the analytical methods used will be documented in the raw data and included in the final report.

Sample Retention

No retention samples (neat test substance or solutions (WAFs)) will be taken for this study.

Dilution Water

Reconstituted water[6] (the dilution water) will be prepared from UV-sterilized, deionized well water and reagent grade chemicals ($NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl), it will be aerated prior to use. The hardness will be >140 mg/L (as $CaCO_3$).

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 I – PROTOCOL AND PROTOCOL REVISIONS

MATERIALS and METHODS (CONT'D)

Husbandry and Acclimation

Eight to ten daphnids are kept in 1-liter glass culture beakers with approximately 800 mL of reconstituted water (study dilution water). The culture chamber is maintained at 20 ± 2°C under a 16 hour light 8 hour dark photoperiod (108 - 215 Lux). Two sets of Day 0 cultures are started at least five days a week. The neonates should be less than 24 hours old and come from a day 12-18 culture which experienced less than an estimated 10% neonate mortality and less than or equal to 20% adult mortality.

Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately 4.5 - 6.0 x 10^5 cells/mL). They are also fed 25µL/L of Vita chem Fresh formula mixed on a magnetic stir plate with the reconstituted water prior to feeding with algae. 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 Vita chem is manufactured by Boyd Enterprises, Inc. and supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin.

Cultures are transferred every other day, with exceptions on holidays or weekends when staff is not present, the brood stock health is evaluated and any mortality, production of males or ephippia is documented as well as any mitigation procedures.

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

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

MATERIALS and METHODS (CONT'D)

Selection (cont'd)

To ensure that quality organisms are used for the study, neonates from parents 12-18 days old (with ≤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 ≤10% daily mortality on the experimental start day. The study director or her designee will determine organism suitability.

Feed

Once test solutions settle following mixing for 1 hour (±30 minutes), two liters of each WAF will be removed and 50 µL of Vita chem fresh water formula (Boyd Enterprises, Inc.) will be added to provide a concentration of 25 µL/L. Additionally, daphnids will be fed at the initiation of the test and during renewals by adding 0.350 mL of a 1.3×10^8 cells/mL suspension of *Pseudokirchneriella subcapitata* to provide approximately 3.3×10^5 cells/mL. Beginning on Day 7, they will be fed an additional 0.200 mL 1.3×10^8 cells/mL suspension of *Pseudokirchneriella subcapitata* on non-renewal days to provide an additional 1.9×10^5 cells/mL (approximate) of algae. The algae is supplied by Aquatic Biosystems, Inc., Fort Collins, Colorado and the Vita chem fresh feed is supplied by Foster and Smith Aquatics, Rhinelander, Wisconsin.

Contaminants

There are no known contaminants in the feed used in culturing and testing the organisms, or the dilution water believed to be at levels high enough to interfere with this study. The algae is not analyzed, it is prepared in deionized/distilled water with reagent grade chemicals. 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. The deionized water is monitored for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties by Accutest®, 2235 Route 130, Dayton, NJ 08810. Contaminant analyses are not performed in a GLP compliant manner. This is not believed to affect the results of the analyses. Contaminant analysis results are maintained at the testing facility.

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

EXPERIMENTAL PROCEDURE

WAF Equilibration and Stability Trial

A WAF equilibration trial to determine the appropriate mixing duration will not be performed specifically for this study. Equilibrium and stability tests were performed as part of the *Daphnia* acute immobilization study (1057742). 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. Additionally, once the WAF solutions were created, they were found to be acceptably stable over a 48-hour period. Results of the equilibrium testing will be appended to the final report.

Range Finding Test

A range finding test will not be performed specifically for this study; the EC50 determined in the acute phase of testing (1057742) was used to estimate loading rates.

Definitive Test Design

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	10 (1 per replicate)
2	0.04	10
3	0.08	10
4	0.16	10
5	0.32	10
6	0.64	10

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 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 24 hours (±2 hour) at test temperature (20°±1°C) or room temperature (22°±2°C). The temperature will be indicated in the raw data. At the end of mixing, the solutions will be allowed to settle for 1 hour (±30 minutes). Once test solutions settle, two liters of each WAF will be removed and 50 µL of Vita chem fresh water formula (Boyd Enterprises, Inc.) will be added to provide a concentration of 25 µL/L. The solutions will then be distributed to the individual test chambers. New WAF solutions will be prepared every other day for the renewals.

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance (cont'd)

Test chambers will be completely filled with the appropriate solution such that zero or minimal headspace exists in the test chambers. Renewals will be performed by transferring each parent daphnid, via glass pipette, to fresh solution every 48 hours. 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.

Test Chamber and Volume of Solution

The test chambers will be 130mL clear glass containers sealed with screw type lids to minimize contamination, evaporation and/or volatilization and will contain no headspace. Test chamber details will be noted in the raw data and reported.

Exposure Duration

21 days

Continuous Measurements

Range of acceptable test water temperatures: 20° ± 1°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. Should Watchdog be unavailable, manual measurements will be taken twice daily.

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 every 48 hours. 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.

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

EXPERIMENTAL PROCEDURE (CONT'D)

Experimental Evaluation (cont'd)

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.

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.

Also, the coefficient of variation around the mean number of living offspring produced per parent animal in the control(s) should be ≤ 25%.

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 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 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 from the control group.

Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as EC, NOEC, and LOEC 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 gas oil components measured in the WAFs differs from the parent gas oil, owing to the differing solubilities of individual gas oil hydrocarbons.

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EXPERIMENTAL PROCEDURE (CONT'D)

Calculations (cont'd)

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's[7] or Wilcoxon Rank Sum[8] with Bonferroni Adjustment[9] using TOXSTAT software[10] may be used to determine the LOELR/LOEC and NOELR/NOEC, the Benchmark Dose (BMD) method[11] may be used to determine the EL_x/EC_x values. A maximum acceptable toxicant concentration (MATC) will also be calculated based on EPA requirements. 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.

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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, 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;
- dilution water source and chemical characteristics (pH, temp. dissolved oxygen, TOC, hardness, alkalinity);
- 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 including examples of chromatography (blank, low and high loading rate WAF, and standards) and a graphical representation of the standard curve
- 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}, MATC values (reproduction and growth, if possible) with confidence limits, if possible;
- statistical procedures followed;
- graph of the loading rate/concentration reproduction-response curve at the end of the test.

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

REPORT (CONT'D)

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 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.

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:

> Consistent with the OECD document on aquatic toxicity testing of complex substances[12], it is 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.

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

REFERENCES

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9. Bland, M. J.., "Multiple significance tests: the Bonferroni method", British Medical Journal, v310, pg. 170, 1995.

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APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

DISTRIBUTION

EMBSI - Clinton:

Study Director..T. M. Knarr-Cousineau
Environmental Sciences, Section Head......................T. F. Parkerton
Environmental Toxicology and Fate Coordinator ....G. E. Bragin
Environmental Chemistry / Principal Investigator
for Characterization/Analysis of Test Solutions ....D. J. Letinski
Study Technicians ....J. D. Butler
....M. J. Connelly
....C. A. Sutherland
....B. A. Kelley
....R. G. Manning
Contract Administrator..B. J. Foster
QAU ....D.M. McDougall

API:

Sponsor Representative ....Paula Podhasky
Sponsor's Study Monitor...Jim Swigert

APPENDIX I – PROTOCOL AND PROTOCOL REVISIONS

PROTOCOL CHANGE RECORD Page 1 of 1

This record must be approved by the Sponsor Representative and the Study Director for all protocol changes made subsequent to initial distribution. Upon completion, a copy of this record must be distributed to all recipients of the protocol and the original submitted to the Archivist.

Study Numbers: 1057746 Revision Number: 1 Date: 07Nov11

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Previous Statement: Sponsor Representative – Paula Podhasky

Revised Statement: Sponsor Representative – Russell White

Justification: Paula Podhasky has retired from American Petroleum Institute and has been replaced with Russell White.

Page 12 / PERSONNEL:

Previous Statement: Environmental Sciences, Section Head - T. F. Parkerton

Revised Statement: Environmental Sciences, Section Head – R.A. Barter

Justification: T. F. Parkerton has been replaced with R.A. Barter as Section Head effective July 1, 2011

DISTRIBUTION

EMBSI - Clinton:

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Environmental Sciences, Section Head.....................R. A. Barter
Environmental Toxicology and Fate Coordinator....G.E. Bragin
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....B. A. Kelley
....R. G. Manning
Contract Administrator ....B. J. Foster
QAU ....D.M. McDougall

API:

Sponsor Representative ....Russell White
....Jim Swigert

Required signatures:

Russell White 29 Nov 2011 Tricia M. Knarr-Cousineau 01Dec11
Russell White Tricia M. Knarr-Cousineau
Sponsor Representative Date Study Director Date