ExxonMobil BIOMEDICAL SCIENCES, INC.

EMBSI 2010-104821

***Oncorhynchus mykiss,* Fish Acute Toxicity Test on Water Accommodated Fractions of a Light Hydrocracked Gas Oil**

Final Report

Study Number: 1057758

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: December 22, 2011

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APPROVAL SIGNATURES

[redacted] 22 Dec 11
Date

Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, NJ 08801-3059

[redacted] 22Dec11
Date

Environmental Toxicology & Fate
Laboratory Coordinator

[redacted] 22 Dec., 2011
Date

Section Head, Environmental Sciences

The final report was accepted by the Sponsor

[redacted] 20 Dec. 2011
Date

Sponsor Representative
American Petroleum Institute
1220 L Street, NW
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GLP COMPLIANCE STATEMENT

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

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

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

The above exception is not believed to have an adverse effect on the study results.

[redacted]

22 Dec 11
Date

Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
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[redacted]

20 Dec. 2011
Date

Sponsor Representative
American Petroleum Institute
1220 L Street, NW
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QUALITY ASSURANCE STATEMENT

STUDY NUMBER: 1057758

TEST SUBSTANCE: MRD-10-577

STUDY SPONSOR: American Petroleum Institute

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

Study Phase Inspected	Date(s) of Inspection	Reported to Study Director	Reported to Management
Protocol	May 16, 2011	May 17, 2011	June 13, 2011 July 12, 2011
Preparation of Day 1 WAF	June 13 & July 29, 2011	July 29, 2011	October 6, 2011 October 7, 2011
First review of Final Report & Raw Data	October 12 & October 14, 2011	October 14, 2011	November 1, 2011 November 8, 2011
Second Review of Final Report & Raw Data	October 25 & October 26, 2011	October 26, 2011	November 1, 2011 November 8, 2011
Third Review of Final Report: Appendix A only	December 6, 2011	December 6, 2011	December 6, 2011

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

[redacted]
Quality Assurance Unit Coordinator

19 Dec 2011
Date

PERSONNEL

Study Director:

Sponsor Representative:

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

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

Environmental Toxicology & Fate
Laboratory Coordinator:

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

Quality Assurance Unit Coordinator:

[redacted]

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 acute toxicity of the water accommodated fractions (WAFs) of light hydrocracked gas oil (CAS No. 64741-77-1) to the rainbow trout, *Oncorhynchus mykiss*. This study was performed as a 96-hour static-renewal test.

A single treatment WAF was prepared by adding the appropriate amount of test substance to dilution water in a 20L glass aspirator bottle and stirring on a magnetic stir plate 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 the WAF solution was removed for testing. A fresh WAF was prepared daily for test solution renewals. The loading rates tested were 0 (control) and 2.6 mg/L.

One test chamber was prepared for the treatment group and control. Each test chamber contained seven rainbow trout. The test chambers were 8 L glass aspirator bottles containing approximately 8500 mL of solution (no headspace) and closed with foil covered stoppers. Water quality (temperature, pH, and dissolved oxygen) measurements of each new and old solution were measured. Observations for mortality and abnormal behavior or appearance were performed at 6, 24, 48, 72 and 96 ±1 hour intervals after the beginning of the test.

Concentrations of the test substance hydrocarbon components were quantified against gas oil standards 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 geometric mean measured hydrocarbon concentrations were ND (Not Detected; control) and 0.54 mg/L. The old solutions retained 65 to 73% of the new initial measured hydrocarbon concentrations.

Acute toxicity results are expressed as percent mortality. The 50% Lethal Loading (LL50) is the calculated loading rate of the test substance which would cause 50% mortality in a population of test organisms over a specified exposure period. Results expressed as the 50% Lethal Concentration (LC50) represent the concentration of hydrocarbons that solubilized from the test substance into the WAF for the treatment group. This test was performed at a single "UTC" (upper threshold concentration). No mortality or sub-lethal effects were observed. As such, the results are reported as a "greater than" value.

The 96-hour LL50 and LC50 values were determined to be > 2.6 mg/L and > 0.54 mg/L, based on loading rate and geometric mean measured hydrocarbon concentration, respectively.

INTRODUCTION

Objective

This study was conducted for the Sponsor to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of light hydrocracked gas oil (CAS No. 64741-77-1) to the rainbow trout, *Oncorhynchus mykiss*, in a 96-hour 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 Date

31 May 2011

WAF Equilibration and Stability Trial Start (Mixing)

13 September 2010

WAF Mixing Start (Definitive Study)

12 June 2011

Experimental Start (Definitive Study)

13 June 2011

Experimental Termination (Definitive Study)

17 June 2011

INTRODUCTION (CONT'D)

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 4. The study was performed in general agreement with the OECD[3] and EPA[4] guidelines with the exceptions listed on page 17. The general methodology of the test was based on the step-down approach proposed by Jeram et.al[5]

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) [6].

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

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.

MATERIALS and METHODS (CONT'D)

Dilution Water

Reconstituted moderately hard water[7] (Batch #644) was prepared with deionized well water and reagent grade chemicals (salts) ($NaHCO_3$, $CaSO_4$, $MgSO_4$, and KCl), and 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.

Contaminants

There are no known contaminants in the feed used for acclimation or in the dilution water believed to be at levels high enough to interfere with this study. The feed was analyzed for minerals and pesticides residues by New Jersey Feed Lab Inc., 1686 Fifth Street, Trenton, NJ 08638. The deionized water is monitored annually 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.

Characterization of the Test Substance

The neat test substance was characterized and the stability determined by the testing facility both prior to and after completion of the study using the following analyses: Ultraviolet/Visible and Infrared Spectrophotometry, density, physical–state, miscibility in water, methanol and /or hexane and a GC-MS Total Ion Chromatogram ("fingerprint") of the neat test substance. The GC-MS fingerprint was 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 E. 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

Samples were collected from each new water-accommodated fraction (WAF) and control solution on Day 0 and Day 3. Samples of the old solutions were collected on Day 1 and Day 4. Samples were collected with no headspace in 40 mL VOA vials and refrigerated pending analysis. (Additional samples were collected, but were not required by the protocol. These additional samples were not analyzed.)

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 the sample 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

Oncorhynchus mykiss (rainbow trout)

Justification for Selection of Test System

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

Supplier

The fish were supplied by Thomas Fish Company, Anderson, California and received in good condition on 26 May 2011.

Husbandry and Acclimation

Upon arrival from the supplier, fish were quarantined and observed for parasites and disease for 18 days prior to use in the test. Fish were held for at least 7 days in dilution water at test temperature of 15 ± 1□C, continuously aerated to maintain the dissolved oxygen level at ≥ 80% of the saturation value. Fish were held under a light regime of 16 hours light: 8 hours dark with a light intensity similar to that of the definitive test. No treatment for disease was administered during the course of the acclimation. The lot of fish was considered acceptable for use, since there was no mortality during acclimation.

MATERIALS and METHODS (CONT'D)

Test System (cont'd)

Husbandry and Acclimation (cont'd)

The remaining fish from the stock population were maintained for a reasonable time for use in method development, training purposes, etc. Fish were euthanized following the most appropriate and humane technique. A tricaine methane sulphonate (MS-222) solution was prepared in laboratory dilution water. The MS-222 solution was prepared at a concentration of 500 mg/L and pH buffered to be in the range of 7.0 - 7.5. Fish were held under static conditions using biological and mechanical filtration and were fed daily with Finfish Starter supplied by Zeigler Bros. Inc., Gardners, PA.

Number and Sex

Number: 14; Sex: not applicable

Age at Initiation of Exposure

Juveniles. The fish hatched on 30 April 2011 and were 45 days old at test initiation.

Test System Identification

Organisms were not individually identified. Each replicate, containing seven fish, was labeled to show study number, loading level, replicate and chamber number.

Feed

Fish were not fed during the study and 50 hours and 25 minutes prior to the study.

Humane Treatment / Euthanasia

All fish were treated humanely in accordance with published guidance. The study design and personnel training was sufficient to minimize animal pain within the confines of the study objective. A tricaine methane sulphonate (MS-222) solution was prepared in laboratory dilution water. The MS-222 solution was prepared at a concentration of 500 mg/L with a pH of 7.4. The Institute for Laboratory Animal Research Journal V37(4), Fish, Amphibians and Reptiles states that "Several methods of euthanasia have been used in fish including hypothermia, electrocution, overdosing with tricaine (MS-222) or carbon dioxide, and a sharp blow to the head. Of these, tricaine administered at 500 mg/L is most desirable (humane) as it does not alter blood cortisol, catecholamine, or glucose levels commonly associated with stress".

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. The equilibration trial was performed as part of the *Daphnia* acute immobilization study (1057742). Stability of the WAF solutions also was evaluated. 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, the soluble components were found to be stable (≥80% retention) over a 48-hour period. Results of the equilibrium and stability trials are presented in Appendix B.

Definitive Test Design

GROUP	LOADING RATE* (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0 (Control)	7 (7 per 1 replicate)
2	2.6	7

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

The definitive test design generally conformed to the Upper Threshold Concentration (UTC) tiered testing strategy described by Jeram, et al.[5] The loading rate for this study was the UTC, defined as the lowest EL50 value generated from either the *Daphnia* Acute Immobilization study (1057742) or the Algal Growth Inhibition Test (1057767). The loading rate for this study was based on the EL50 value (yield) from the Algal test (1057767).

Preparation and Administration of Test Substance

The test substance was added to the 20L aspirator bottles using stainless steel and glass syringes. The loading rate was determined from the volume of test substance added and converted to mass per unit volume (mg/L) based on its density. A control WAF was prepared in the same manner without the addition of test substance. The vessels were closed using foil covered stoppers. The solutions were mixed using a vortex ≤ 10% (of the static liquid depth) for 24 hours ± 1 hour on a magnetic stir plate with Teflon®-coated stir bar at test temperature (15 ± 1°C). At the end of mixing, the solutions were allowed to settle for 40 minutes to 2 hours at the test temperature. At the end of the settling period, the solutions were removed from the mixing vessels through the outlet at the bottom of the vessels and placed into the test chambers.

EXPERIMENTAL PROCEDURE (CONT'D)

Preparation and Administration of Test Substance (cont'd)

One replicate for the treatment group and the control group were prepared by completely filling the test chambers with the test solution (no headspace). Approximately 80 to 90% renewal of the test solutions was performed at approximately 24-hour intervals. Super saturation with oxygen was employed prior to test substance addition in order to meet minimum dissolved oxygen level guideline expectations throughout the exposure. Fresh WAFs were prepared daily for test solution renewals.

Test Chamber / Organism Loading

The test chambers were 8 L glass aspirator bottles containing approximately 8500 mL of solution (no headspace). The test chambers were closed with foil covered stoppers to minimize contamination, evaporation and/or volatilization.

At test termination, organism loading in the control was 0.43 grams of fish per liter of solution.

Length / Weight of Test System

The mean total length of the fish in the control replicate at test termination was 4.1 cm. The longest fish was not more than twice the size in length of the shortest fish. See Appendix D for individual fish weights and lengths for the control replicate.

Selection

Organisms were randomly assigned to test chambers using a computer generated randomization schedule (SAS 9.2)[8]. All the randomization data is included in the raw data.

To ensure that quality organisms are used for the study, fish were selected from a pool of organisms larger than that needed for the study. The study director determined organism suitability.

Exposure Duration

96 hours (± 1 hour)

EXPERIMENTAL PROCEDURE (CONT'D)

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.

The temperature in the environmental chamber ranged from 14.4°C to 15.3°C, continuously monitored by computer in the test area.

Diurnal light: approximately 16 hours light and 8 hours dark. Daylight intensity ranged from 407 – 468 lux during full daylight periods.

Experimental Evaluation

Observations for mortality were performed and recorded at 6, 24, 48, 72 and 96 hours (±1 hour) after the beginning of the test. During observations, organisms were also examined for abnormal behavior and coloration.

Observations of test substance insolubility (surface slicks, precipitates, and adherence to test chamber) were recorded daily at the time of organism observations. Feces from the fish were removed upon the 24 and 48 hour observation points and fish were placed in new solution at approximately 24 hour intervals.

Temperature, dissolved oxygen, and pH were measured for each treatment and the control on Day 0. On Days 1 through 4, samples for temperature, dissolved oxygen, and pH measurements were collected from each "new" treatment and the control, prior to renewals; and from each "old" treatment and control solution following renewals. Samples were held under refrigeration until analysis on Day 4.

Calculations

Calculations were not required for this limit test. Since no mortality was observed, the LL50 is reported as greater than the loading level tested; and the LC50 is reported as greater than the geometric mean measured concentration of hydrocarbons that solubilized from the test substance into the WAF.

Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as the LC50 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 WAF differs from the parent gas oil, owing to the differing solubilities of individual gas oil hydrocarbons.

RESULTS AND DISCUSSION

The WAF loading rates for this study were 0 (control) and 2.6 mg/L. The measured hydrocarbon concentrations for the treatment WAF solutions (new) at test initiation and on Day 3 were 0.63 and 0.67 mg/L, respectively. The measured hydrocarbon concentrations for old treatment solutions were 0.41 and 0.49 mg/L, on Day 1 and at test termination, respectively. The old solution measured hydrocarbon concentrations retained 65 to 73% of the measured concentration in the respective new solutions. 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. The geometric mean measured hydrocarbon concentrations were ND (control) and 0.54 mg/L. The analytical results are presented in Table 1.

At WAF stirring initiation and termination, all treatments appeared transparent with transparent test substance visible on the surface. Water quality measurements were consistent throughout the exposure (Table 2). Dissolved oxygen concentrations for the treatment and control remained above 60% of the air saturation value (6.0 mg/L). pH measurements ranged from 7.38 to 8.25 and 7.00 to 7.53 for the new solutions and old solutions, respectively.

The fish were observed daily for mortality, behavior and appearance during the 96 hour exposure. A summary of daily observations (percent survival and observed normal) for the treatment group and control is presented in Table 3. No mortality or abnormal behavior/appearance was observed in either the treatment group or control group throughout the entire exposure. Daily in-life observations for each treatment group are presented 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.

This study met the acceptability criteria for control mortality, abnormal behavior and dissolved oxygen concentration. None of the control fish died or exhibited abnormal behavior. Dissolved oxygen remained above 60% of the air saturation value at the exposure temperature of 15 ± 1 °C.

This test was performed at a single "UTC" (upper threshold concentration). No mortality or sub-lethal effects were observed. As such, the results are reported as a "greater than" value.

The 96-hour LL50 and LC50 values were determined to be > 2.6 mg/L and > 0.54 mg/L, based on loading rate and geometric mean measured hydrocarbon concentration, respectively.

PROTOCOL DEVIATIONS

The WAFs were allowed to settle for 40 minutes (Day 2) to 2 hours (Day 0) after mixing instead of the protocol specified 1 hour.

On Day 0, the pH measurement was 8.25 for both the treatment and control WAF solutions. The protocol states the pH level should remain between 6 and 8.

The USEPA OPPTS 850.1075 guideline recommends that the concentration of the test substance in solutions be determined at every renewal period. In this study, analytical samples were collected from each "new" treatment WAF on Day 0, 1, 2 and Day 3 and on Day 1 and day 4 for the "old" solutions. Analysis on days 1 and 2 “new” solutions were not required by the protocol and analysis was not conducted.

Except for the Day 0 Water Accommodated Fraction (WAF), the water quality measurements were not measured daily, but measured on Day 4. However, the samples analyzed on Day 4 were taken on each respective day of the test (e.g., Day 1,2,3,4) and stored in the LE 343/345 refrigerator until analysis on day 4 or the 17 of June 2011.

Hardness was measured during discrete measurements. In addition, length and weight measurements were made at test initiation (additional fish from the lot and not used in the study) and the treatment group at test termination. These data were not required by the protocol, but are maintained in the raw data.

The above deviations are not believed to have affected the outcome or integrity of the study.

GUIDELINE EXCEPTIONS

Due to the limited solubility and potential volatility of the test substance the following exception will apply for this study:

> Consistent with the OECD document on aquatic toxicity testing of complex substances[9], 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.

The USEPA OPPTS 850.1075 guideline recommends that the concentration of the test substance in solutions be determined at every renewal period. In this study, analytical samples were collected from each "new" treatment WAF on day 0, 1, 2 and day 3 and on day 1 and day 4 for the "old" solutions. Analysis on days 1 and 2 “new” solutions were not required by the protocol.

The OPPTS Guideline 850.1075 prescribes a number of chemical analyses be performed on the test water. While many of the required tests are consistent with those performed by Accutest®, 2235 Route 130, Dayton, NJ 08810. However chemical oxygen demand (COD), boron, fluoride, residual chlorine, aluminum, cobalt and iron analyses were not performed.

These minor exceptions did not affect the integrity of the data generated.

RECORDS

All appropriate materials, methods and experimental measurements required in the protocol were recorded and documented in the raw data.

The protocol, final report, raw data or computer generated listings of raw data, and supporting documentation 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. In addition, a non-study specific retention sample of the test substance was collected.

REFERENCES

1. OECD Principles of Good Laboratory Practice (GLP), C(97)186 (Final), 1997.

2. United States Environmental Protection Agency (USEPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice Standards, 40 CFR Part 792, 1989.

3. OECD Guidelines for Testing of Chemicals: Fish, Acute Toxicity Test (OECD Guideline 203, adopted 17 July 1992).

4. U.S. Environmental Protection Agency, Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (Draft, 1996).

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

6. API. Petroleum process stream terms included in the chemical substances inventory under the Toxic Substances Control Act (TSCA). American Petroleum Institute, Washington, DC. February, 1985. 40 pp.

7. American Public Health Association, American Water Works Association and Water Environment Federation. 2005. *Standard Methods for the Examination of Water and Wastewater*, 21th ed. American Public Health Association, Washington, D.C. Method 8010E (Table 8010-I).

8. SAS Version 9.2, Copyright(c) 2002-2008 by SAS Institute Inc., Cary, NC, USA.

9. OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment, no. 23. Organisation for Economic Co-operation and Development, Paris.

Table 1. Analytical Results

Loading Rate* (mg/L)	Measured Hydrocarbon Concentration[1] (mg/L)				Geometric Mean Measured Concentration (mg/L)
	Day 0 (new[2])	Day 1 (old) / Retention[3] (%)	Day 3 (new[2])	Day 4 (old) / Retention[3] (%)	
0 (Control)	ND	ND	ND	ND	--
2.6	0.63	0.41 (65 %)	0.67	0.49 (73 %)	0.54

* Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1] Duplicate analytical samples from the treatment and control solutions were analyzed and the two values for the WAF treatment were averaged.
[2] Samples were collected from each freshly prepared WAF.
[3] Percent retention was determined by dividing the concentration of the old solution to the new solution concentration x 100.
ND = Non Detectable
PQL (Practical Quantitation Limit) = 0.013 mg/L

Table 2. Water Quality Measurements

Loading Rate* (mg/L)	Measurement	Hours							
		0 (new)	24		48		72		96
			old	new	old	new	old	new	old
Control (0)	Temperature[1] (°C)	16.1	15.7	14.4	15.7	14.9	15.6	15.4	16.1
	Dissolved Oxygen[2] (mg/L)	22.22	13.32	20.24	11.05	19.85	11.64	19.14	14.49
	pH	8.25	7.00	7.89	7.48	7.79	7.53	7.49	7.05
2.6	Temperature[1] (°C)	16.3	16.0	14.7	15.8	15.1	15.3	15.4	16.1
	Dissolved Oxygen[2] (mg/L)	22.27	15.53	23.12	8.65	17.23	12.70	21.20	16.65
	pH	8.25	7.01	7.67	7.51	7.83	7.39	7.38	7.11

*Loading rate is defined by the amount of light hydrocracked gas oil per unit volume of dilution water.
[1]All water quality measurements were conducted on an aliquot of each respective test solution collected for water quality and not measured in exposure chamber.
[2]Dilution water was super saturated with oxygen prior to test substance addition in order to achieve acceptable dissolved oxygen concentrations throughout the exposure.

Table 3. Summary of Mortality and Observations

Loading Rate* (mg/L)	Observation (%)	Hours				
		6	24	48	72	96
0 (Control)	Mortality	0	0	0	0	0
	Observed Normal	100	100	100	100	100
2.6	Mortality	0	0	0	0	0
	Observed Normal	100	100	100	100	100

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

APPENDIX A - ANALYTICAL METHOD and RESULTS

Standards and samples of light hydrocracked gas oil (CAS No. 64741-77-1) were analyzed by static headspace gas chromatography with flame ionization detection (HS GC-FID). Analysis was performed on a Perkin Elmer Autosystem XL gas chromatograph with a 30 m x 0.53 mm id, 1.5 µm film DB-5 (J&W Scientific) analytical column. The transfer line of a Perkin-Elmer TurboMatrix 40 Trap Headspace Sampler was connected directly to the analytical column. The headspace sampler was operated in the trap mode using a sorbent tube packed with 50:50 Carbopack C and Tenax. The low trap temperature was 35^{o}C and high (desorb) trap temperature was 290^{o}C. 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. Vial pressure was 40 psi, column pressure 30 psi and desorb pressure 20 psi. 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 setting 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. For "new" solutions 2.0 mL water sample aliquots were 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 and 8.0 mL diluent water to yield a final volume of 10 mL. As with the headspace gas oil standards, WAF sample vials were crimp sealed and shaken to solubilize the sodium sulfate prior to analysis. The same procedure was followed for "old" WAF solutions except that 3.0 mL were sampled and added to vials containing 7.0 mL diluent water.

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

APPENDIX A - ANALYTICAL METHOD and RESULTS (CONT'D)

Light hydrocracked gas oil 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 upper plot displays a 197 ng/mL concentration gas oil standard. The middle plot represents a Day 0 "new" control sample and bottom chromatogram represents a Day 0, 2.6 mg/L sample. The total area integrated for the detected hydrocarbons was used for quantification. The o-xylene internal standard eluted at about three minutes under the analytical conditions utilized. The practical quantitation limit (PQL) was approximately 13 ng/mL (0.013 µg/mL) 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.

Table A1. Individual Analytical Results

Sample	Day 0 (new)	Day 1 (old)	Day 3 (new)	Day 4 (old)
Control D1	ND	ND	ND	ND
Control D2	ND	ND	ND	ND
2.6 mg/L D1	0.619	0.426	0.702	0.522
2.6 mg/L D2	0.641	0.399	0.636	0.453

D1 & D2 represent duplicate analyses of each exposure solution.
ND = Not Detected.
PQL = is 0.013 µg/mL (lowest analytical standard)
Results expressed as µg/mL.

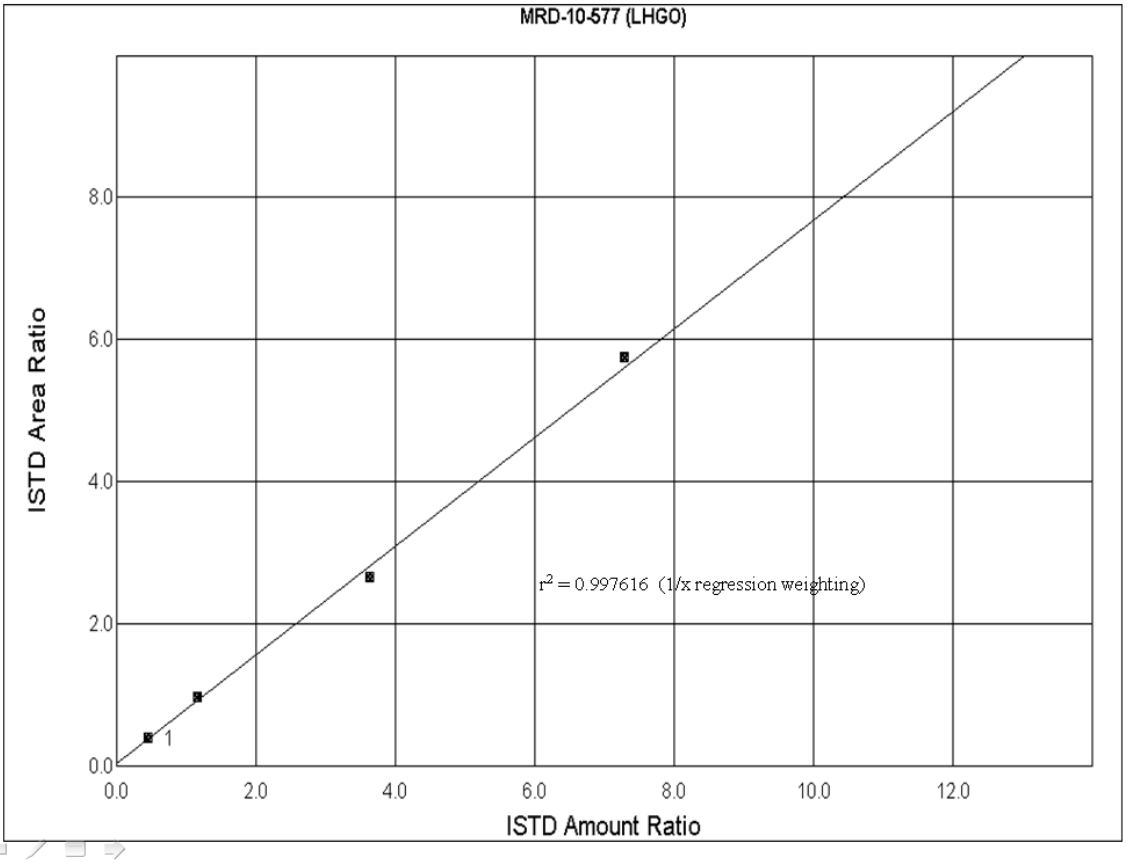
[redacted]
Laboratory Coordinator; Principal Investigator for
Characterization & Analysis of Test Solutions

19 Dec 2011
Date

APPENDIX A - ANALYTICAL METHOD and RESULTS (CONT'D)

Figure A-1

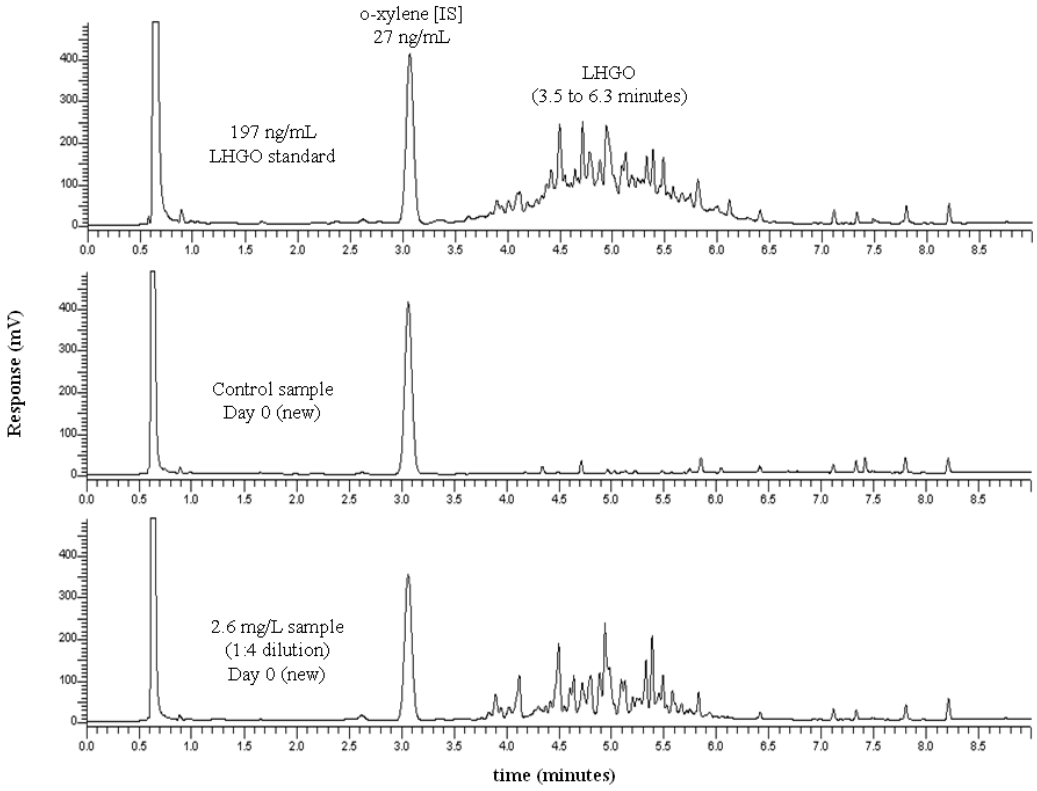
Gas Oil Standard Curve



APPENDIX A - ANALYTICAL METHOD and RESULTS (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 definitive testing. The purpose of the equilibration trial was to confirm the analytical method to be used in subsequent testing, to determine the optimum mixing duration to use in WAF preparation and to evaluate the stability of the WAF solutions once they were produced. The stability information was used to establish the renewal interval for a chronic test with *Daphnia magna*, and to determine whether or not a renewal was needed for the acute test with *D. magna*.

Mixtures of hard reconstituted water and test substance were prepared at loading levels of 0.1, 0.5 and 5.0 mg/L. 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. 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 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 consistent 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 24 hours, the measured concentrations for the 0.1 and 0.50 mg/L WAF solutions represent 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 samples 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.

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

Table B1. WAF Equilibration Trial Results

Loading Rate*	Measured Hydrocarbon Concentration in WAF (mg/L)					
	24 hour mix	% solubility[1]	48 hour mix	% solubility	72 hour mix	% solubility
0.1 mg/L - 1	0.028	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.

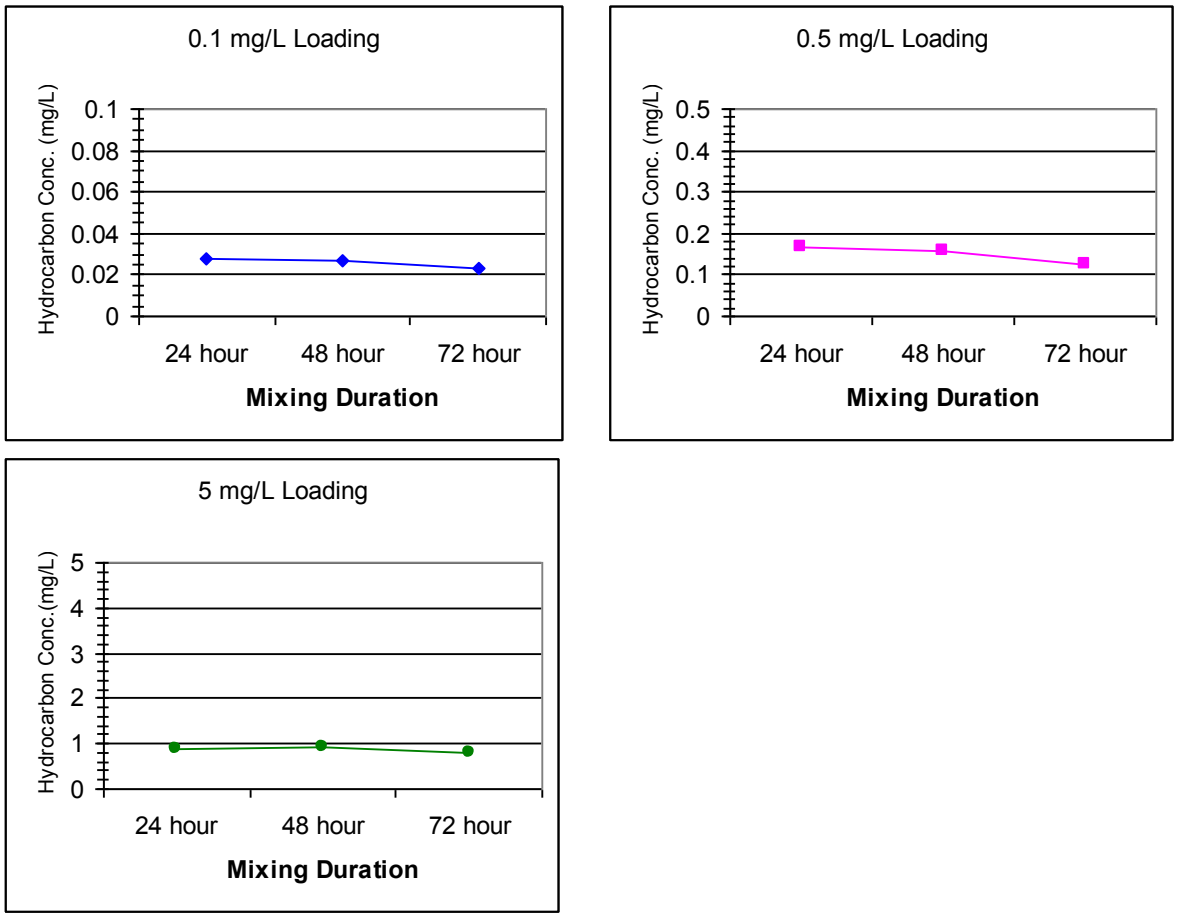


Figure B1. Concentration plots of measured hydrocarbons in WAFs at different mixing times and loading rates.

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

Assessment of WAF Stability

The WAF stability was assessed primarily to establish the renewal interval to be used in the chronic test with *Daphnia magna*, and determine whether a renewal was necessary for the acute *D. magna* test. 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, samples were collected at each loading level directly into screw-top sealed test chambers (130 mL, no headspace) identical to those anticipated for use in the definitive *D. magna* acute study.

For WAF stability related to a 21-day chronic exposure, 2 L of WAF was removed from the 48 hour mix of the 0.1 and 0.5 mg/L loading level WAFs and 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 *D. magna* life cycle study.

All test chambers were set aside under environmental conditions similar to that used for testing. At 24 and 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 (81%)	0.025 (93%)	0.024 (89%)	0.023 (85%)
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 a 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, it was determined that a renewal is not necessary for the 48-hour daphnid acute testing and that a 48-hour renewal period will suffice for the chronic test.

APPENDIX C - DILUTION WATER ANALYSIS

The dilution water was prepared from UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless-steel pipes. Batches of 500 L of this deionized water are reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater, 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 $CaCO_3$[1] (mg/L)	Hardness as $CaCO_3$ (mg/L)[2]	pH	Temperature (°C)	Dissolved Oxygen (mg/L)	Total Organic Carbon (ppm)[3]
Batch 644	62	100	7.9	23.1	8.76	0.26

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 - IN-LIFE OBSERVATIONS

Test Day: 0 6 Hours Date: 13 June 2011

Loading Rate* (mg/L)	0 (Control)	2.6
Daily Mortality	0	0
Cumulative Mortality	0	0
Normal	7	7
Survival	7	7

Test Day: 1 24 Hours Date: 14 June 2011

Loading Rate* (mg/L)	0 (Control)	2.6
Daily Mortality	0	0
Cumulative Mortality	0	0
Normal	7	7
Survival	7	7

Test Day: 2 48 Hours Date: 15 June 2011

Loading Rate* (mg/L)	0 (Control)	2.6
Daily Mortality	0	0
Cumulative Mortality	0	0
Normal	7	7
Survival	7	7

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

APPENDIX D – IN-LIFE OBSERVATIONS (CONT'D)

Test Day: 3 72 Hours Date: 16 June 2011

Loading Rate* (mg/L)	0 (Control)	2.6
Daily Mortality	0	0
Cumulative Mortality	0	0
Normal	7	7
Survival	7	7

Test Day: 4 96 Hours Date: 17 June 2011

Loading Rate* (mg/L)	0 (Control)	2.6
Daily Mortality	0	0
Cumulative Mortality	0	0
Normal	7	7
Survival	7	7

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

Length and Weight of the Control Fish at Test Termination

Organism #	Total Length (mm)	Weight (g)
1	4.2	0.584
2	4.0	0.491
3	4.5	0.637
4	4.0	0.405
5	3.9	0.450
6	4.2	0.555
7	4.1	0.550
Mean	4.1	0.525

APPENDIX E

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.

[redacted] Principal Investigator for Characterization (located at the testing facility)

8 Nov 2014
Date

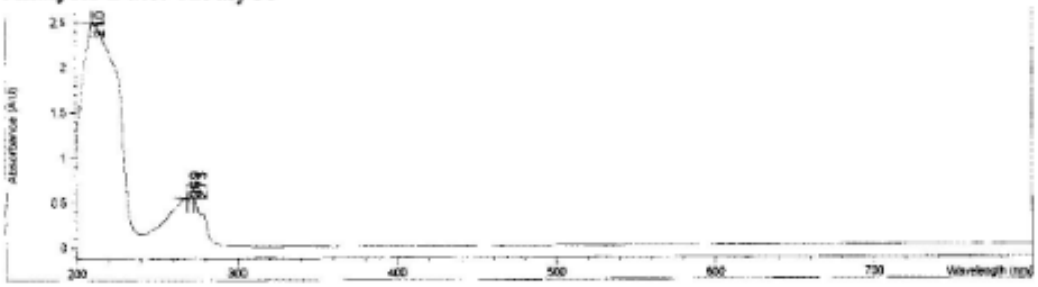
APPENDIX E

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure: UV-VIS-1
Initial

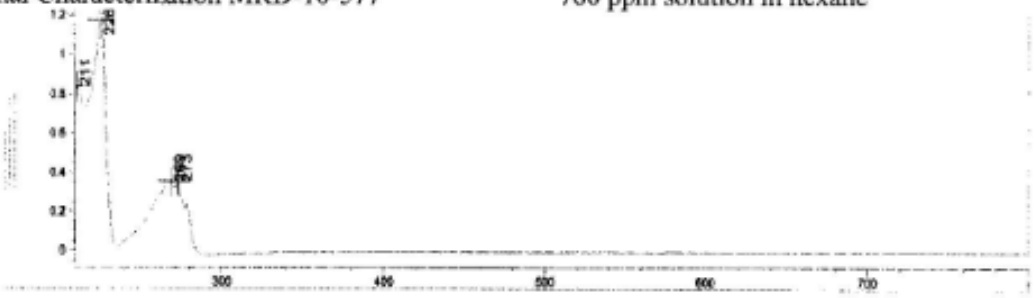
Initial Characterization MRD-10-577 908 ppm solution in hexane
Analysis Date: 12July10



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 E

TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FT-IR SPECTRA

Figure: FTIR-1
Initial

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

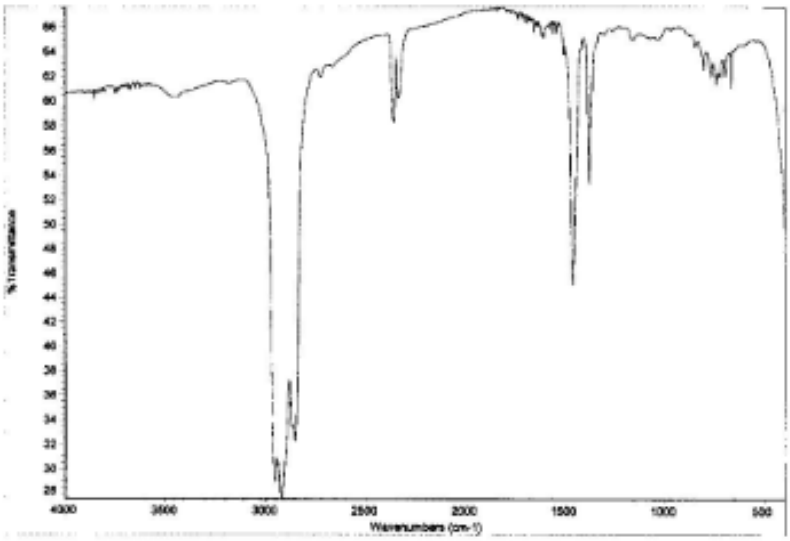
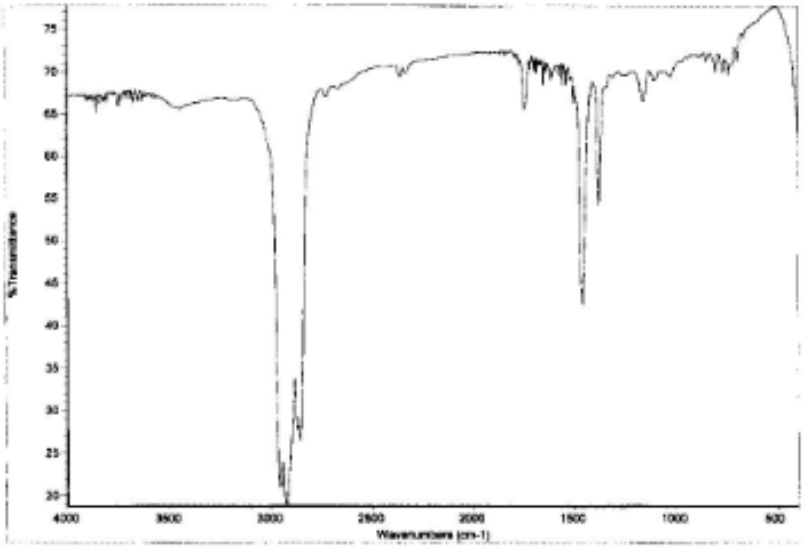


Figure: FTIR-2
Final

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



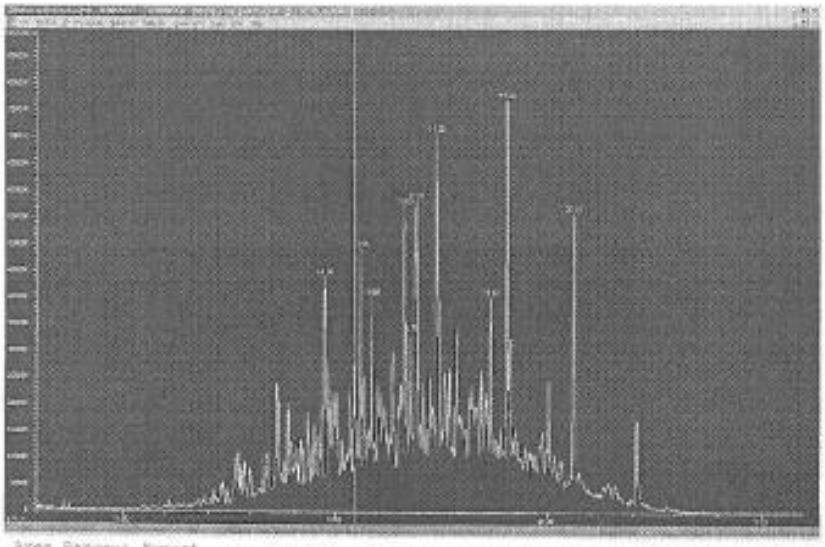
APPENDIX E

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	67.65%	11.848%
2	15.589	1597	1603	1608	VV 3	396816	10077761	50.99%	8.930%
3	15.845	1631	1636	1643	VV	300934	6897086	34.91%	6.115%
4	16.630	1729	1733	1745	VV 3	456236	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	462340	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	19.060	2026	2037	2048	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

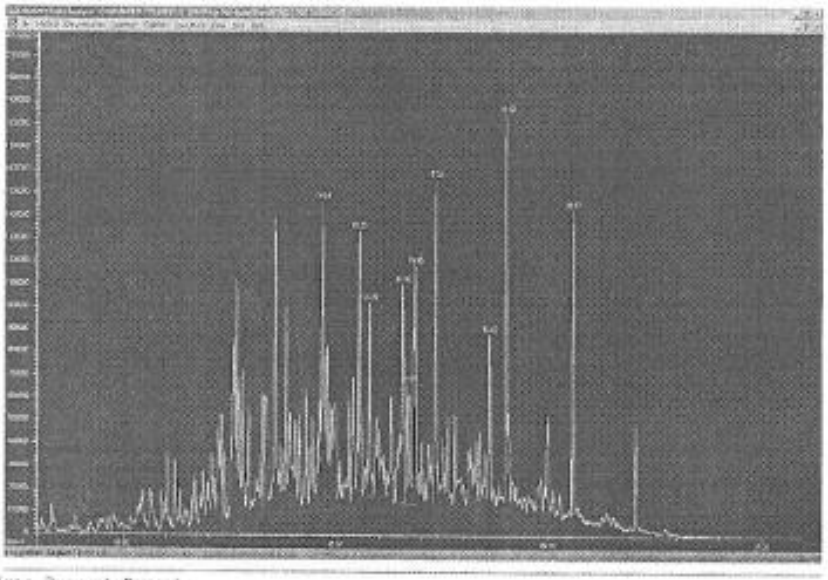
APPENDIX E

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 Inst
Misc : distillates (petroleum) light hydrocrack Multiplr: 1.00
Sample Amount: 0.00

MS Integration Params: 577.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	14.681	2263	2353	2363	VV 5	1083640	31865216	73.62%	11.691%
2	15.554	2399	2614	2421	BV 2	1139110	25500505	58.92%	9.356%
3	15.788	2455	2462	2472	VV	851466	17619089	40.71%	6.465%
4	16.566	2603	2609	2614	VV 2	957842	20580868	47.57%	7.551%
5	16.686	2624	2632	2646	VVA5	526058	16422951	37.95%	6.026%
6	16.938	2666	2664	2682	VV 7	1050447	43281045	100.00%	15.879%
7	17.340	2764	2755	2772	VV 3	1376660	38921803	89.93%	14.279%
8	18.614	2989	2996	3011	VV 3	718156	13237548	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: 272573833

26JUL03.D CHAR2010.M Tue Jul 26 14:20:51 2011

APPENDIX F – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION

Intertek

Report of Analysis **INTERIM**

[redacted]

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 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 %

Page 4 of 14
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 F – SPONSOR SUPPLIED TEST SUBSTANCE INFORMATION (CONT'D)

Intertek

Report of Analysis **INTERIM**

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 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 %

Page 5 of 14
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 F – 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 G – PROTOCOL AND PROTOCOL REVISIONS

PROTOCOL

Contract Number:	EMBSI 2010-104821
Study Title:	*Oncorhynchus mykiss,* Fish Acute Toxicity Test on Water Accommodated Fractions of a Light Hydrocracked Gas Oil
EMBSI Study Number:	1057758
Test Substance:	Gas oil; CAS RN 64741-77-1, Distillates (petroleum), light hydrocracked
EMBSI Test Substance Code:	MRD-10-577
Date:	April 15, 2011
Room Number:	LE-337

Proposed Key Dates:

WAF Equilibration and Stability Trial	Sep 13, 2010
Initial Characterization	Jul 12, 2010
Experimental Start	Jun 13, 2011
Experimental Termination	Jun 17, 2011
Draft Report Completion	Jul 29, 2011
Final Report Completion	Sep 8, 2011

Approved By:

[redacted] 31 May 11
Date

Study Director
ExxonMobil Biomedical Sciences, Inc.
1545 US Highway 22 East
Annandale, New Jersey 08801-0971

[redacted] 26 May 2011
Date

Sponsor Representative
American Petroleum Institute
Washington DC

SAFETY FIRST

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

INTRODUCTION

Objective

This study will be conducted for the sponsor to evaluate the acute toxicity of the water accommodated fractions (WAFs) of a light hydrocracked gas oil, CAS 64741-77-1 (MRD-10-577) to the rainbow trout, *Oncorhynchus mykiss* in a 96-hour semi-static 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 OECD[1] and EPA[2] guidelines, and will be performed in compliance with OECD[3] and USEPA[4] GLP standards. The general methodology of the test will be based upon the Upper Threshold Concentration (UTC) approach proposed by Jeram et. al[5].

Justification for Selection of Test System

Oncorhynchus mykiss 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 G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

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)[6].

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

Characterization of Test Substance

Pre-test and post-test characterization and stability analysis will include the following determinations: FT-IR and UV-Vis spectra, density, physical-state, miscibility in water, methanol and/or hexane and GC-MS "fingerprint" of the neat test substance. The GC-MS fingerprint is run against an ASTM hydrocarbon standard mixture. The ASTM D2887 standard will be applied for higher boiling mixtures with compounds eluting between approximately n-octane (n-C8) and n-triacontane (n-C30). Due to the complex nature of the test substance, no reporting will be made of 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. A statement will be provided by the testing facility specifically addressing whether the test substance was stable over the course of the testing period based on the set of analyses.

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.

Analysis of Test Solutions

Samples will be taken from each "new" treatment WAF and control solution on Day 0 and Day 3. Samples will also be taken on Day 1 and Day 4 (composite of replicates) of the "old" solutions. The samples will be taken with no headspace and refrigerated pending analysis. 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. Due to the complex nature of the test substance, no reporting will be made of specific hydrocarbon components. A detailed description of the analytical methods used will be documented in the raw data and included in the final report.

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS CONT'D

Sample Retention

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

Dilution Water

Reconstituted moderately hard water[7] will be prepared using reagent grade chemicals ($NaHCO_3$, $CaSO_4$,$MgSO_4$, and KCl), and will be aerated prior to use. The hardness range will be between approximately 80-100 mg $CaCO_3$/L.

Test System

Oncorhynchus mykiss

Supplier

The test organisms will be obtained from a commercial fish hatchery. The supplier will be documented in the raw data and final report.

Husbandry and Acclimation

Upon arrival from an outside supplier, fish will be quarantined, observed for parasites and disease for at least 12 days prior to use in the test. Fish will be held for at least 7 days in dilution water at test temperature of 15±1°C, continuously aerated to maintain the dissolved oxygen level at ≥ 80% of the saturation value. Fish will also be held under a light regime of 16light:8dark with a light intensity similar to that of the definitive test. If needed, treatment for disease is administered as per laboratory procedures. No fish will be used for testing for a minimum of 14 days after treatment. The batch is considered acceptable as long as mortalities of less than 5% are seen prior to the initiation of testing.

Any remaining fish from the stock population will be maintained for a reasonable time, so that they may be used for method development, training purposes, etc., following that they will be euthanized following the most appropriate and humane technique. An example of such a method is to use a tricaine methane sulphonate (MS 222) solution, prepared in laboratory dilution water (an MS222 concentration of 500 mg/L in laboratory dilution water pH buffered to 7.0-7.5 could be used).

Fish are held under static conditions using biological and mechanical filtration and are fed daily with Salmon Starter and/or Tetramin®.

Feed Supplier: Finfish Starter - Zeigler Bros. Inc., Gardners, PA
Tetramin® - That Fish Place, Lancaster, PA

Number and Sex

Number: 14 Sex: Not Applicable

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

MATERIALS and METHODS (CONT'D)

Age at Initiation of Exposure

Juveniles of the same age; actual age will be noted in the raw data and final report.

Test System Identification

Organisms will not be individually identified. All test chambers will be labeled to show study number, loading level, replicate, and chamber number.

Selection

Organisms will be randomly assigned to test chambers using a computer generated randomization schedule. A printout of the randomization schedule will be included in the raw data.

To ensure that quality organisms are used for the study, fish will be selected from a pool of organisms larger than that needed for the study. The study director or his designee determines organism suitability.

Feed

Fish are not fed at least 48 hours prior to, or during the study.

Contaminants

There are no known contaminants in the feed used for acclimation or the dilution water believed to be at levels high enough to interfere with this study. The feed will be analyzed for minerals and pesticide residues by New Jersey Feed Lab Inc., 1686 Fifth Street, Trenton, NJ 08638. 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 G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE

Equilibration Trial

A WAF equilibration trial to determine the appropriate WAF mixing duration will not be performed specifically for this study. An equilibrium test was performed as part of the *Daphnia* acute immobilization study (1057742). Stability of the WAF solutions was also evaluated. 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.

Definitive Test Design

GROUP	LOADING LEVEL (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	7 (7 per 1 replicate)
2	2.6	7

The definitive test design will generally conform to the Upper Threshold Concentration (UTC) tiered testing strategy described by Jeram, et al. (2005)[5]. The definitive test loading level was dependent upon the EL50 values generated in the Daphnia Acute Immobilization Test (1057742) and the Algal Growth Inhibition Test (1057767). The lower of the two EL50 values, which was the Algal Growth Inhibition Test (1057767), is defined as the Upper Threshold Concentration (UTC) and is the concentration tested in this study. If the fish LL50 is determined to be less that the UTC, then this protocol will be amended to describe the next phase of testing.

Preparation and Administration of Test Substance

Individual WAFs will be prepared for each loading level by adding the appropriate amount of the test substance to 20 L of dilution water in 20 L glass aspirator bottles. The vessels will be closed using 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 (±1 hour) at test temperature (15±1°C). At the end of mixing, the solutions will be allowed to settle for approximately 1 hour at the test temperature. At the end of the settling period the solutions will be removed from the mixing vessels through the outlet at the bottom of the vessels and placed into the test chamber. An approximately 80% to 90% renewal of the test solutions will be performed at approximately 24-hour intervals. A method such as super saturation with oxygen may be employed prior to chemical addition in order to meet minimum dissolved oxygen level guideline expectations. New WAFs will be prepared daily for the renewals.

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Test Chamber and Volume of Solution

Test chambers will be 4 L or 8 L glass aspirator bottles containing no headspace. The aspirator bottles will be closed using foil covered stoppers.

Exposure Duration

96 hours (± 1 hour)

Environmental Conditions

Acceptable test water conditions: 15±1°C.
Diurnal light: 323-1076 lux (~30-100 lumens/sq. ft.) with a 16 hours light : 8 hours dark photoperiod.

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. If Watchdog is unavailable, manual temperatures and light readings will be taken.

Experimental Evaluation

Observations for mortality will be performed and recorded at 6, 24, 48, 72 and 96 hours (±1 hour) after the beginning of the test. Additional observations may be performed. Fish are considered dead if touching the caudal peduncle produces no reaction and/or no breathing movements are visible. During observations, organisms will also be examined for abnormal behavior or coloration and any dead organisms will be removed.

Observations of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) will be recorded daily at the time of organism observations. If feces are observed in the chambers, they will be removed on a daily basis.

All fish will be euthanized according to the most appropriate and humane method. An example is the use of a tricaine methane sulphonate (MS 222) solution, prepared in laboratory dilution water (an MS222 concentration of 500 mg/L in laboratory dilution water pH buffered to 7.0-7.5 could be used).

All fish will be treated humanely in accordance with published guidance. The study design and personnel training must be sufficient to minimize animal pain within the confines of the study objective.

The Institute for Laboratory Animal Research Journal V37(4), Fish, Amphibians and Reptiles states that "Several methods of euthanasia have been used in fish including hypothermia, electrocution, overdosing with tricaine or carbon dioxide, and a sharp blow to the head. Of these, tricaine administered at 500 mg/L is most desirable (humane) as it does not alter blood cortisol, catecholamine, or glucose levels commonly associated with stress".

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

Control fish will be individually weighed and their total lengths measured.

EXPERIMENTAL PROCEDURE (CONT'D)

Discrete Measurements

Temperature, dissolved oxygen, and pH: measured in each "new" treatment and the control on Day 0 and daily, prior to renewals. Temperature, dissolved oxygen and pH will be measured in each "old" treatment and control solutions daily. Dissolved oxygen levels should remain above 60% of the air-saturation value, however, due to the nature of the test substance the exposure chambers will contain very limited or no headspace. The study will be deemed acceptable if the levels drop below 60%, as long as the control fish show no signs of stress (gasping, surfacing, dark color, etc.). The minimum acceptable air-saturation value will be 50%. The pH level should remain between 6 - 8.

Loading

Loading will not exceed 0.8 g of fish per liter of solution.

Length / Weight of Test System

Length and weight of the organisms used in a study is approximated from measurements of the control organisms at the end of the study. Total length of the fish will be measured. Total length of the fish may be <4.0 cm (the minimum recommended size). The longest fish will not be more than twice the size in length of the shortest fish. Smaller fish may be used to minimize the likelihood of dissolved oxygen depletion due to the nature of the test substance. Past experience with these types of substances has indicated or has shown oxygen depletion in treatment solutions.

Test Acceptability

A test may not be acceptable if more than 10% of the control fish die or exhibit abnormal behavior during the study. The dissolved oxygen level must not drop to a level causing sub-lethal or lethal effects on the control fish, the minimum acceptable level will be 50% of the air-saturation value.

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

EXPERIMENTAL PROCEDURE (CONT'D)

Calculations

Calculations are not required for the threshold test. If less than 50% mortality is observed, the LL50 will be reported as greater than the loading level tested; and the LC50 will be reported as greater than the concentration of hydrocarbons that solubilized from the test substance into the WAF.

If further testing is required, test results will be used to derive the LL50, (or other appropriate statistical value) defined as the loading level of the test substance estimated to kill 50% of the test organisms within a specified period of exposure.

Measured concentrations do not represent all hydrocarbons constituting the test substance. Results expressed as the LC50 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 WAF differs from the parent gas oil, owing to the differing solubilities of individual gas oil hydrocarbons.

The statistical method used to calculate the LL50 and LC50 values will be either a maximum likelihood analysis based on D. J. Finney (1971)[8], a Trimmed Spearman-Karber Method[9], a Binomial Method[10] or a graphical method[11].

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

REPORT

After termination of the study, a final report that includes the following information will be submitted:

Test substance:

- physical nature, and where relevant, physiochemical properties
- identification data

Test fish:

- scientific name, strain (if applicable), size, supplier, any pretreatment, etc.

Test conditions:

- test procedure used, equilibration test results determined in study number 1057742
- dilution-water source, water quality characteristics (pH, hardness, temperature)
- dissolved oxygen concentration, pH values and temperature of the test solutions at 24-hour intervals, lighting regime
- methods of preparation of test solutions
- loading levels/concentrations used
- information on concentrations of the test substance in the test solutions
- number of fish in each test solution
- description of the test chambers, and volume of solution

Results:

- results of chemical analysis and methods used including examples of chromatography (blank, low WAF and standards) and a graphical representation of the standard curve
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative mortality, survival and abnormal responses of the fish

In addition, if further testing is required:

- methods used and results obtained from chemical analysis
- maximum loading level/concentration causing no mortality
- minimum loading level/concentration causing 100% mortality
- LL50 and/or LC50 at each observation interval, if possible.
- statistical procedures followed
- graph of the loading level/concentration-response curve at the end of the test, if applicable

Study Conduct:

- GLP 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

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

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, and supporting documentation 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. In addition, a non-study specific retention sample of the test substance has been taken.

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 and the appropriate guidelines and Good Laboratory Practice Regulations.

GUIDELINE EXCEPTIONS

Due to the limited solubility and potential volatility of the test substance the following exceptions 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.

The OECD Guideline[1] states that the dissolved oxygen concentration must have been at least 60% of the air saturation value throughout the test. While we will strive to meet guideline expectations, we have set our minimum air saturation value to 50%. This is not expected to affect the integrity of the data generated.

The OPPTS Guideline[2] recommends that the concentration of the test substance in solutions be determined at every renewal period. Samples will be taken from each "new" treatment WAF and control solution on Day 0 and Day 3. Samples will also be taken on Day 1 and Day 4 (composite of replicates) of the "old" solutions.

The OPPTS Guideline[2] prescribes a number of chemical analyses be performed on the test water. While many of the tests required are consistent with those performed by Accutest®, 2235 Route 130, Dayton, NJ 08810, not all are performed. This is not expected to affect the integrity of the data generated.

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

REFERENCES

1. OECD Guidelines for Testing of Chemicals: Fish, Acute Toxicity Test (OECD Guideline 203,adopted 17 July 1992).

2. U.S. Environmental Protection Agency, Ecological Effects Test Guidelines, OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine (Draft, 1996).

3. OECD Principles of Good Laboratory Practice (GLP), C(97)186 (Final), 1997.

4. United States Environmental Protection Agency (USEPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice Standards, 40 CFR Part 792, 1989.

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

6. API. Petroleum process stream terms included in the chemical substances inventory under the Toxic Substances Control Act (TSCA). American Petroleum Institute, Washington, DC. February, 1985. 40 pp.

7. American Public Health Association, American Water Works Association and Water Environment Federation. 1999. *Standard Methods for the Examination of Water and Wastewater*, 21st. American Public Health Association, Washington, D.C. Method 8010E. (Table8010-I).

8. Finney, D.J., 1971. Probit Analysis, 3rd Edition, London: Cambridge University Press.

9. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.

10. Stephan, C. E., Methods for Calculating an LC_{50}, *Aquatic Toxicology and Hazard Evaluation, ASTM STP 634*, F. L. Mayer and J. L. Hamelink, Eds., American Society for Testing and Materials, 1977, pp. 65-84.

11. Weber, C.I., 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fourth Edition EPA/600/4-90/027. U.S. Environmental Protection Agency, Cincinnati, OH.

12. OECD, 2000. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment, no. 23. Organisation for Economic Co-operation and Development, Paris.

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

DISTRIBUTION

EMBSI - Clinton:
- Study Director
- Environmental Sciences, Section Head
- Environmental Toxicology and Fate Coordinator
- Environmental Chemistry / Principal Investigator for Characterization/Analysis of Mixtures
- Study Technicians
- Contract Administrator
- QAU

API:
- Sponsor Representative
- Sponsor's Study Monitor

APPENDIX G – PROTOCOL AND PROTOCOL REVISIONS (CONT'D)

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: 1057758 Revision Number: 1 Date: 7 November 2011

Page 1 / Sponsor Representative:

[redacted]

Revised: [redacted]

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

Page 12 / PERSONNEL:

Section Head, Env. Sciences..... [redacted]

Revised:
Section Head, Env. Sciences..... [redacted]

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

DISTRIBUTION

Study Director..... [redacted]
Sponsor Representative .....
Sponsor's Study Monitor .....
Section Head, Env. Sciences .....
Lab Coordinator, Env. Toxicology and Fate.....
Lab Coordinator, Environmental Chemistry,
Principal Investigator Characterization (EMBSI) ..
Study Technicians.....
.....
.....
.....
Quality Assurance Unit.....
Contract Administrator .....

Required signatures:

[redacted]
Sponsor Representative

28 Nov 2011
Date

[redacted]
Study Director

2 Dec 11
Date