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Lyondell Chemical Company  
One Houston Center  
1227 McKinney Street, Suite 700  
P.O. Box 3646  
Houston, Texas 77253-3646

04 JAN 14 AM 7:12  
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VIA COURIER

December 14, 2004

8EHQ-1204-15536

US Environmental Protection Agency  
EPA East Mall/Mail Code 7407M  
1201 Constitution Avenue, NW  
Washington DC 20004

CONTAIN NO CBI

Attention: TSCA 8(e) Coordinator

RE: **8EHQ Number: 8EHQ-04-15536 (Submission of Final Report)  
Heavy Pyrolysis Fuel Oil (Residues, petroleum, steam-cracked; CASRN 64742-90-1)  
Ecotoxicity and Environmental Fate Studies**

Dear Sir or Madam:

In March 2004, Lyondell Chemical Company (Lyondell) submitted information to EPA in accordance with Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide. In that correspondence, Lyondell reported environmental effects information resulting from ecotoxicity and environmental fate studies with Heavy Pyrolysis Fuel Oil stream, a hydrocarbon stream which encompasses the substance identified by CAS Registry Number 64742-90-1 (Residues, petroleum, steam-cracked). This submission was assigned 8EHQ Number 8EHQ-04-15536. At that time, the final reports for these studies were not available. Lyondell has now received the final reports and is hereby providing copies to EPA.

Should you have any questions or require additional details, please do not hesitate to call me at 713-309-2136. I may also be reached by facsimile at 713-951-1574 or by e-mail at patrick.gibson@lyondell.com.

Sincerely,

Patrick L. Gibson  
Product Safety Specialist - Regulatory  
Corporate TSCA Coordinator  
Lyondell Chemical Company



Enclosures

Cc: TSCA 8(e) Files



2005 JAN 31 AM 7:21

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282350

# **ExxonMobil** BIOMEDICAL SCIENCES, INC.

**OLF-105.0-HPV10-EMBSI**

***Daphnia sp.*, ACUTE IMMOBILIZATION TEST on  
HEAVY PYROLYSIS FUEL OIL**

**FINAL REPORT**

**STUDY NUMBER: 176842**

**TEST SUBSTANCE: MRD-03-768**

**PERFORMED FOR:**

**American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209**

**PERFORMED AT:**

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

**COMPLETION DATE: October 29, 2004**

**04TP 115**

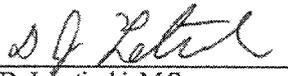
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APPROVAL SIGNATURES

  
\_\_\_\_\_  
J. J. Freeman, Ph.D., D.A.B.T.  
Acting Section Head, Laboratory Operations

13 Oct 04  
Date

  
\_\_\_\_\_  
D. J. Letinski, M.S.  
Supervisor, Environmental Chemistry

15 Oct 04  
Date

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<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards except as outlined on pages 12-14.

  
\_\_\_\_\_  
E. J. Febbo, M.S.  
Study Director  
ExxonMobil Biomedical Sciences, Inc.  
1545 Route 22 East, P.O. Box 971  
Amundale, New Jersey 08801-0971

29 Oct 04  
Date

The final report was accepted by the Sponsor.

  
\_\_\_\_\_  
E. J. Moran, Ph.D.  
Sponsor's Representative

10/21/04  
Date

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QUALITY ASSURANCE STATEMENT

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STUDY NUMBER: 176842

TEST SUBSTANCE: MRD-03-768

STUDY SPONSOR: American Chemistry Council

---

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.

<u>Study Phase Inspected</u>	<u>Date(s) of Inspection</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
Protocol	07 Aug 03	07 Aug 03	13,14 Aug 03
24-Hour Observations	10 Dec 03	10 Dec 03	11,15 Dec 03
Final Report	06 May 04	07 May 04	11,13 May 04
Second Review of Final Report	10 May 04	10 May 04	11,14 May 04

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

  
\_\_\_\_\_  
W. James Bover, Ph.D.  
Data Integrity & Quality Assurance / Archives  
Section Head

12 Oct 04  
Date

**PERSONNEL**

Study Director:	E. J. Febbo, M.S.
Sponsor Representative:	E. J. Moran, Ph.D.
Acting Section Head, Laboratory Operations:	J. J. Freeman, Ph.D., D.A.B.T.  R. L. Rucker, Ph.D. (Prior to December 9, 2003)
Data Integrity & Quality Assurance / Archives Section Head:	W. J. Bover, Ph.D.
Supervisor, Environmental Toxicology & Compound Preparation:	E. J. Febbo, M.S.
Supervisor, Environmental Chemistry:	D. J. Letinski, M.S.

### SUMMARY

This study was performed to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of MRD-03-768 (Heavy Pyrolysis Fuel Oil), to the daphnid, *Daphnia magna* in a 48-hour static test, conducted according to OECD guideline 202.

Individual treatments were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes, to 4.0 L of vehicle/dilution water in glass aspirator bottles (capacity 4.5 L) and stirring on magnetic stirplates using a 5% (of the static liquid depth) vortex for 24 hours. After an additional hour without stirring the aqueous portions (WAFs) were removed for testing. The following table defines the target loading rates, actual loading rates and measured concentrations.

TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	MEASURED CONCENTRATION† (mg/L)
0	0	0
0.41	0.50	0.18
0.90	1.0	1.6
2.0	2.3	1.8
4.5	4.8	4.1
10	10	7.8

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

† Concentration based on mean (Day 0 and termination) measured concentrations.

Four replicates per treatment were tested. Each replicate contained five daphnids. Replicate chambers were 125 mL glass Erlenmeyer flasks containing approximately 140 mL of solution (no headspace) closed with ground glass stoppers. Water quality (temperature, pH, and dissolved oxygen) measurements were recorded in each treatment at the start of the test and in a composite of the replicates at termination (termination includes complete immobilization in a treatment). Observations for immobilization and abnormal behavior or appearance were performed at 24 and 48 hours  $\pm$  1 hour after the beginning of the test.

**SUMMARY (CONT'D)**

Acute toxicity results are expressed as the Effect Loading / Effect Concentration 50 (EL/EC<sub>50</sub>); that is, the actual loading rate or measured concentration of test substance in dilution medium which is calculated to result in 50% immobilization compared to the control for the specified time of exposure. The maximum actual loading rate causing no immobilization after 48-hours was 2.3 mg/L. The maximum measured concentration causing no immobilization after 48-hours was 1.8 mg/L. The minimum actual loading rate causing 100% immobilization after 48-hours was 4.8 mg/L. The minimum measured concentration causing 100% immobilization after 48-hours was 4.1 mg/L.

Hours	EL50 mg/L	EC50 mg/L
24	3.7 (3.3 - 4.2*)	3.0 (2.7 - 3.4*)
48	3.3 (2.3 - 4.8**)	2.7 (1.8 - 4.1**)

\* 95% Confidence Interval

\*\* 99% Confidence Interval

Summary of In-Life Observations		
Loading Rate* (Concentration)** mg/L	24 hour (% Immobilization)	48 hour (% Immobilization)
Control (0)	0	0
0.50 (0.18)	0	0
1.0 (1.6)	0	0
2.3 (1.8)	0	0
4.8 (4.1)	85	100
10 (7.8)	100	†

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and termination) measured concentrations.

† Complete immobilization occurred on Day 1.

No abnormal behavior or appearance was observed in the daphnids that were alive.

## INTRODUCTION

### ***Objective***

This study was conducted for the Sponsor to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of the test substance, to the daphnid, *Daphnia magna*, in a 48-hour static test.

### ***Sponsor***

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209

### ***Testing Facility***

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

### ***Study Initiation Date***

September 15, 2003

### ***In-life Test Period***

December 9, 2003 to December 11, 2003

### ***Experimental Termination***

December 16, 2003

### ***Compliance***

This test was conducted in compliance with OECD<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards with the exceptions listed on pages 12-14 and in agreement with OECD<sup>3</sup> Guideline 202 with the exceptions listed on pages 19.

## MATERIALS and METHODS

### *Test Substance Identification*

EMBSI Identification: MRD-03-768  
Industry Stream Name (acronym): Heavy Pyrolysis Fuel Oil

CAS Number:  
68513-69-9  
64741-62-4  
69013-21-4  
8002-05-9

CAS Inventory Name:  
Residues, petroleum, steam-cracked light  
Clarified oils, petroleum, catalytic cracked  
Fuel oil, pyrolysis  
Petroleum

Supplier: Dow Chemical Company  
Freeport, TX  
Date Received: July 29, 2003  
Expiration Date: July 2008  
Description: Dark Amber liquid

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

### *Stream Derivation*

In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.

### *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.

### *Vehicle / Dilution Water*

Reconstituted water<sup>4</sup> (Batch 137A). Dilution water was aerated prior to the addition of the test substance. Ca/Mg ratio 1.2:1; Na/K ratio 12.5:1. See Appendix A, page 27 for the vehicle/dilution water analysis.

## MATERIALS and METHODS (CONT'D)

### *Characterization of the Test Substance*

The neat test substance was characterized and the stability determined by the testing facility using the following analyses: UltraViolet/Visible and Infrared Spectrophotometry, Gas Chromatography with Mass Selective Detection and Density. The test substance was used in a number of studies at the testing facility. Characterization of the test substance was performed at the testing facility prior to its use in the first of these studies and after completion of the final study. Documentation of characterization and stability assessment is maintained at the testing facility (see Appendix B, page 28).

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, was considered the "pure" substance.

### *Analysis of Mixtures*

Samples of each treatment WAF and the control were taken on Day 0 prior to the start of the study and on a composite of the replicates at termination (termination includes complete immobilization in replicate or treatment). The method of analysis was gas chromatography with flame ionization detection (GC-FID). Samples were analyzed using a Perkin Elmer Autosystem XL Gas Chromatograph. Analytical standards were prepared and analyzed at concentrations bracketing the sample concentrations. The methods and results of the analyses are included in Appendix A starting on page 25.

### *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*

Cultured at the test facility. Original culture supplied by Aquatic Biosystems, Inc., Fort Collins, Colorado. Starter culture received 11-Apr-02.

## MATERIALS and METHODS (CONT'D)

### *Husbandry and Acclimation*

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

Cultures of *Daphnia magna* are fed *Pseudokirchneriella subcapitata* (approximately  $4.5 \times 10^5$  cells/mL) and 4.0 mL of a yeast / salmon starter / wheat grass (YTC) mixture per 800 mL daily (five days per week at a minimum). YTC and algae were supplied by Aquatic Biosystems, Inc., Fort Collins, Colorado.

### *Number and Sex*

Number: 120

Sex: Not applicable

### *Age at Initiation of Exposure*

<24 hours old, taken from 13-day old parents.

### *Test System Identification*

Each replicate, containing five daphnids, was labeled to show study number, loading rate, replicate and randomization number.

### *Selection*

Organisms were randomly assigned to intermediate chambers using a computer-generated randomization schedule, they were then transferred to their respective test chambers. The test chambers were randomly positioned within the test area. A printout of the randomization schedule is included in the raw data.

To ensure that quality organisms were used for the study, neonates from parents 13 days old (with  $\leq 20\%$  adult mortality) were selected. Neonates were selected from a pool of organisms larger than that needed for the study. The pool of neonates had  $\leq 10\%$  daily mortality on the experimental start day. The study director or his designee determined organism suitability.

## MATERIALS and METHODS (CONT'D)

### *Feed*

Daphnids were not fed during the study.

### *Contaminants*

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

## EXPERIMENTAL PROCEDURE

### *Equilibrium Test*

An equilibrium study was performed prior to testing to determine the most appropriate mixing duration. Gas chromatography with flame ionization detection (GC-FID) was used to detect the test substance. Two sets of individual WAFs at approximately 1 mg/L and 10 mg/L were prepared and sampled after approximately 24 and 48 hours. The solutions were stirred using a  $\leq 10\%$  (of the static liquid depth) vortex. All mixing vessels were closed using foil covered rubber stoppers during mixing. Three replicates were analyzed at each sampling interval. The equilibrium phase did not extend beyond 48-hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test (to be used for the fish study). The goal was to achieve equilibrium of the soluble components without compromising a loss of the lighter components (it is the lighter components that usually contribute to toxicity). The results indicate that a mixing duration of 24 hours was sufficient for the soluble components of the test substance to achieve equilibrium. This phase of the study was not subject to GLP standards.

Summary of Equilibrium Test Results		
Loading Rate (mg/L)	24 Hour Stirring* (mg/L)	48 Hour Stirring* (mg/L)
1	0.455 (SD = 0.013)	0.435 (SD = 0.018)
10	6.79 (SD = 0.15)	6.70 (SD = 0.049)

1 mg /L PQL (Practical Quantitation Limit) = 0.1 mg/L

10 mg /L PQL (Practical Quantitation Limit) = 0.7 mg/L

\* Average of three replicates.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Range Finding Test*

A 48-hour range finding test was performed to determine the loading rates of Heavy Pyrolysis Fuel Oil (MRD-03-768) for the definitive *Daphnia sp.* Acute Immobilization Test.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.1 mg/L, 1 mg/L, 10 mg/L and 100 mg/L. The actual loading rates were determined to be 0.11 mg/L, 1.0 mg/L, 9.8 mg/L and 98 mg/L. A control treatment consisting only of the vehicle/dilution water also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes, to the vehicle/dilution water in glass aspirator bottles (mixing vessels) containing Teflon<sup>®</sup> coated stir bars. The syringes were weighed with and without test substance to determine the actual loading rates. The mixing vessels were sealed with foil covered rubber stoppers and the treatments were stirred using an 5% (of the static liquid depth) vortex at room temperature (approximately 22°C) on magnetic stirplates for approximately 23.5 hours. As stirring initiated and after stirring, all treatments appeared clear and colorless with test substance evident on the surface. The mixtures were allowed to equilibrate to test temperature for one hour without stirring before removing the aqueous portions (WAFs) for testing.

Two replicates per treatment were tested. Each replicate contained five daphnids. Replicate chambers were 125 mL glass Erlenmeyer flasks containing approximately 140 mL of solution (no headspace) closed with ground glass stoppers. Water quality (temperature, pH, and dissolved oxygen) measurements were recorded in each treatment at the start of the test and in a composite of the replicates at termination (termination includes complete immobilization in a treatment). Observations for immobilization and abnormal behavior or appearance were performed at 24 and 48 hours  $\pm$  1 hour. This phase of the study was not subject to GLP standards.

**EXPERIMENTAL PROCEDURE (CONT'D)**

<b>Summary of Water Quality Measurements at study start and termination</b>			
<b>Loading Rate (mg/L)</b>	<b>pH</b>	<b>Dissolved Oxygen (mg/L)</b>	<b>Temperature (°C)</b>
<b>0 (Control)</b>	7.9 - 8.1	8.0 - 8.2	20.5 - 20.9
<b>0.11</b>	8.1	8.0 - 8.2	20.4 - 20.8
<b>1.0</b>	8.0 - 8.1	8.0 - 8.3	20.4 - 20.8
<b>9.8</b>	8.0	7.9 - 8.2	20.7 - 20.9
<b>98</b>	8.0	8.0 - 8.3	20.6 - 20.9

<b>Summary of In-Life Observations</b>		
<b>Loading Rate (mg/L)</b>	<b>24 hour (% Immobilization)</b>	<b>48 hour (% Immobilization)</b>
<b>0 (Control)</b>	0	0
<b>0.11</b>	0	0
<b>1.0</b>	0	0
<b>9.8</b>	100	*
<b>98</b>	100	*

\* Complete immobilization occurred on Day 1.  
 No abnormal behavior or appearance was observed in the Daphnids that were alive.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Definitive Test Design*

GROUP	TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	0	20 (5 per 4 replicates)
2	0.41	0.50	20
3	0.90	1.0	20
4	2.0	2.3	20
5	4.5	4.8	20
6	10	10	20

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

### *Preparation and Administration of Test Substance*

Individual treatments were prepared by adding the appropriate amount of test substance to 4.0 L of laboratory vehicle/dilution water in glass aspirator bottles (capacity 4.5 L). The test substance was added to the water in the aspirator bottles using stainless steel and glass syringes. The syringes were weighed before and after adding the test substance to determine the actual loading rate. The mixing vessels were sealed with foil covered rubber stoppers. The mixtures were stirred using a 5% (of the static liquid depth) vortex for 24 hours on magnetic stirplates with Teflon<sup>®</sup> coated stirbars at room temperature (23.1-23.4°C). As stirring initiated and after stirring, all treatments appeared clear/colorless with the test substance floating at the surface. The mixtures were allowed to equilibrate to test temperature in a waterbath at 20.2°C for 1 hour without stirring before removing the aqueous portions (WAFs) for testing.

Four replicates were prepared for each treatment by completely filling the test chambers with the WAF (no headspace). Four replicates of the control were prepared in the same manner using laboratory dilution water.

### *Test Chamber / Volume of Solution*

The test chambers were 125 mL size glass Erlenmeyer flasks containing approximately 140 mL of solution (no headspace). The test chambers were closed with ground glass stoppers to minimize contamination, evaporation and/or volatilization.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Exposure Duration*

48 hours ( $\pm$  1 hour)

### *Exposure Conditions*

Mean test temperature: 20.1°C (S.D. = 0.1), continuously monitored by computer in the test area.

Diurnal light: approximately 16 hours light and 8 hours dark. Daylight intensity ranged from approximately 100 to 113 lux during full daylight periods of the study.

An environmental conditions study was activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

### *Experimental Evaluation*

Observations for immobilization of the daphnids were made on each replicate at 24 and 48 hours ( $\pm$  1 hour). 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 daphnid behavior or appearance were made.

Water quality measurements (pH, dissolved oxygen and temperature) were performed on a sub-sample of the WAFs from each treatment and control on Day 0 and in a composite of the replicates from each treatment at termination (termination includes complete immobilization in a treatment). No undissolved test substance was observed in any of the test chambers. After completion of the study, the test organisms were discarded and monitoring of the environmental conditions was discontinued.

### *Organism Loading During the Definitive Study*

Approximately 28 mL of solution per daphnid.

### *Calculations*

The 24-hour  $EL_{50}$  and  $EC_{50}$  values were determined using a Trimmed Spearman-Kärber Method<sup>5</sup>. A Binomial Method<sup>6</sup> was used to determine the 48-hour  $EL_{50}$  and  $EC_{50}$  values.

## RESULTS

Acute toxicity results are expressed as the Effect Loading / Effect Concentration 50 (EL/EC<sub>50</sub>); that is, the actual loading rate or measured concentration of test substance in dilution medium which is calculated to result in 50% immobilization compared to the control for the specified time of exposure. The maximum actual loading rate causing no immobilization after 48-hours was 2.3 mg/L. The maximum measured concentration causing no immobilization after 48-hours was 1.8 mg/L. The minimum actual loading rate causing 100% immobilization after 48-hours was 4.8 mg/L. The minimum measured concentration causing 100% immobilization after 48-hours was 4.1 mg/L.

Hours	EL50 mg/L	EC50 mg/L
24	3.7 (3.3 - 4.2*)	3.0 (2.7 - 3.4*)
48	3.3 (2.3 - 4.8**)	2.7 (1.8 - 4.1**)

\* 95% Confidence Interval

\*\* 99% Confidence Interval

Summary of In-Life Observations		
Loading Rate* (Concentration)** mg/L	24 hour (% Immobilization)	48 hour (% Immobilization)
Control (0)	0	0
0.50 (0.18)	0	0
1.0 (1.6)	0	0
2.3 (1.8)	0	0
4.8 (4.1)	85	100
10 (7.8)	100	†

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and termination) measured concentrations.

† Complete immobilization occurred on Day 1.

No abnormal behavior or appearance was observed in the Daphnids that were alive.

Table 1 presents the in-life observation data for the replicates in each treatment. Table 2 presents the water quality values measured during the test. Figure 1 presents the loading rate/concentration response curve at 24 and 48 hours. Appendix A presents the analytical chemistry methods and results.

The test was considered acceptable as none of the control organisms were immobilized or trapped at the surface and the dissolved oxygen levels remained above 60% of the air saturation value at the temperature tested.

### CONCLUSION

After *Daphnia magna* were exposed to WAFs prepared from Heavy Pyrolysis Fuel Oil for 48-hours, the EL<sub>50</sub> was 3.3 mg/L and the EC<sub>50</sub> was 2.7 mg/L.

### GUIDELINE EXCEPTIONS

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

The concentration of the test substance in solutions was not determined prior to use as indicated in the guideline. The initial concentration of the test substance was not maintained at 80% in the lowest loading rate throughout the test, 74% of the initial concentration was maintained.

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

### PROTOCOL DEVIATIONS

No protocol deviations occurred for this study.

### 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.

## REFERENCES

1. Organization for Economic Cooperation and Development (OECD), Principles of Good Laboratory Practice, C(97)186 (Final), 1997.
2. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
3. OECD, Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 202: *Daphnia sp.* Acute Immobilisation Test. Adopted 4 April 1984.
4. American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 8010E (Table 8010:I).
5. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.
6. Stephan, C. E., Methods for Calculating an LC<sub>50</sub>, *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.

**TABLE 1 - DEFINITIVE STUDY, IN-LIFE OBSERVATIONS**

Test Day: 1      Date: 10-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)				0.50 (0.18)				1.0 (1.6)			
	1	2	3	4	1	2	3	4	1	2	3	4
Replicate	0	0	0	0	0	0	0	0	0	0	0	0
Daily Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Immobilization	5	5	5	5	5	5	5	5	5	5	5	5
Normal	5	5	5	5	5	5	5	5	5	5	5	5
Survival	5	5	5	5	5	5	5	5	5	5	5	5

Loading Rate* (Concentration**) mg/L	2.3 (1.8)				4.8 (4.1)				10 (7.8)			
	1	2	3	4	1	2	3	4	1	2	3	4
Replicate	0	0	0	0	3	4	5	5	5	5	5	5
Daily Immobilization	0	0	0	0	3	4	5	5	5	5	5	5
Cumulative Immobilization	5	5	5	5	2	1	0	0	0	0	0	0
Normal	5	5	5	5	2	1	0	0	0	0	0	0
Survival	5	5	5	5	2	1	0	0	0	0	0	0

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and termination) measured concentrations.

TABLE 1 - DEFINITIVE STUDY, IN-LIFE OBSERVATIONS (CONT'D)

Test Day: 2

Date: 11-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)				0.50 (0.18)				1.0 (1.6)			
	1	2	3	4	1	2	3	4	1	2	3	4
Replicate	0	0	0	0	0	0	0	0	0	0	0	0
Daily Immobilization	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Immobilization	5	5	5	5	5	5	5	5	5	5	5	5
Normal	5	5	5	5	5	5	5	5	5	5	5	5
Survival	5	5	5	5	5	5	5	5	5	5	5	5

Loading Rate* (Concentration**) mg/L	2.3 (1.8)				4.8 (4.1)				10 (7.8)			
	1	2	3	4	1	2	3	4	1	2	3	4
Replicate	0	0	0	0	2	1	0	0	0	0	0	0
Daily Immobilization	0	0	0	0	5	5	5	5	5	5	5	5
Cumulative Immobilization	5	5	5	5	0	0	0	0	0	0	0	0
Normal	5	5	5	5	0	0	0	0	0	0	0	0
Survival	5	5	5	5	0	0	0	0	0	0	0	0

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and termination) measured concentrations.

**TABLE 2 - DEFINITIVE STUDY, WATER QUALITY VALUES**

Test Day: 0      Date: 9-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)	0.50 (0.18)	1.0 (1.6)	2.3 (1.8)	4.8 (4.1)	10 (7.8)
Dissolved Oxygen (mg/L)	8.6	8.5	8.5	8.4	8.4	8.3
pH	8.0	7.9	7.8	7.8	7.8	7.8
Temperature (°C)	20.0	20.1	20.1	20.0	20.1	20.1

Test Day: 2      Date: 11-Dec-03      composite of remaining replicates

Loading Rate* (Concentration**) mg/L	Control (0)	0.50 (0.18)	1.0 (1.6)	2.3 (1.8)	4.8† (4.1)†	10‡ (7.8)‡
Dissolved Oxygen (mg/L)	8.0	8.0	8.0	8.0	8.3	8.3
pH	8.1	8.0	8.0	8.0	8.0	8.0
Temperature (°C)	20.3	20.3	20.4	20.4	20.4	20.5

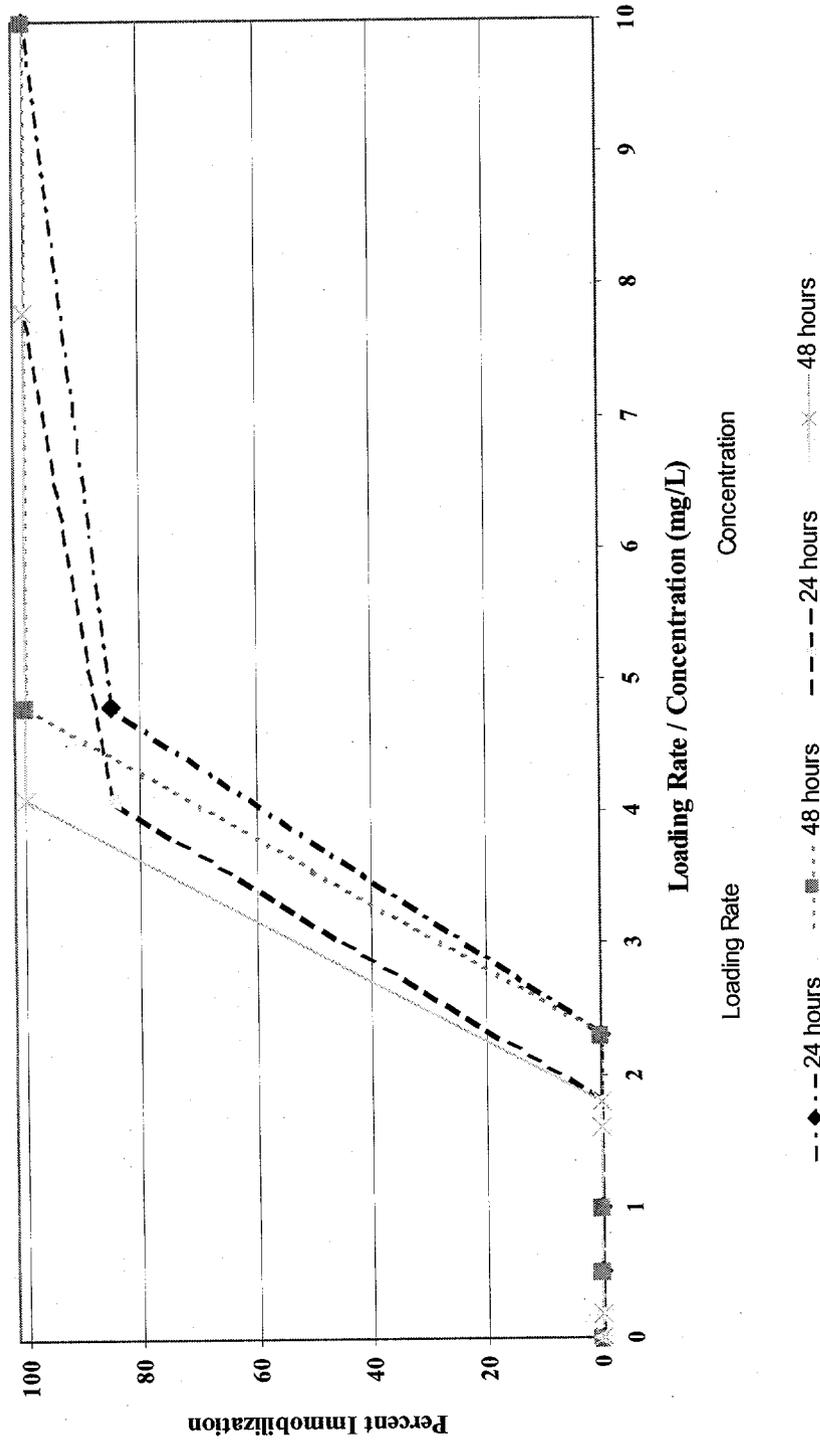
\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and termination) measured concentrations.

† Water quality performed on Day 1 (composite of replicates 3 and 4) due to complete immobilization.

‡ Water quality performed on Day 1 (composite of replicates 1 - 4) due to complete immobilization.

FIGURE 1 - LOADING RATE / CONCENTRATION RESPONSE CURVE



## APPENDIX A - ANALYTICAL METHODS and RESULTS

Heavy Pyrolysis Fuel Oil WAF samples (ca. 125 mL) were collected with no headspace in amber glass bottles with Teflon<sup>®</sup> lined caps. Samples were refrigerated pending extraction with hexane and analysis by gas chromatography with flame ionization detection (GC-FID). Samples of WAFs prepared at each loading level were taken on Day 0 from the stirring vessels and from the "old" WAFs of each treatment (composite of replicates) at termination.

Samples were allowed to come to room temperature prior to extraction. Depending on the nominal sample concentration, approximately 25, 50 or 125 mL sample volumes were transferred to glass extraction bottles and 4.0 or 5.0 mL of hexane was added as extraction solvent. The exact amount of water extracted was determined by using tared extraction bottles and re-weighing the bottles after the appropriate volume of sample was dispensed.

After the addition of hexane, the extraction bottles were crimp sealed with Teflon<sup>®</sup> faced septum caps and extracted by hand for approximately one minute followed by 60 minutes on a mechanical shaker. The contents were then allowed to settle for at least 30 minutes before the hexane (upper) layer was transferred to small (ca. 4 mL) glass vials. An aliquot was removed from the 4 mL vial to a GC autosampler vial for analysis. Hexane extracts were stored in a freezer pending analysis.

Heavy Pyrolysis Fuel Oil standards in hexane and hexane extracts were analyzed on a Perkin Elmer Autosystem XL Gas Chromatograph with a 15m x 0.53mm id Rtx-5 capillary column with 1.5  $\mu$ m film thickness (Restek) and 5 m Integra guard column. The carrier gas was helium at 15 mL/min. The oven temperature was programmed from 55°C for 6 minutes up to 250°C at 30°C/minute. Automated large volume injections of 30  $\mu$ L were made in the programmable split/splitless (PSS) mode. The FID temperature was 275°C and the detector attenuation setting was -5.

Heavy Pyrolysis Fuel Oil standards were analyzed at concentrations of 3.84, 12.8, and 64  $\mu$ g/mL. The extraction method provided quantitative recoveries and was validated by spiking Heavy Pyrolysis Fuel Oil in water at 0.961  $\mu$ g/mL. Five replicate spikes were extracted and the mean recovery (standard deviation) was 108% (6.7%).

Data were acquired and processed with Perkin Elmer Totalchrom Workstation software. Heavy Pyrolysis Fuel Oil eluted as a complex mixture at approximate retention time of 8.5 minutes under the instrumental conditions employed.

The Practical Quantitation Limit (PQL) for water samples was approximately 0.12  $\mu$ g/mL taking into account the concentration of the lowest analyzed standard (3.84  $\mu$ g/mL), the maximum sample volume (approximately 125 mL) and the minimum extract solvent volume (4.0 mL).

**APPENDIX A - ANALYTICAL METHODS and RESULTS (CONT'D)**

**TABLE A-1**

Loading Rate* (mg/L)	Measured Concentration (mg/L)		
	Day 0	Day 2**	Average
Control	ND	ND	ND
0.50	0.212	0.156	0.18
1.0	1.70	1.53 <sup>ⓐ</sup>	1.6
2.3	1.89	1.77	1.8
4.8	4.29	3.87 <sup>ⓑ</sup>	4.1
10	7.94	7.75 <sup>ⓒ</sup>	7.8

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Composite of replicates.

ND = Not Detected

ⓐ Average of two values, sample re-extracted for confirmation (1.51 mg/L and 1.54 mg/L)

ⓑ Average of two values (3.90 mg/L - sampled Day 1, composite of replicates 3 and 4 due to complete immobilization and 3.84 mg/L - from Day 2, composite of replicates 1 and 2)

ⓒ Sample taken on day 1 due to complete immobilization.

PQL (Practical Quantitation Limit) = 0.12 mg/L

**APPENDIX A - ANALYTICAL METHODS and RESULTS (CONT'D)  
 DILUTION WATER (VEHICLE) ANALYSIS**

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. Batches of 500 to 1000 L of this deionized water are then reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater*, 18th edition.

The following water quality data is most representative of the dilution water used during the in-life period of the study. Table A-2 presents analyses performed on the reconstituted water (RW) on a batch basis. Water quality analyses (dissolved oxygen, pH, alkalinity, hardness and specific conductance) are performed by environmental toxicology laboratory personnel. Total Organic Carbon analysis is performed by the laboratory's environmental chemistry and fate group. The quality of the feed water for the dilution water system is monitored at least semi-annually for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties. Results of semi-annual analyses are maintained at the testing facility.

**Table A-2  
 Results of Water Quality Analysis**

Sample	Sample Date	Alkalinity as CaCO <sub>3</sub> (mg/L)*	Hardness as CaCO <sub>3</sub> (mg/L)**	Specific Conductance (µmhos)	pH	Dissolved Oxygen (mg/L)	Temp. (°C)
Batch 137A	9-Dec-03	90	134	380	8.1	8.0	20.2

Sample	Sample Date	Total Organic Carbon (ppm) <sup>†</sup>
Batch 137A	9-Dec-03	0.2983

\* 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).

\*\* 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).

† American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 5310C, Persulfate- Ultraviolet Oxidation Method.

## APPENDIX B - TEST SUBSTANCE CHARACTERIZATION

The test substance was initially characterized on August 19 and 21, 2003. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and GC-MS analysis. Stability of the neat test substance was confirmed by repeating these same analyses on February 5, 2004 after the completion of this study.

UV-VIS spectra are presented in Figures B-1 and B-2 representing, the initial and final spectrum at concentrations of 10 and 12 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 B-3 and B-4 representing the initial and final spectra. FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of  $4\text{ cm}^{-1}$ , gain of 1 and scan number of 32.

The test substance was also characterized by GC-MS using a Varian Saturn 2000 GC-MS system with a Varian 3800 gas chromatograph. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, Heavy Pyrolysis Fuel Oil was analyzed against an ASTM D2887 calibration mixture. Figures B-5 and B-6 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components detected between retention times 13 and 26 minutes. This corresponds to bracketing by the standard hydrocarbons n-decane and n-octadecane under the analytical conditions employed. The single most abundant component eluted at approximately 16.3 minutes.

The test substance's initial and final density was measured at 20°C using an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 1.068 g/mL and the final density was 1.069 g/mL. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water, methanol and hexane. All test substance solutions were prepared with methylene chloride as the solvent.

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.

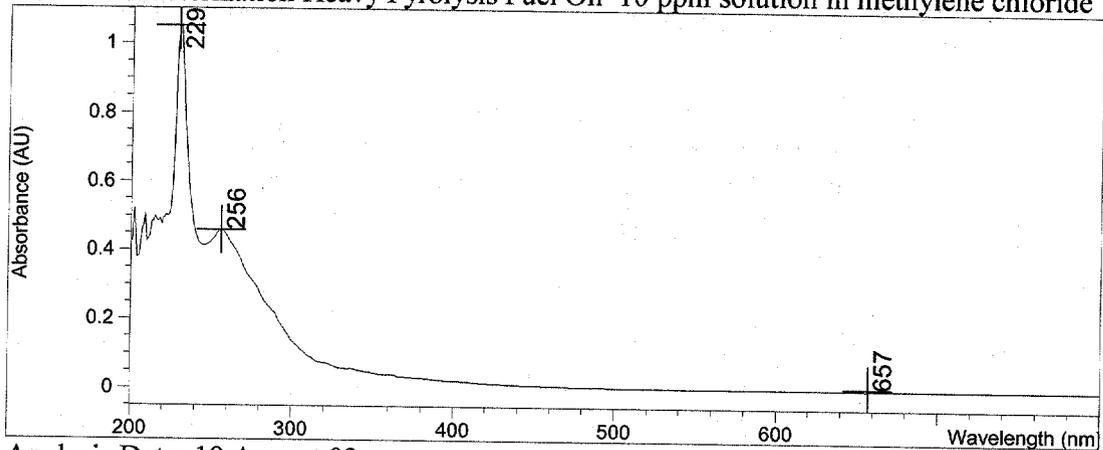
**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**UV-VIS SPECTRA**

**Figure B-1**

**Initial**

Initial Characterization Heavy Pyrolysis Fuel Oil 10 ppm solution in methylene chloride



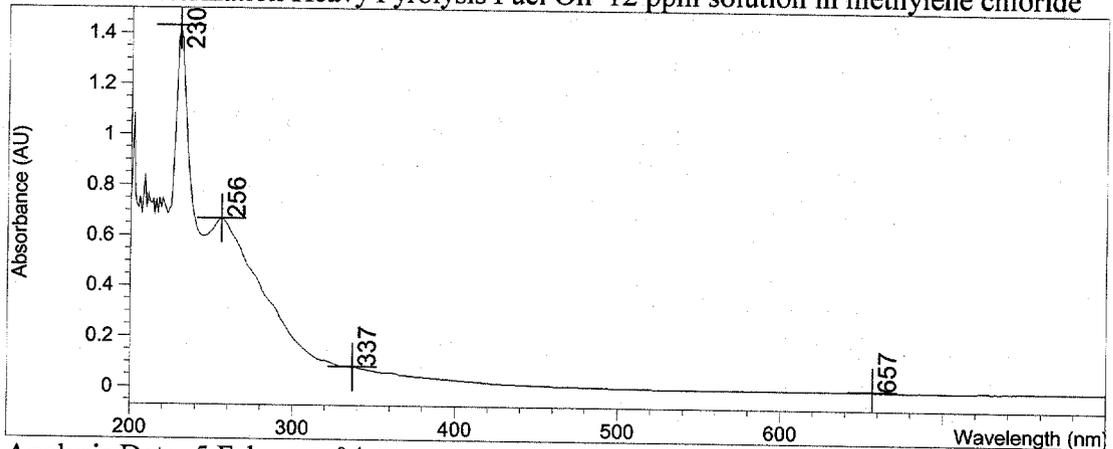
Analysis Date: 19 August 03

Peak 229nm Absorbance = 1.051  
Peak 256nm Absorbance = 0.4597  
Peak 657nm Absorbance = 0.0095

**Figure B-2**

**Final**

Final Characterization Heavy Pyrolysis Fuel Oil 12 ppm solution in methylene chloride



Analysis Date: 5 February 04

Peak 230nm Absorbance = 1.4298  
Peak 256nm Absorbance = 0.6677  
Peak 337nm Absorbance = 0.0832  
Peak 657nm Absorbance = 0.0068

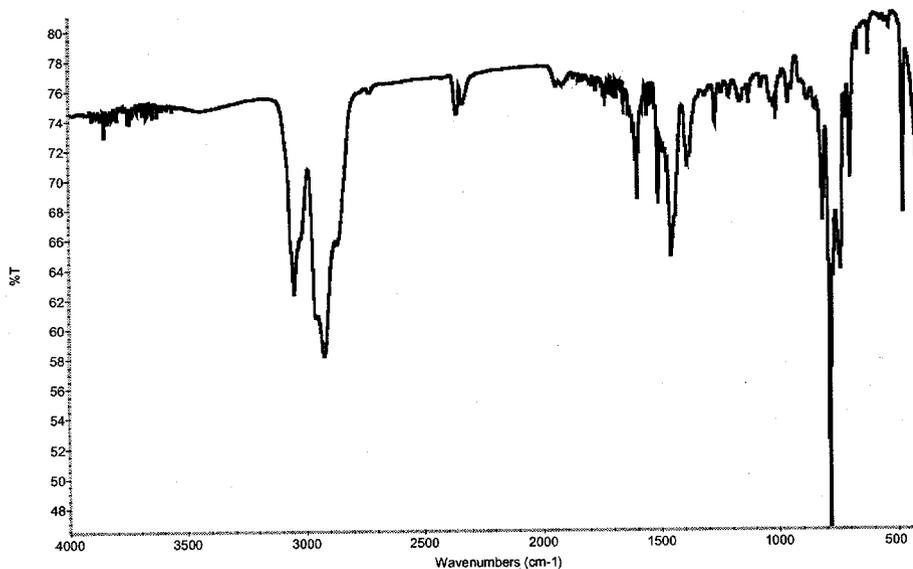
**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**FT-IR SPECTRA**

**Figure B-3**

**Initial**

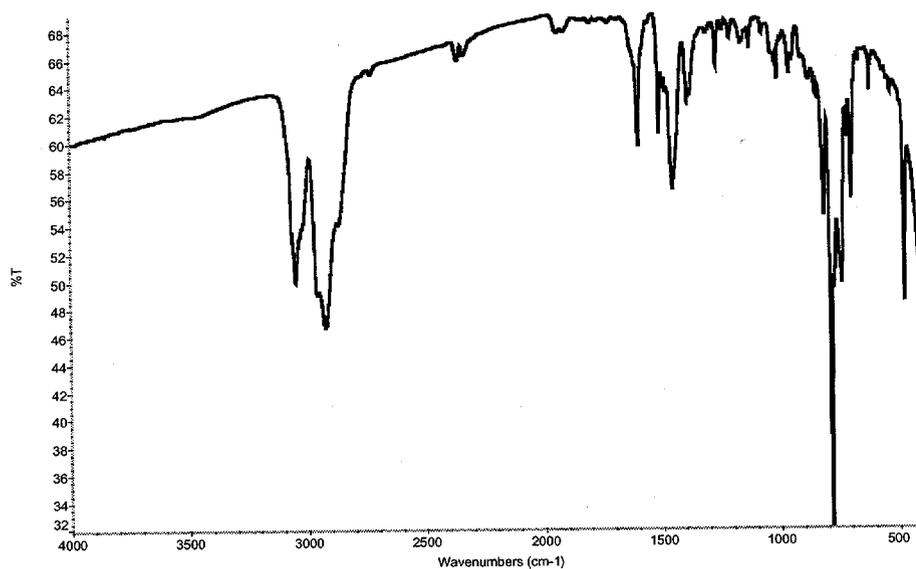
Initial Characterization Heavy Pyrolysis Fuel Oil      Analysis Date: 19 Aug 03



**Figure B-4**

**Final**

Final Characterization Heavy Pyrolysis Fuel Oil      Analysis Date: 5 Feb 04



%T = % Transmittance

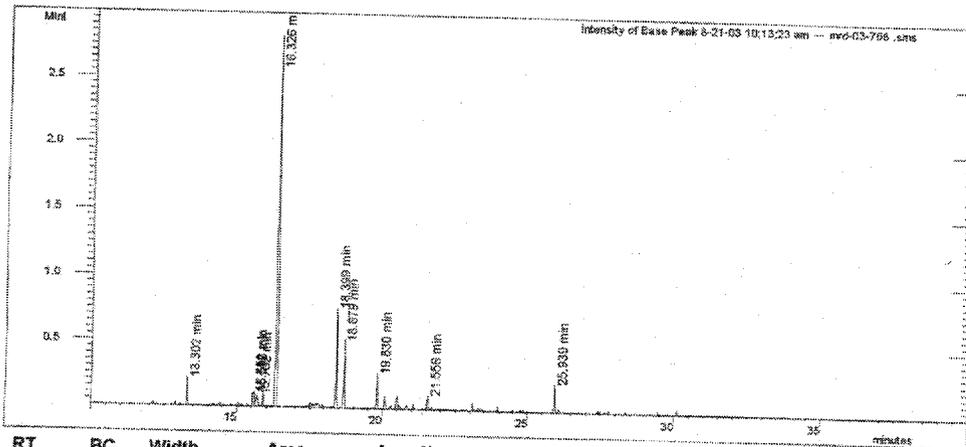
**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**INITIAL TOTAL ION CHROMATOGRAM**

**Figure B-5**

**AREA PERCENT REPORT**

Data File Name: c:\saturaws\8-21-03 10:13;23 am - mrd-03-768 .sms Acquisition Date: 8/21/03 10:13:24 AM  
 Inst. Method: C:\SaturWS\Crude Oil Charac.mth Instrument ID: Saturn GC/MS #1  
 Sample Name: MRD-03-768 Inj. Notes: MRD-03-768 (heavy fuel oil)  
 initial characterization (10% in CS2)  
 0.5uL injection



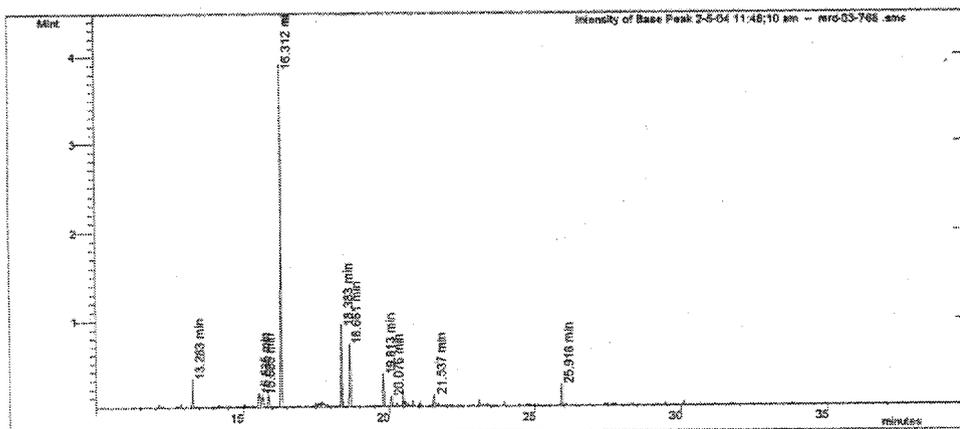
RT	BC	Width	Area	Area %
13.302	BB	1.9	1213022	4.86
15.552	BV	2.1	1037451	4.16
15.586	VV	2.2	895935	3.59
15.702	VV	2.7	1011392	4.05
16.326	VB	1.9	9296601	37.27
18.399	VB	1.7	4306416	17.26
18.679	BB	1.9	3339778	13.39
19.830	BB	1.7	1375559	5.51
21.556	VV	2.2	1524481	6.11
25.939	BB	1.8	942704	3.78

**APPENDIX B- TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**FINAL TOTAL ION CHROMATOGRAM**  
**Figure B-6**

**AREA PERCENT REPORT**

Data File Name:	c:\saturnws\2-5-04 11:48:10 am -- mrd-03-768 .sms	Acquisition Date:	2/5/04 11:48:11 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) final characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.283	BB	1.8	1973177	5.02
15.535	BB	2.0	3045234	7.75
15.585	TF	0.0	1853164	4.21
16.312	TF	0.0	13855800	35.25
18.383	VV	0.0	6504165	16.55
18.661	VV	0.0	5061242	12.88
19.813	TF	0.0	2145104	5.46
20.076	VB	0.0	1399923	3.56
21.537	TF	0.0	2322773	5.91
25.918	MV	0.0	1346340	3.43

## APPENDIX C - PROTOCOL

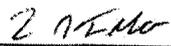
### PROTOCOL

OLF-105.0-HPV10-EMBSI

**Study Title:** *Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil  
**EMBSI Study Number:** 176842  
**Test Substance:** MRD-03-768  
**Date:** August 11, 2003  
**Room Number:** LE-337/343  
**Proposed Key Dates:**

Experimental Start .....	15-Oct-03
Experimental Termination .....	17-Oct-03
Draft Report Completion .....	14-Nov-03
Final Report Completion .....	27-Feb-04

**Approved By:**

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

15 Sep 03  
Date

  
E. J. Moran, Ph.D.  
Sponsor Representative

9/19/03  
Date

SAFETY FIRST

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### INTRODUCTION

#### *Objective*

This study will be conducted for the Sponsor to evaluate the acute toxicity of the water accommodated fractions (WAFs) of MRD-03-768 to the daphnid, *Daphnia magna*. This study will be performed as a 48-hour static test.

#### *Sponsor*

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, VA 22209

#### *Testing Facility*

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

#### *Compliance*

This test will be conducted in general agreement with the OECD<sup>1</sup> guidelines, and will be conducted in compliance with OECD<sup>2</sup> and USEPA<sup>3</sup> 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 C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### MATERIALS and METHODS

#### *Test Substance Identification*

EMBSI Code: MRD-03-768

Industry Stream Name: Heavy Pyrolysis Fuel Oil

CAS Number	CAS Inventory Name
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum

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

#### *Characterization of Test Substance*

The neat test substance will be characterized as described in SOP A.3.4.10 using the following analyses: Ultra-violet/Visible and Infrared Spectrophotometry and Gas Chromatography with Mass Selective Detection; density will also be determined. The test substance will be used in a number of studies at the testing facility. Characterization of the test substance will be performed at the testing facility prior to its use in the first of these studies. Stability assessment will span the duration of all studies. The results of the characterization and stability assessment will be appended to the final report. Characterization and stability documentation will be maintained at the testing facility.

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, will be considered the "pure" substance for dosing purposes.

#### *Analysis of Mixtures*

Samples will be taken from each water-accommodated fraction (WAF) and control solution on Day 0 and Day 2 (composite of replicates). If complete immobilization is observed in any treatment on Day 1, a sample will be taken (composite of replicates if applicable). The samples will be taken with no headspace. The method of analysis will be automated static headspace gas chromatography with flame ionization detection (HS GC-FID). Samples will be analyzed using a Perkin-Elmer HS 40 Headspace Sampler connected to a Perkin Elmer AutoSystem XL Gas Chromatograph with flame ionization detector. The gas chromatograph will be equipped with a 30m x 0.53mm id DB-5 capillary column with 1.5um film thickness (or equivalent). Analytical standards will be prepared and analyzed at concentrations bracketing the sample concentrations except in the case of those samples below the method's limit of quantification.

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *Vehicle/Dilution Water*

Reconstituted water<sup>4</sup>: Vehicle/Dilution water will be aerated prior to use.

#### *Test System*

*Daphnia magna* Straus

#### *Supplier*

Cultured in the Environmental Toxicology Laboratory. Original culture supplied by Aquatic Biosystems, Inc., Fort Collins, CO.

#### *Husbandry and Acclimation*

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

Cultures of *Daphnia magna* Strauss are fed *Pseudokirchneriella subcapitata* (approximately  $4.5 \times 10^5$  cells/mL) and 4.0 mL of a yeast / salmon starter / wheat grass (YTC) mixture per 800 mL daily (five days per week at a minimum). YTC and algae were supplied by Aquatic Biosystems, Inc., Fort Collins, CO.

#### *Number and Sex*

Number: 120    Sex: Not Applicable

#### *Age at Initiation of Exposure*

<24 hours; 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, loading rate, replicate and randomization number.

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *Selection*

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

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

#### *Feed*

Daphnids are not fed during the study.

#### *Contaminants*

There are no known contaminants in the feed used in culturing the organisms or the vehicle/dilution water believed to be at levels high enough to interfere with this study. Contaminant analysis for the YTC is provided by the supplier. The algae is not analyzed, it is prepared from UV-sterilized, deionized water and reagent grade chemicals. It is believed to contain no contaminants at levels high enough to affect the daphnids used for studies. 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. 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 C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil;  
176842; MRD-03-768

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### EXPERIMENTAL PROCEDURE

#### *Equilibrium Test*

An equilibrium study will be performed prior to testing to determine the most appropriate mixing duration. Specific analytical (e.g. GC analysis) will be used to detect soluble components of the substance. Individual WAFs at 1 mg/L and 10 mg/L will be prepared and sampled after 24 and 48 hours of mixing. The vortex will be set at  $\leq 10\%$  of the static liquid depth. All mixing vessels will be closed using foil covered stoppers during mixing. The equilibrium phase will not extend beyond 48 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. This phase of the study will not be subject to GLP standards.

#### *Range Finding Test*

A 48-hour range finding test will be performed to determine definitive test loading rates. WAFs at the following loading rates will be tested: 0.1 mg/L, 1 mg/L, 10 mg/L, 100 mg/L and a control. The WAFs will be prepared by adding the appropriate amount of test substance to vehicle/dilution water in glass vessels. The vessels will be sealed with foil covered stoppers, and will mix on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by equilibrium testing ( $\pm 1$  hour). The vortex will be set at  $\leq 10\%$  of the static liquid depth. The treatments will be allowed to settle for 1 hour ( $\pm 15$  min.) after mixing. Two replicates at each loading rate will be prepared containing 5 organisms. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

#### *Definitive Test Design*

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	TBD	20
3	TBD	20
4	TBD	20
5	TBD	20
6	TBD	20

TBD = To Be Determined

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia* sp., Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Preparation and Administration of Test Substance*

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

#### *Test Chamber and Volume of Solution*

The test chambers will be 125mL glass Erlenmeyer flasks containing ~140mL of solution (no headspace). Each chamber will be closed with ground glass stoppers to minimize contamination, evaporation and/or volatilization.

#### *Exposure Duration*

48 hours (±1 hour)

#### *Environmental Conditions*

Range of acceptable test water temperatures: 20°± 1°C.  
Diurnal light: 16 hours light: 8 hours dark.

An environmental condition study will be activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

#### *Experimental Evaluation*

Observations for immobilization will be performed and recorded at 24 and 48 hours (±1 hour) after the beginning of the test. Additional observations may be performed. Immobilization is the lack of swimming ability or movement within 15 seconds after gentle agitation of the test container. Any abnormal behavior or appearance will also be recorded.

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

Organisms will be discarded at termination. The monitoring of environmental conditions will be discontinued after completion of the study.

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia* sp., Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Discrete Measurements*

Temperature, dissolved oxygen, and pH: measured in each treatment and control at the start of the test and in a composite of replicates at termination (termination includes complete immobilization in a treatment).

#### *Loading*

At least 25 mL of test solution will be provided for each organism.

#### *Test Acceptability*

In the control, not more than 10% of the *Daphnia* should have been immobilized or trapped at the surface of the water. Dissolved oxygen should be  $\geq 60\%$  of the air saturation value at the temperature tested.

#### *Calculations*

Test results are used to derive the EC/EL<sub>50</sub>, defined as the concentration or loading rate of the test substance estimated to immobilize 50% of the test organisms within a specified period of exposure. The statistical method used to calculate the EC/EL<sub>50</sub> values and their associated 95% confidence limits will be either a maximum likelihood analysis based on D. J. Finney, 1971<sup>5</sup>, a Trimmed Spearman-Kärber Method<sup>6</sup>, a Binomial Method<sup>7</sup> or a graphical method<sup>8</sup>.

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia* sp., Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### 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 *Daphnia*:

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

Test conditions:

- test procedure used, equilibration test results
- vehicle/dilution water source and water quality characteristics (pH, hardness, temperature, alkalinity)
- Ca/Mg ratio and Na/K ratio in the dilution water
- Light quality, intensity and periodicity
- dissolved oxygen concentration, and pH values and temperature of the test solutions at study initiation and termination
- methods of preparation of test solutions
- loading rates/concentrations used
- information on concentrations of the test substance in the test solutions
- number of organisms in each test vessel
- description of the test chambers, and volume of solution

Results:

- maximum loading rate/concentration causing no immobilization
- minimum loading rate/concentration causing 100% immobilization
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative immobilization, survival and abnormal responses of the *Daphnia*
- EL<sub>50</sub> or EC<sub>50</sub> with 95% confidence limit at each observation interval, if possible
- statistical procedures followed
- graph of the loading rate/concentration-response curve at the end of the test, if applicable

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

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia* sp., Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### 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 and the appropriate guidelines and Good Laboratory Practice Regulations.

### GUIDELINE EXCEPTIONS

Due to the limited solubility of the test substance the following exceptions will apply for this study:

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

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

## APPENDIX C - PROTOCOL (CONT'D)

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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### REFERENCES

1. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 202: *Daphnia sp.* Acute Immobilisation Test. Adopted 4 April 1984.
2. OECD Principles of Good Laboratory Practice, C(97)186 (Final), 1997.
3. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
4. American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*. 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 8010E (Table 8010-I).
5. Finney, D.J., 1971. Probit Analysis, 3rd Edition, London: Cambridge University Press.
6. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.
7. Stephan, C. E., Methods for Calculating an LC<sub>50</sub>, *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.
8. 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.

**APPENDIX C - PROTOCOL (CONT'D)**

*Daphnia sp.*, Acute Immobilization Test on Heavy Pyrolysis Fuel Oil:  
176842; MRD-03-768

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**DISTRIBUTION**

Study Director .....	E. J. Febbo
Sponsor Representative .....	E. J. Moran
Section Head, Laboratory Operations .....	R. L. Rucker
Study Technicians .....	R. F. Blattenberger
.....	P. N. Unanka
.....	J. Yarusinsky
Analytical Chemistry .....	D. J. Letinski
Quality Assurance .....	Staff
Contract Administrator .....	B. J. Foster

### APPENDIX C - PROTOCOL (CONT'D)

PROTOCOL CHANGE RECORD  
*Daphnia* sp., ACUTE IMMOBILIZATION TEST on HEAVY PYROLYSIS FUEL OIL 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 Number: 176842

Revision Number: 1

Date: 29-Oct-03

#### Pg. 6 / Definitive Test Design

Previous Statement:

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	TBD	20
3	TBD	20
4	TBD	20
5	TBD	20
6	TBD	20

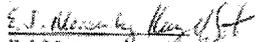
TBD = To Be Determined

Revised Statement:

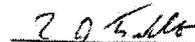
GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	20 (5 per 4 replicates)
2	0.41	20
3	0.90	20
4	2.0	20
5	4.5	20
6	10	20

Justification: addition of definitive loading rates

Required signatures:

  
E. J. Moran  
Sponsor Representative

11/29/03  
Date

  
E. J. Febbo  
Study Director

1 Dec 03  
Date

**Robust Summary  
Invertebrate Acute Toxicity**

<b>Test Substance:</b>	<p>Industry Stream Name (acronym): Heavy Pyrolysis Fuel Oil</p> <table border="0"> <tr> <td><u>CAS Number:</u></td> <td><u>CAS Inventory Name:</u></td> </tr> <tr> <td>68513-69-9</td> <td>Residues, petroleum, steam-cracked light</td> </tr> <tr> <td>64741-62-4</td> <td>Clarified oils, petroleum, catalytic cracked</td> </tr> <tr> <td>69013-21-4</td> <td>Fuel oil, pyrolysis</td> </tr> <tr> <td>8002-05-9</td> <td>Petroleum</td> </tr> </table> <p>In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.</p>	<u>CAS Number:</u>	<u>CAS Inventory Name:</u>	68513-69-9	Residues, petroleum, steam-cracked light	64741-62-4	Clarified oils, petroleum, catalytic cracked	69013-21-4	Fuel oil, pyrolysis	8002-05-9	Petroleum
<u>CAS Number:</u>	<u>CAS Inventory Name:</u>										
68513-69-9	Residues, petroleum, steam-cracked light										
64741-62-4	Clarified oils, petroleum, catalytic cracked										
69013-21-4	Fuel oil, pyrolysis										
8002-05-9	Petroleum										
<b>Method/Guideline:</b>	OECD Guideline 202										
<b>Year (guideline):</b>	1984										
<b>Type (test type):</b>	Daphnid Acute Toxicity Test										
<b>GLP (Y/N):</b>	Yes										
<b>Year (study performed):</b>	2003										
<b>Species:</b>	<i>Daphnia magna</i> Straus										
<b>Analytical Monitoring:</b>	Yes										
<b>Exposure Period:</b>	48 hours										
<b>Statistical Method:</b>	<p>The 24-hour EL<sub>50</sub> and EC<sub>50</sub> values were determined using a Trimmed Spearman-Kärber Method (Hamilton et al., 1977). A Binomial Method (Stephan, 1977) was used to determine the 48-hour EL<sub>50</sub> and EC<sub>50</sub> values.</p> <p>Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. <i>Environmental Science and Technology</i>, Vol. 11, No. 7, p.714-719.</p> <p>Stephan, C. E., Methods for Calculating an LC<sub>50</sub>, <i>Aquatic Toxicology and Hazard Evaluation</i>, ASTM STP 634, F. L. Mayer and J. L. Hamelink, Eds., American Society for Testing and Materials, 1977, pp. 65-84.</p>										
<b>Test Conditions:</b> <ul style="list-style-type: none"> <li><b>Note: Concentration preparation, vessel type, volume, replication, environmental conditions, organisms supplier, loading, deviations from guideline or protocol.</b></li> </ul>	<p>Individual Water Accommodated Fractions (WAF's) were prepared for each treatment. The test substance was added to 4.0 L of reconstituted water in glass aspirator bottles (capacity 4.5 L). The solutions were mixed for 24 hours using a 5% vortex (of the static liquid depth). The test solutions were removed through the outlet at the bottom of each mixing vessel into four replicates of approximately 140 mL in 125 mL Erlenmeyer flasks (no headspace). Five daphnids were added to each replicate and the replicates were closed. The test was performed under static conditions with no aeration.</p> <p>Mean test temperature: 20.1°C (S.D. = 0.1), diurnal light: approximately 16 hours light and 8 hours dark with 100 to 113 lux during full daylight periods. Dissolved oxygen ranged from 8.0 to 8.6 mg/L and pH ranged from 7.8 to 8.1 during the study. Water hardness was 134 mg/L as CaCO<sub>3</sub>.</p> <p>The Daphnids were cultured in-house. Age was &lt;24 hours old from 13-day old parents.</p> <p>Due to the relatively complex nature and limited water solubility of the test</p>										

	<p>substance, the following exceptions to the guideline apply for this study:  The concentration of the test substance in solution was not determined prior to use. The initial concentration of the test substance was not maintained at 80% in the lowest loading rate throughout the test, 74% of the initial concentration was maintained. It was deemed more appropriate to prepare individual treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.</p>																																							
<p><b>Results:</b>  <b>Units/Value:</b>  <b>Note: Analytical method, biological observations, control survival.</b></p>	<p>Effect Loading (EL<sub>50</sub>) / Effect Concentration (EC<sub>50</sub>) Values (mg/L)</p> <table border="1"> <thead> <tr> <th></th> <th>EL<sub>50</sub></th> <th>EC<sub>50</sub></th> </tr> </thead> <tbody> <tr> <td>24 hours</td> <td>3.7 (3.3-4.2*)</td> <td>3.0 (2.7-3.4*)</td> </tr> <tr> <td>48 hours</td> <td>3.3 (2.3-4.8**)</td> <td>2.7 (1.8-4.1**)</td> </tr> </tbody> </table> <p>* 95% Confidence Interval  ** 99% Confidence Interval</p> <p>The maximum actual loading rate causing no immobilization after 48-hours was 2.3 mg/L. The minimum actual loading rate causing 100% immobilization after 48 hours was 4.8 mg/L.</p> <p>The maximum measured concentration causing no immobilization after 48-hours was 1.8 mg/L. The minimum measured concentration causing 100% immobilization after 48-hours was 4.1 mg/L.</p> <p>The method of analysis was gas chromatography with flame ionization detection (HS GC-FID).</p> <table border="1"> <thead> <tr> <th rowspan="2">Loading Rate (mg/L)</th> <th rowspan="2">Measured Conc. (mg/L)</th> <th colspan="2">% Immobilization</th> </tr> <tr> <th>24-hour</th> <th>48-hour</th> </tr> </thead> <tbody> <tr> <td>Control</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>0.50</td> <td>0.18</td> <td>0</td> <td>0</td> </tr> <tr> <td>1.0</td> <td>1.6</td> <td>0</td> <td>0</td> </tr> <tr> <td>2.3</td> <td>1.8</td> <td>0</td> <td>0</td> </tr> <tr> <td>4.8</td> <td>4.1</td> <td>85</td> <td>100</td> </tr> <tr> <td>10</td> <td>7.8</td> <td>100</td> <td>100</td> </tr> </tbody> </table>		EL <sub>50</sub>	EC <sub>50</sub>	24 hours	3.7 (3.3-4.2*)	3.0 (2.7-3.4*)	48 hours	3.3 (2.3-4.8**)	2.7 (1.8-4.1**)	Loading Rate (mg/L)	Measured Conc. (mg/L)	% Immobilization		24-hour	48-hour	Control	0	0	0	0.50	0.18	0	0	1.0	1.6	0	0	2.3	1.8	0	0	4.8	4.1	85	100	10	7.8	100	100
	EL <sub>50</sub>	EC <sub>50</sub>																																						
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2.3	1.8	0	0																																					
4.8	4.1	85	100																																					
10	7.8	100	100																																					
<b>Conclusion:</b>	<p>After <i>Daphnia magna</i> were exposed to WAFs prepared from Heavy Pyrolysis Fuel Oil for 48-hours, the EL<sub>50</sub> was 3.3 mg/L and the EC<sub>50</sub> was 2.7 mg/L.</p>																																							
<b>Reliability:</b>	1-Reliable without restrictions.																																							
<b>Reference:</b>	ExxonMobil Biomedical Sciences, Inc. 2004. <i>Daphnia sp.</i> , ACUTE IMMOBILIZATION TEST on HEAVY PYROLYSIS FUEL OIL. Study # 176842																																							
<b>Other (source):</b>	Olefins Panel, American Chemistry Council																																							

# **ExxonMobil** BIOMEDICAL SCIENCES, INC.

**OLF-105.0-HPV10-EMBSI**

**FISH, ACUTE TOXICITY TEST on  
HEAVY PYROLYSIS FUEL OIL**

**FINAL REPORT**

**STUDY NUMBER: 176858**

**TEST SUBSTANCE: MRD-03-768**

**PERFORMED FOR:**

**American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209**

**PERFORMED AT:**

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

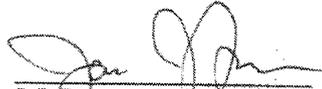
**COMPLETION DATE: October 29, 2004**

**04TP 116**

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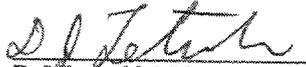
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APPROVAL SIGNATURES



J. J. Freeman, Ph.D., D.A.B.T.  
Acting Section Head, Laboratory Operations

13 Oct 04  
Date



D. J. Lefinski, M.S.  
Supervisor, Environmental Chemistry

15 Oct 04  
Date

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<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards except as outlined on page 13-15.



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

29 Oct 04  
Date

The final report was accepted by the Sponsor.



E. J. Moran, Ph.D.  
Sponsor's Representative

10/20/04  
Date

---

QUALITY ASSURANCE STATEMENT

---

STUDY NUMBER: 176858

TEST SUBSTANCE: MRD-03-768

STUDY SPONSOR: American Chemistry Council

---

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.

<u>Study Phase Inspected</u>	<u>Date(s) of Inspection</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
Protocol	06 Aug 03	06 Aug 03	13,14 Aug 03
Environmental Conditions Study Activation	15 Dec 03	17 Dec 03	09 Jan 04
Final Report	30 Apr 04 & 04 May 04	04 May 04	10,14 May 04
Second Review of Final Report	05 May 04	05 May 04	10,25 May 04

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

  
\_\_\_\_\_  
W. James Bover, Ph.D.  
Data Integrity & Quality Assurance / Archives  
Section Head

12 Oct 04  
Date

**FISH, ACUTE TOXICITY TEST on HEAVY PYROLYSIS FUEL OIL**  
Study No. 176858; MRD-03-768

**PERSONNEL**

Study Director:	E. J. Febbo, M.S.
Sponsor Representative:	E. J. Moran, Ph.D.
Acting Section Head, Laboratory Operations:	J. J. Freeman, Ph.D., D.A.B.T.
	R. L. Rucker, Ph.D. (Prior to December 9, 2003)
Data Integrity & Quality Assurance / Archives Section Head:	W. J. Bover, Ph.D.
Supervisor, Environmental Toxicology & Compound Preparation:	E. J. Febbo, M.S.
Supervisor, Environmental Chemistry:	D. J. Letinski, M.S.

### SUMMARY

This study was performed to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of MRD-03-768 (Heavy Pyrolysis Fuel Oil), to rainbow trout, *Oncorhynchus mykiss*, in a 96-hour semi-static (renewal) test, conducted according to OECD guideline 203.

Individual treatments were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes, to 18 L of vehicle/dilution water in glass aspirator bottles (capacity 22 L) and stirring on magnetic stirplates using a 3% (of the static liquid depth) vortex for 24 hours. The mixtures were allowed to cool to test temperature in a waterbath for one hour without stirring before removing the aqueous portions (WAFs) for testing. WAFs were prepared daily, the day preceding each renewal. The following table defines the target loading rates, actual loading rates and measured concentrations.

TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	MEASURED CONCENTRATION† (mg/L)
0	0	0
0.63	0.63	0.30
1.3	1.4	1.2
2.5	2.6	2.5
5.0	5.8	4.1
10	11	9.1

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

† Concentration based on mean (new and old) measured concentrations.

Three replicates per treatment were tested. Each replicate contained four fish. Replicate chambers were 4 L size glass aspirator bottles containing approximately 4.5 L of solution (no headspace) closed with foil covered neoprene stoppers. Water quality (temperature, pH, and dissolved oxygen) measurements were recorded per treatment for each new solution prior to renewals and in a composite of the replicates after each renewal. Observations for mortality and abnormal behavior or coloration were performed at 3, 6, 24, 48, 72 and 96 hours  $\pm$  1 hour after the beginning of the test.

**SUMMARY (CONT'D)**

Acute toxicity results are expressed as the Lethal Loading / Lethal Concentration 50 (LL/LC<sub>50</sub>); that is, the actual loading rate or measured concentration of test substance in dilution medium which is calculated to result in 50% mortality compared to the control for the specified time of exposure. The maximum actual loading rate causing no mortality after 96-hours was 2.6 mg/L. The maximum measured concentration causing no mortality after 96-hours was 2.5 mg/L. The minimum actual loading rate causing 100% mortality after 96-hours was 11 mg/L. The minimum measured concentration causing 100% mortality after 96-hours was 9.1 mg/L.

Hours	LL50 mg/L	LC50 mg/L
3 and 6	>11*	>9.1*
24	7.5 (6.7-8.4)	5.8 (5.2-6.4)
48	5.9 (4.8-7.3)	4.7 (3.9-5.6)
72 and 96	5.6 (4.5-6.9)	4.4 (3.7-5.3)

\* Not a calculated value, no mortality was observed in the highest loading rate/concentration at 3 hours, 42% mortality was observed in the highest loading rate/concentration at 6 hours therefore the EL/EC<sub>50</sub> is greater than the highest loading rate/concentration tested.

Values in parentheses are 95% confidence intervals.

SUMMARY (CONT'D)

Summary of In-Life Observations						
Loading Rate* (Concentration)** mg/L	3 hours	6 hours	24 hours	48 hours	72 hours	96 hours
0 (0)	0 <sup>①</sup>	0	0	0	0	0
	0 <sup>②</sup>	0	0	0	0	0
0.63 (0.30)	0	0	0	0	0	0
	0	0	0	0	0	0
1.4 (1.2)	0	0	0	0	0	0
	0	0	0	0	0	0
2.6 (2.5)	0	0	0	0	0	0
	100	100	100	100	100	100
5.8 (4.1)	0	0	8	42	58	58
	100	100	100	100	100	100
11 (9.1)	0	42	100	†	†	†
	100	100				

① Cumulative % Mortality

② % Sublethal Effects (includes lethargy, dark pigmentation and side swimming)

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

\*\* Concentration based on mean (new and old) measured concentrations.

† Indicates that 100% mortality occurred prior to the exposure period.

## INTRODUCTION

### *Objective*

This study was conducted for the Sponsor to evaluate the acute toxicity of the water-accommodated fractions (WAFs) of the test substance, to rainbow trout, *Oncorhynchus mykiss*, in a 96-hour semi-static (renewal) test.

### *Sponsor*

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209

### *Testing Facility*

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

### *Study Initiation Date*

September 15, 2003

### *In-life Test Period*

December 15, 2003 to December 19, 2003

### *Experimental Termination*

December 24, 2003

### *Compliance*

This test was conducted in compliance with OECD<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards with the exceptions listed on page 13-15 and in agreement with OECD<sup>3</sup> Guideline 203 with the exceptions listed on page 22.

## MATERIALS and METHODS

### *Test Substance Identification*

EMBSI Identification:	MRD-03-768
Industry Stream Name (acronym):	Heavy Pyrolysis Fuel Oil
<u>CAS Number:</u>	<u>CAS Inventory Name:</u>
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum
Supplier:	Dow Chemical Company Freeport, TX
Date Received:	July 29, 2003
Expiration Date:	July 2008
Description:	Dark Amber liquid

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

### *Stream Derivation*

In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.

### *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.

### *Vehicle / Dilution Water*

Reconstituted water<sup>4</sup> (Batch 283). Dilution water was aerated prior to the addition of the test substance. See Appendix A, page 34 for the vehicle/dilution water analysis.

## MATERIALS and METHODS (CONT'D)

### *Characterization of the Test Substance*

The neat test substance was characterized and the stability determined by the testing facility using the following analyses: UltraViolet/Visible and Infrared Spectrophotometry, Gas Chromatography with Mass Selective Detection and Density. The test substance was used in a number of studies at the testing facility. Characterization of the test substance was performed at the testing facility prior to its use in the first of these studies and after completion of the final study. Documentation of characterization and stability assessment is maintained at the testing facility (see Appendix B, page 35).

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, was considered the "pure" substance.

### *Analysis of Mixtures*

Samples were taken from each treatment WAF and control solution on Day 0 and Day 3. Samples were also taken on Day 1 and Day 4 (composite of replicates) of the "old" solutions. The samples were taken with no headspace. The method of analysis was gas chromatography with flame ionization detection (GC-FID). Samples were analyzed using a Perkin Elmer Autosystem XL Gas Chromatograph. Analytical standards were prepared and analyzed at concentrations bracketing the sample concentrations. The methods and results of the analyses are included in Appendix A starting on page 32.

### *Test System*

*Oncorhynchus mykiss* (Rainbow trout)

Lot 481, Receipt date: 3-Dec-03

### *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*

Thomas Fish Company  
Anderson, CA

## MATERIALS and METHODS (CONT'D)

### *Husbandry and Acclimation*

The rainbow trout were quarantined and observed for parasites and disease for 12 days prior to use in the test (in dilution water) at approximately 12.8°C. The water was continuously aerated to provide a dissolved oxygen concentration of at least 80% of the air saturation value. No mortality was observed and the fish were not treated for disease or parasites before use in this study. Fish were not treated for disease or parasites before use in this study. Fish were held under static conditions using biological and mechanical filtration, a 16-hour photoperiod, and were fed at least five days per week with Finfish Starter (formerly referred to as Salmon Starter).

Feed Supplier: Finfish Starter - Zeigler Bros., Inc., Gardners, PA

### *Number and Sex*

Number: 72

Sex: Not applicable

### *Age at Initiation of Exposure*

29 days post hatch

### *Test System Identification*

Fish were not individually identified. Each replicate, containing four fish, was labeled to show study number, loading rate, replicate and chamber number.

### *Selection*

Organisms were randomly assigned to intermediate chambers and then transferred to their respective test chambers using a computer-generated randomization schedule. A printout of the randomization schedule is included in the raw data.

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

### *Feed*

Test fish were not fed at least 24 hours prior to, or during the study.

## MATERIALS and METHODS (CONT'D)

### *Contaminants*

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

## EXPERIMENTAL PROCEDURE

### *Equilibrium Test*

An equilibrium study was performed prior to testing to determine the most appropriate mixing duration. Gas chromatography with flame ionization detection (GC-FID) was used to detect the test substance. Two sets of individual WAFs at approximately 1 mg/L and 10 mg/L were prepared and sampled after approximately 24 and 48 hours. The solutions were stirred using a  $\leq 10\%$  (of the static liquid depth) vortex. All mixing vessels were closed using foil covered rubber stoppers during mixing. Three replicates were analyzed at each sampling interval. The equilibrium phase did not extend beyond 48-hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test (to be used for the fish study). The goal was to achieve equilibrium of the soluble components without compromising a loss of the lighter components (it is the lighter components that usually contribute to toxicity). The results indicate that a mixing duration of 24 hours was sufficient for the soluble components of the test substance to achieve equilibrium. This phase of the study was not subject to GLP standards.

Summary of Equilibrium Test Results		
Loading Rate (mg/L)	24 Hour Stirring* (mg/L)	48 Hour Stirring* (mg/L)
1	0.455 (SD = 0.013)	0.435 (SD = 0.018)
10	6.79 (SD = 0.15)	6.70 (SD = 0.049)

1 mg /L PQL (Practical Quantitation Limit) = 0.1 mg/L

10 mg /L PQL (Practical Quantitation Limit) = 0.7 mg/L

\* Average of three replicates.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Rangefinding Test*

A 48-hour semi static (renewal) range finding test was performed for the Sponsor to determine the loading rates of Heavy Pyrolysis Fuel Oil (MRD-03-768) for the definitive Fish, Acute Toxicity Test.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.1 mg/L, 1 mg/L, 10 mg/L and 100 mg/L. The mean actual loading rates were determined to be 0.08 mg/L, 1.0 mg/L, 10 mg/L and 98 mg/L. A control treatment consisting only of the vehicle/dilution water also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via stainless steel and glass syringes, to the vehicle/dilution water in glass aspirator bottles (mixing vessels) containing Teflon<sup>®</sup> coated stir bars. The syringes were weighed with and without test substance to determine the actual loading rates. The mixing vessels were closed with foil covered neoprene stoppers and the treatments were stirred using a 3% (of the static liquid depth) vortex at room temperature (approximately 22°C) on magnetic stirplates for 24 hours. As stirring initiated and after stirring, all treatments appeared clear and colorless with test substance evident on the surface. After stirring, a small amount of the test substance was also observed at the bottom of the mixing vessel for the 100 mg/L treatment. The mixtures were allowed to cool to test temperature in a waterbath for two hours without stirring before removing the aqueous portions (WAFs) for testing. New WAFs were prepared for the renewal.

One replicate per treatment was tested. Each replicate contained five fish. Replicate chambers were 4 L size aspirator bottles containing approximately 4.5 L of test solution (no headspace) closed with foil covered neoprene stoppers. A piece of Nitex<sup>®</sup> screening was placed in the outlet at the bottom of the bottles to prevent fish from becoming trapped in the narrow space. An approximately 90% renewal of the test solution was performed at 24 hours. Water quality (temperature, pH, and dissolved oxygen) measurements were recorded daily on the new and old solutions. The fish were observed for mortality and abnormal behavior or coloration at 3, 24 and 48 hours  $\pm$  1 hour. This phase of the study was not subject to GLP standards.

**EXPERIMENTAL PROCEDURE (CONT'D)**

*Range-finding Test (Cont'd)*

<b>Summary of In-Life Observations</b>			
<b>Loading Rate (mg/L)</b>	<b>3 hour (% Mortality)</b>	<b>24 hour (% Mortality)</b>	<b>48 hour (% Mortality)</b>
<b>0 (Control)</b>	0	0	0
<b>0.08</b>	0	0	0
<b>1.0</b>	0	0	0
<b>10</b>	0†	100	*
<b>98</b>	100	*	*

\* Indicates that 100% mortality occurred prior to the exposure period.

No abnormal behavior or coloration was observed in the fish that were alive.

† All fish were lethargic and exhibited dark pigmentation.

<b>Summary of Water Quality Measurements, New and Old (Day 0 - Termination)</b>			
<b>Loading Rate (mg/L)</b>	<b>pH</b>	<b>Dissolved Oxygen (mg/L)</b>	<b>Temperature (°C)</b>
<b>0 (Control)</b>	6.8 - 7.9	6.4 - 8.8	12.5 - 13.8
<b>0.08</b>	6.9 - 7.9	6.5 - 8.9	12.5 - 13.8
<b>1.0</b>	6.9 - 7.7	6.5 - 8.8	12.5 - 13.9
<b>10</b>	7.0 - 7.8	7.5 - 8.8	12.8 - 13.8
<b>98</b>	7.7 - 7.8*	8.8 - 8.9*	12.8 - 13.9*

\* Day 0 values, complete mortality occurred within 3 hours of the experimental start time.

The protocol indicated that "the loading level (g of fish per liter of solution) with regards to oxygen depletion would be assessed during the range finder. A loading between 0.2 - 0.6 g of fish per liter of solution was the target for the range finder".

The study resulted in a loading of 0.25 g of fish/L of solution. The dissolved oxygen levels remained above 60% of the air-saturation value (approximately 6.2 mg/L).

### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Definitive Test Design*

GROUP	TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	0	12 (4 per 3 replicates)
2	0.63	0.63	12
3	1.3	1.4	12
4	2.5	2.6	12
5	5.0	5.8	12
6	10	11	12

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

#### *Preparation and Administration of Test Substance*

Individual treatments were prepared by adding the appropriate amount of test substance to 18.0 L of laboratory vehicle/dilution water in glass aspirator bottles (capacity 22 L). The test substance was added to the water in the aspirator bottles using stainless steel and glass syringes. The syringes were weighed with and without the test substance to determine the actual loading rate. The mixing vessels were closed with foil covered neoprene stoppers. The mixtures were stirred using a 3% (of the static liquid depth) vortex for 24 hours on magnetic stirplates with Teflon<sup>®</sup> coated stirbars at room temperature (23.4°C, S.D. 0.2°C). As stirring initiated and after stirring, all treatments appeared clear/colorless with the test substance floating at the surface. The mixtures were allowed to cool to test temperature in a waterbath at 11.5°C to 11.6°C for one hour without stirring before removing the aqueous portions (WAFs) for testing. New WAFs were prepared daily for the renewals.

Three replicates were prepared for each treatment by completely filling the test chambers with the WAF (no headspace). Three replicates of the control were prepared in the same manner using laboratory dilution water. Approximately 90% of the test solution was renewed on days 1, 2, and 3. Renewals were accomplished by draining 4 L of old solution from each replicate and adding 4 L of fresh solution.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Test Chamber / Volume of Solution*

The test chambers were 4 L size glass aspirator bottles containing approximately 4.5 L of solution (no headspace). The test chambers contained a small piece of Nitex<sup>®</sup> screening in the outlet to prevent fish from being trapped in the small area. Test chambers were closed with foil covered neoprene stoppers to minimize contamination, evaporation and/or volatilization.

### *Exposure Duration*

96 hours

### *Exposure Conditions*

Mean test temperature: 13.6°C (S.D. = 0.1), continuously monitored by computer in the test area.

Diurnal light: approximately 16 hours light and 8 hours dark. The intensity during full daylight hours was 644 to 653 lux.

An environmental conditions study was activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

### *Experimental Evaluation*

Each test and control chamber was observed for mortality of the fish at 3, 6, 24, 48, 72 and 96 hours ( $\pm 1$  hour). Fish were considered dead if touching the caudal peduncle produces no reaction and/or no breathing movements were visible. During observations, organisms were also examined for abnormal behavior or coloration and any dead fish were removed. Feces was observed in the test chambers at each observation interval. Any feces that was not removed while draining the test chambers during renewals was siphoned out using a glass tube and pipette bulb.

Temperature, dissolved oxygen, and pH were measured in each "new" treatment and the control on Day 0 and daily, prior to renewals, and on the "old" solutions (composite of the replicates) daily. The dissolved oxygen levels remained above 60% of the air-saturation value (approximately 6.2 mg/L), throughout the test. No undissolved test substance was observed in any of the test chambers.

After completion of the study, the monitoring of environmental conditions was discontinued. All remaining fish were euthanized using a 2 g/L tricaine methane sulphonate (MS 222) solution, prepared in laboratory dilution water. Control fish were individually weighed and total lengths measured.

### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Weight & Length of Control Fish at Termination*

Length and weight of the organisms used in the study are approximated from measurements of the control organisms at the end of the study.

Mean Weight - 0.194 g (S.D. = 0.034)

Mean Total Length - 3.1 cm (S.D. = 0.2)

#### *Organism Loading During the Definitive Study*

Loading of fish was 0.172 grams per liter of solution.

#### *Calculations*

The 24 - 96 hour  $LL_{50}$  and  $LC_{50}$  values were determined using a Trimmed Spearman-Kärber Method<sup>5</sup>.

## RESULTS

Acute toxicity results are expressed as the Lethal Loading / Lethal Concentration 50 (LL/LC<sub>50</sub>); that is, the actual loading rate or measured concentration of test substance in dilution medium which is calculated to result in 50% mortality compared to the control for the specified time of exposure. The maximum actual loading rate causing no mortality after 96-hours was 2.6 mg/L. The maximum measured concentration causing no mortality after 96-hours was 2.5 mg/L. The minimum actual loading rate causing 100% mortality after 96-hours was 11 mg/L. The minimum measured concentration causing 100% mortality after 96-hours was 9.1 mg/L.

Hours	LL50 mg/L	LC50 mg/L
3 and 6	>11*	>9.1*
24	7.5 (6.7-8.4)	5.8 (5.2-6.4)
48	5.9 (4.8-7.3)	4.7 (3.9-5.6)
72 and 96	5.6 (4.5-6.9)	4.4 (3.7-5.3)

\* Not a calculated value, no mortality was observed in the highest loading rate/concentration at 3 hours, 42% mortality was observed in the highest loading rate/concentration at 6 hours therefore the EL/EC<sub>50</sub> is greater than the highest loading rate/concentration tested.

Values in parentheses are 95% confidence intervals.

**RESULTS (CONT'D)**

Summary of In-Life Observations						
Loading Rate* (Concentration)** mg/L	3 hours	6 hours	24 hours	48 hours	72 hours	96 hours
0 (0)	0 <sup>①</sup>	0	0	0	0	0
	0 <sup>②</sup>	0	0	0	0	0
0.63 (0.30)	0	0	0	0	0	0
	0	0	0	0	0	0
1.4 (1.2)	0	0	0	0	0	0
	0	0	0	0	0	0
2.6 (2.5)	0	0	0	0	0	0
	100	100	100	100	100	100
5.8 (4.1)	0	0	8	42	58	58
	100	100	100	100	100	100
11 (9.1)	0	42	100	†	†	†
	100	100				

① Cumulative % Mortality

② % Sublethal Effects (includes lethargy, dark pigmentation and side swimming)

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

\*\* Concentration based on mean (new and old) measured concentrations.

† Indicates that 100% mortality occurred prior to the exposure period.

Table 1 presents the in-life observation data for the replicates in each treatment. Table 2 summarizes the water quality values measured during the test. Figures 1 and 2 present the loading rate/concentration response curves at each observation interval. Appendix A presents the analytical chemistry methods and results.

The test was considered acceptable as none of the control fish died or exhibited abnormal behavior during the study and the dissolved oxygen levels remained above 60% of the air saturation value at the temperature tested (approximately 6.2 mg/L).

## CONCLUSION

After *Oncorhynchus mykiss* were exposed to WAFs prepared from Heavy Pyrolysis Fuel Oil for 96-hours, the LL<sub>50</sub> was 5.6 mg/L and the LC<sub>50</sub> was 4.4 mg/L.

## GUIDELINE EXCEPTIONS

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

The concentration of the test substance in solutions was not determined prior to use as indicated in the guideline.

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

## PROTOCOL DEVIATION

The protocol required that the fish would be held at test temperature (13-15°C) for at least 7 days prior to use in the test. The fish were held at 12.8°C for the 7 days prior to use in the study. This deviation is not believed to have affected the outcome or integrity of the study.

## 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 include 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.

## REFERENCES

1. Organization for Economic Cooperation and Development (OECD), Principles of Good Laboratory Practice, C(97)186 (Final), 1997.
2. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
3. OECD, Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 203: Fish, Acute Toxicity Test. Adopted 17 July 1992.
4. American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 8010E (Table 8010:I).
5. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.

**Table 1 - Definitive Study, In-Life Observations**

Loading Rate* (Concentration**) mg/L	3 Hours			Date: 15-Dec-03			Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)								
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
Replicate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Swimming on Side	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normal	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Loading Rate* (Concentration**) mg/L	6 Hours			Date: 15-Dec-03			Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)								
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
Replicate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark Pigmentation, Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normal	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.  
 \*\* Concentration based on mean (new and old) measured concentrations.

**Table 1 - Definitive Study, In-Life Observations (Cont'd)**

Test Day: 1 Date: 16-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark Pigmentation, Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	4
Normal	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Test Day: 2 Date: 17-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dark Pigmentation, Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	4
Normal	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.  
 \*\* Concentration based on mean (new and old) measured concentrations.

**Table 1 - Definitive Study, In-Life Observations (Cont'd)**

Loading Rate* (Concentration**) mg/L	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	4
Dark Pigmentation, Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	3	2	0
Normal	4	4	4	4	4	4	4	4	4	4	4	4	0	0	0	0	0	0
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2	0

Test Day: 3 Date: 18-Dec-03

Loading Rate* (Concentration**) mg/L	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	Control (0)			0.63 (0.30)			1.4 (1.2)			2.6 (2.5)			5.8 (4.1)			11 (9.1)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Replicate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Daily Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	4
Dark Pigmentation, Lethargic	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	3	2	0
Normal	4	4	4	4	4	4	4	4	4	4	4	4	0	0	0	0	0	0
Survival	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2	0

Test Day: 4 Date: 19-Dec-03

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.  
 \*\* Concentration based on mean (new and old) measured concentrations.

**Table 2 - Definitive Study, Water Quality Values**

Test Day: 0	Date: 15-Dec-03	new solution				
Loading Rate* (Concentration**) mg/L	Control (0)	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Dissolved Oxygen (mg/L)	8.4	8.4	8.3	8.4	8.4	8.4
pH	7.9	7.9	7.9	7.9	7.8	7.9
Temperature (°C)	14.0	13.8	13.7	13.7	13.4	13.4

Test Day: 1	Date: 16-Dec-03	old solution	composite of replicates			
Loading Rate* (Concentration**) mg/L	Control (0)	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Dissolved Oxygen (mg/L)	7.0	7.0	6.9	7.0	7.1	7.5
pH	6.5	6.9	6.9	6.9	7.0	7.0
Temperature (°C)	14.0	13.9	13.8	13.8	13.9	14.0

Test Day: 1	Date: 16-Dec-03	new solution				
Loading Rate* (Concentration**) mg/L	Control (0)	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Dissolved Oxygen (mg/L)	8.2	8.4	8.3	8.5	8.4	Not Present
pH	7.6	7.7	7.7	7.7	7.7	Present
Temperature (°C)	14.0	13.9	13.9	13.3	13.5	

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

\*\* Concentration based on mean (new and old) measured concentrations.

Table 2 - Definitive Study, Water Quality Values (Cont'd)

Test Day: 2	Date: 17-Dec-03	old solution			composite of replicates		
		Control	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Loading Rate* (Concentration**) mg/L							
Dissolved Oxygen (mg/L)		6.8	6.8	6.7	6.8	7.0	Not Present
pH		7.2	7.2	7.2	7.2	7.3	
Temperature (°C)		14.4	14.1	14.1	14.0	14.3	

Test Day: 2	Date: 17-Dec-03	new solution			composite of replicates		
		Control	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Loading Rate* (Concentration**) mg/L							
Dissolved Oxygen (mg/L)		8.2	8.3	8.3	8.4	8.4	Not Present
pH		7.9	7.8	7.9	7.8	7.8	
Temperature (°C)		14.5	14.4	14.4	14.3	14.2	

Test Day: 3	Date: 18-Dec-03	old solution			composite of replicates		
		Control	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Loading Rate* (Concentration**) mg/L							
Dissolved Oxygen (mg/L)		7.0	7.3	7.0	6.9	6.8	Not Present
pH		7.0	7.1	7.1	7.1	7.0	
Temperature (°C)		14.2	14.4	14.4	14.0	13.8	

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

\*\* Concentration based on mean (new and old) measured concentrations.

**Table 2 - Definitive Study, Water Quality Values (Cont'd)**

Test Day: 3	Date: 18-Dec-03	new solution				
Loading Rate* (Concentration**) mg/L	Control (0)	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Dissolved Oxygen (mg/L)	8.5	8.5	8.4	8.4	8.5	Not Present
pH	8.0	7.9	7.8	7.9	7.9	
Temperature (°C)	13.9	13.8	13.6	13.6	13.4	

Test Day: 4	Date: 19-Dec-03	old solution	composite of replicates			
Loading Rate* (Concentration**) mg/L	Control (0)	0.63 (0.30)	1.4 (1.2)	2.6 (2.5)	5.8 (4.1)	11 (9.1)
Dissolved Oxygen (mg/L)	7.3	7.3	7.2	7.1	7.1	Not Present
pH	7.3	7.3	7.3	7.2	7.2	
Temperature (°C)	14.1	14.2	14.0	13.9	13.9	

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

\*\* Concentration based on mean (new and old) measured concentrations.

FIGURE 1 - LOADING RATE RESPONSE CURVE

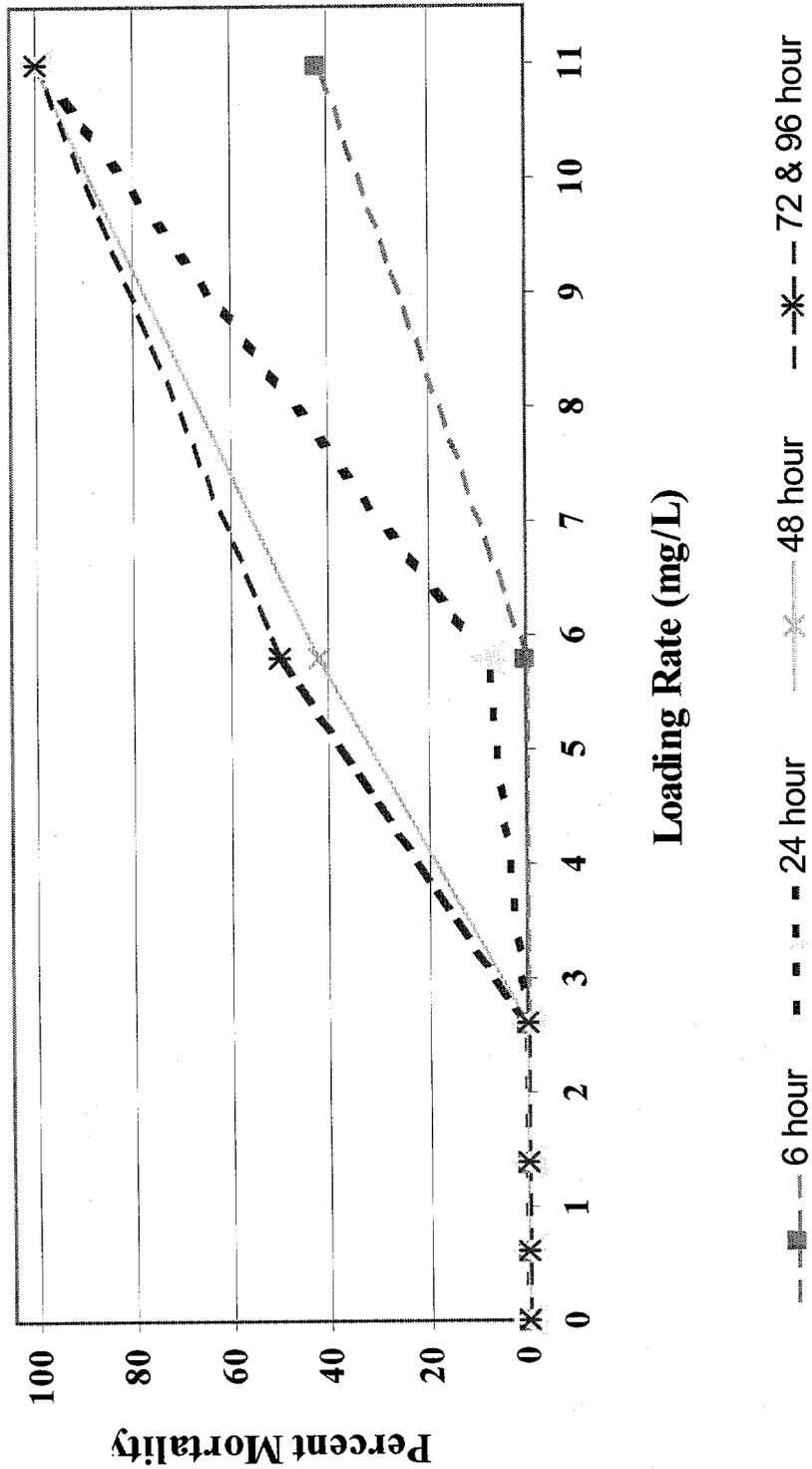
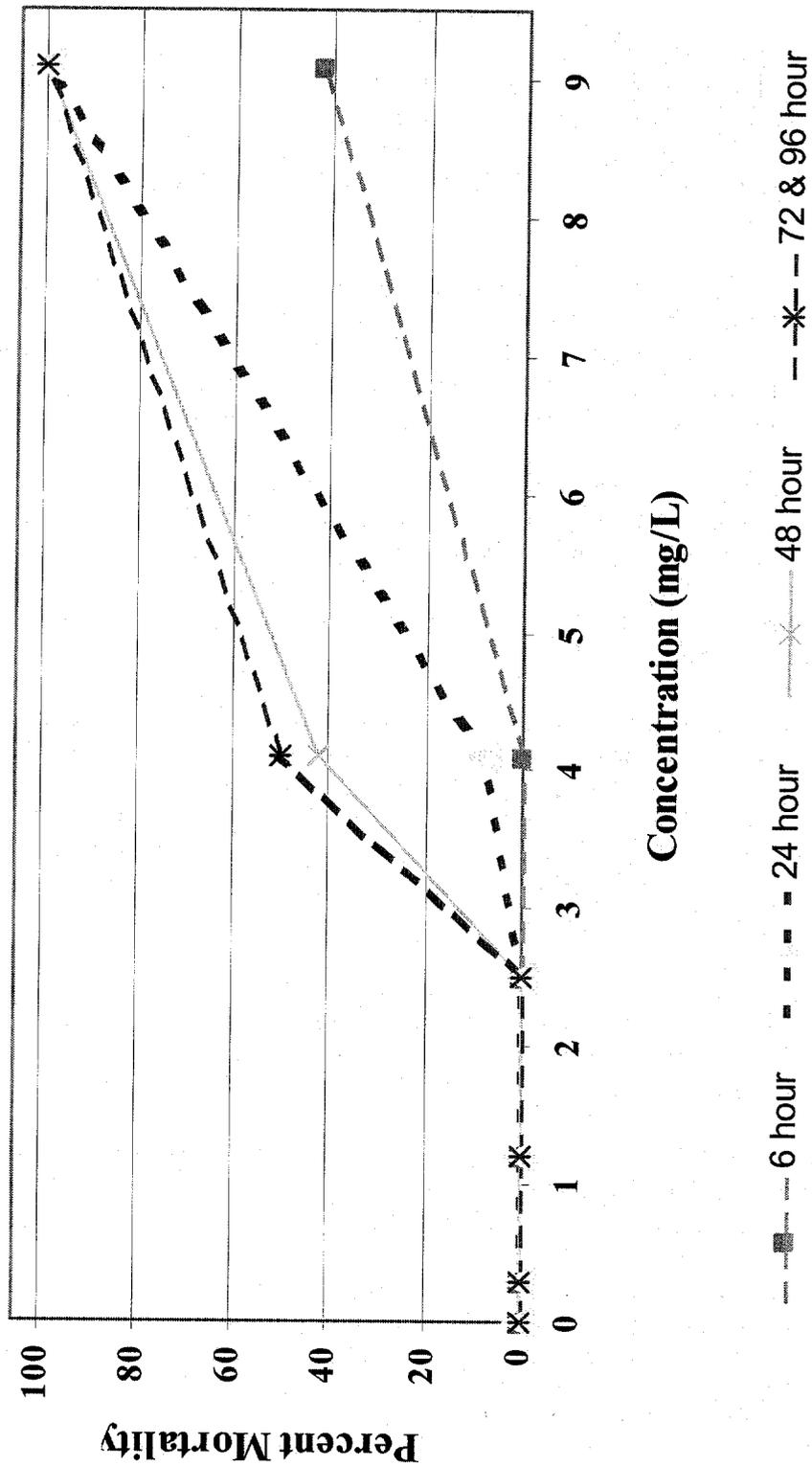


FIGURE 2 - CONCENTRATION RESPONSE CURVE



## APPENDIX A - ANALYTICAL METHODS and RESULTS

Heavy Pyrolysis Fuel Oil WAF samples (ca. 125 mL) were collected with no headspace in amber glass bottles with Teflon<sup>®</sup> lined caps. Samples were refrigerated pending extraction with hexane and analysis by gas chromatography with flame ionization detection (GC-FID). Samples of WAFs prepared at each loading level were taken on Day 0 and 3 from the stirring vessels and from the "old" WAFs of each treatment (composite of replicates) on Day 1 and 4.

Samples were allowed to come to room temperature prior to extraction. Depending on the nominal sample concentration, approximately 25, 50 or 125 mL sample volumes were transferred to glass extraction bottles and 4.0 or 5.0 mL of hexane was added as extraction solvent. The exact amount of water extracted was determined by using tared extraction bottles and re-weighing the bottles after the appropriate volume of sample was dispensed.

After the addition of hexane, the extraction bottles were crimp sealed with Teflon<sup>®</sup> faced septum caps and extracted by hand for approximately one minute followed by 60 minutes on a mechanical shaker. The contents were then allowed to settle for at least 30 minutes before the hexane (upper) layer was transferred to small (ca. 4 mL) glass vials. An aliquot was removed from the 4 mL vial to a GC autosampler vial for analysis. Hexane extracts were stored in a freezer pending analysis.

Heavy Pyrolysis Fuel Oil standards in hexane and hexane extracts were analyzed on a Perkin Elmer Autosystem XL Gas Chromatograph with a 15m x 0.53mm id Rtx-5 capillary column with 1.5  $\mu\text{m}$  film thickness (Restek) and a 5m Integra guard column. The carrier gas was helium at 15 mL/min. The oven temperature was programmed from 55°C for 6 minutes up to 250°C at 30°C/minute. Automated large volume injections of 30  $\mu\text{L}$  were made in the programmable split/splitless (PSS) mode. The FID temperature was 275°C and the detector attenuation setting was -5.

Heavy Pyrolysis Fuel Oil standards were analyzed at concentrations of 3.84, 12.8, and 64  $\mu\text{g/mL}$ . The extraction method provided quantitative recoveries and was validated by spiking Heavy Pyrolysis Fuel Oil in water at 0.961  $\mu\text{g/mL}$ . Five replicate spikes were extracted and the mean recovery (standard deviation) was 108% (6.7%).

Data were acquired and processed with Perkin Elmer Totalchrom Workstation software. Heavy Pyrolysis Fuel Oil eluted as a complex mixture at approximate retention time of 8.5 minutes under the instrumental conditions employed.

The Practical Quantitation Limit (PQL) for water samples was approximately 0.12  $\mu\text{g/mL}$  taking into account the concentration of the lowest analyzed standard (3.84  $\mu\text{g/mL}$ ), the maximum sample volume (approximately 125 mL) and the minimum extract solvent volume (4.0 mL).

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

TABLE A-1

Loading Rate <sup>◇</sup> (mg/L)	Measured Concentration (mg/L)				
	Day 0 (new)	Day 1* (old)	Day 3 (new)	Day 4* (old)	Average
0	ND	ND	ND	ND	ND
0.63	0.511	0.445	0.134	0.127	0.30
1.4	1.04	0.872	1.44	1.28	1.2
2.6	2.86	2.48	2.50	2.21	2.5
5.8	4.67	4.38 4.18**	3.89	3.61	4.1
11	9.22	9.00	†	†	9.1

◇ Actual loading rate (weight) of test substance added to the vehicle/dilution water. The values represent the mean of the individual WAFs prepared for the renewals.

ND = Not Detected

\* Composite of remaining replicates.

\*\* Sampled from replicate 3 due to complete mortality, actually Day 2 "old" solution (value used for average).

† No Sample, due to complete mortality.

PQL (Practical Quantitation Limit) = 0.12 mg/L

**APPENDIX A - ANALYTICAL METHODS and RESULTS (CONT'D)  
 DILUTION WATER (VEHICLE) ANALYSIS**

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. Batches of 500 to 1000 L of this deionized water are then reconstituted in the laboratory to meet aquatic toxicity testing needs, following Method 8010E of *Standard Methods for the Examination of Water and Wastewater*, 18th edition.

The following water quality data is most representative of the dilution water used during the in-life period of the study. Table A-2 presents analyses performed on the reconstituted water (RW) on a batch basis. Water quality analyses (dissolved oxygen, pH, alkalinity, hardness and specific conductance) are performed by environmental toxicology laboratory personnel. Total Organic Carbon analysis is performed by the laboratory's environmental chemistry and fate group. The quality of the feed water for the dilution water system is monitored at least semi-annually for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties. Results of semi-annual analyses are maintained at the testing facility.

**Table A-2  
 Results of Water Quality Analysis**

Sample	Sample Date	Alkalinity as CaCO <sub>3</sub> (mg/L)*	Hardness as CaCO <sub>3</sub> (mg/L)**	Specific Conductance (µmhos)	pH	Dissolved Oxygen (mg/L)	Temp. (°C)
Batch 283	9-Dec-03	61	98	305	7.8	9.0	20.2

Sample	Sample Date	Total Organic Carbon (ppm) <sup>†</sup>
Batch 283	9-Dec-03	0.3032

\* 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).

\*\* 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).

† American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 5310C, Persulfate- Ultraviolet Oxidation Method.

## APPENDIX B - TEST SUBSTANCE CHARACTERIZATION

The test substance was initially characterized on August 19 and 21, 2003. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and GC-MS analysis. Stability of the neat test substance was confirmed by repeating these same analyses on February 5, 2004 after the completion of this study.

UV-VIS spectra are presented in Figures B-1 and B-2 representing, the initial and final spectrum at concentrations of 10 and 12 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 B-3 and B-4 representing the initial and final spectra. FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of  $4\text{ cm}^{-1}$ , gain of 1 and scan number of 32.

The test substance was also characterized by GC-MS using a Varian Saturn 2000 GC-MS system with a Varian 3800 gas chromatograph. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, Heavy Pyrolysis Fuel Oil was analyzed against an ASTM D2887 calibration mixture. Figures B-5 and B-6 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components detected between retention times 13 and 26 minutes. This corresponds to bracketing by the standard hydrocarbons n-decane and n-octadecane under the analytical conditions employed. The single most abundant component eluted at approximately 16.3 minutes.

The test substance's initial and final density was measured at 20°C using an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 1.068 g/mL and the final density was 1.069 g/mL. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water, methanol and hexane. All test substance solutions were prepared with methylene chloride as the solvent.

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.

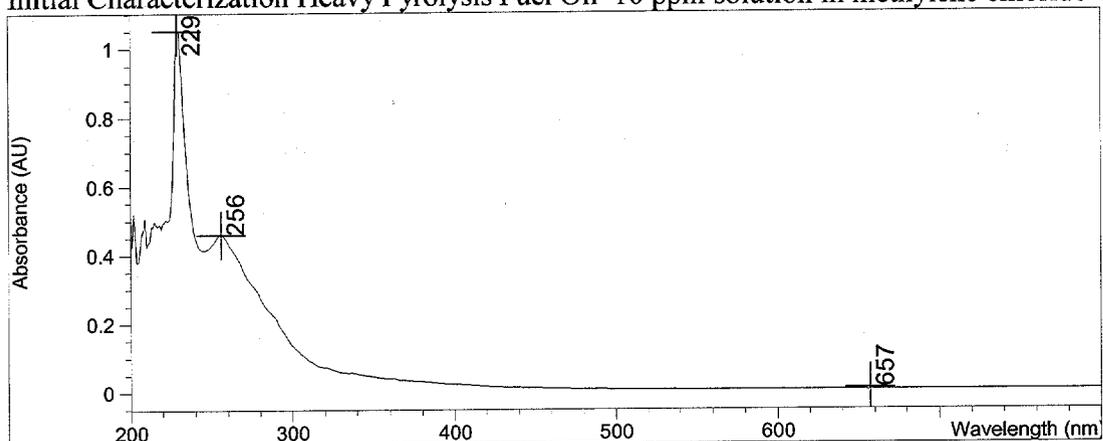
APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure B-1

Initial

Initial Characterization Heavy Pyrolysis Fuel Oil 10 ppm solution in methylene chloride



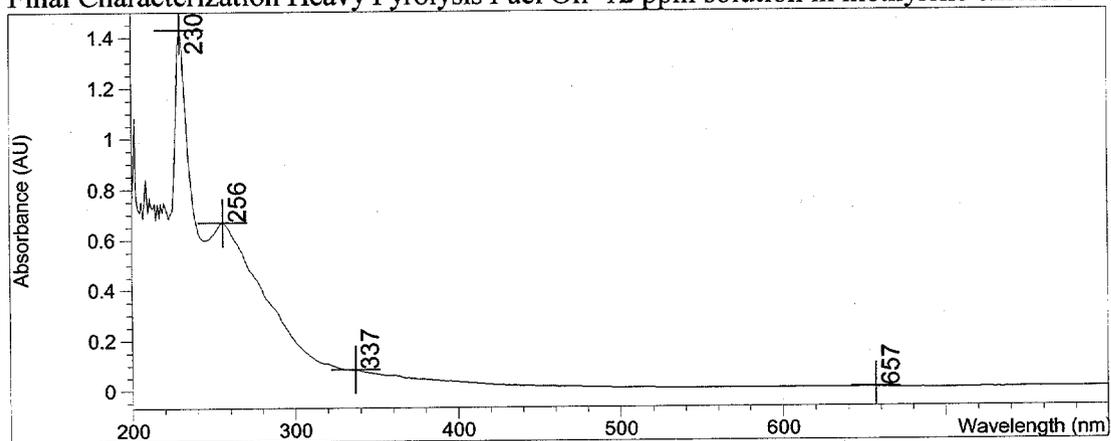
Analysis Date: 19 August 03

Peak 229nm Absorbance = 1.051  
Peak 256nm Absorbance = 0.4597  
Peak 657nm Absorbance = 0.0095

Figure B-2

Final

Final Characterization Heavy Pyrolysis Fuel Oil 12 ppm solution in methylene chloride



Analysis Date: 5 February 04

Peak 230nm Absorbance = 1.4298  
Peak 256nm Absorbance = 0.6677  
Peak 337nm Absorbance = 0.0832  
Peak 657nm Absorbance = 0.0068

APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FT-IR SPECTRA

Figure B-3

Initial

Initial Characterization Heavy Pyrolysis Fuel Oil

Analysis Date: 19 Aug 03

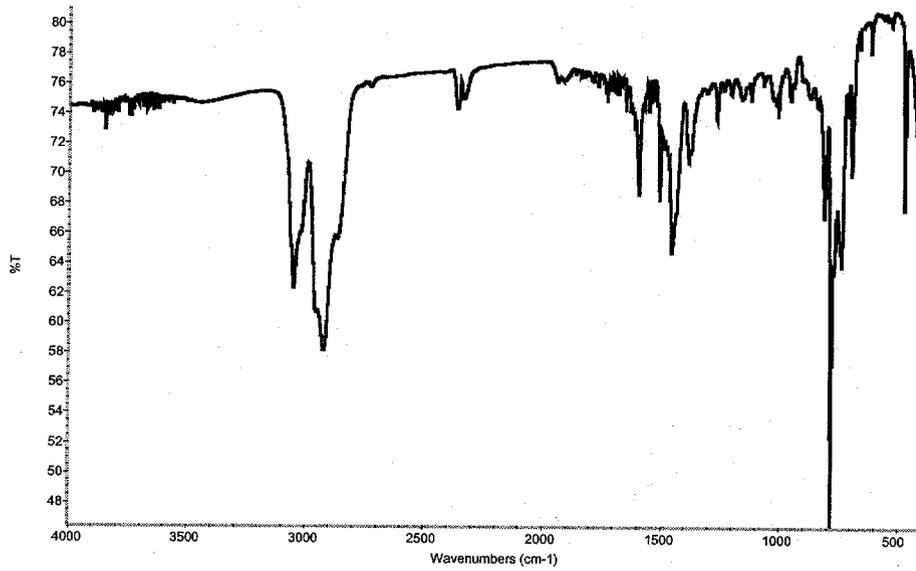
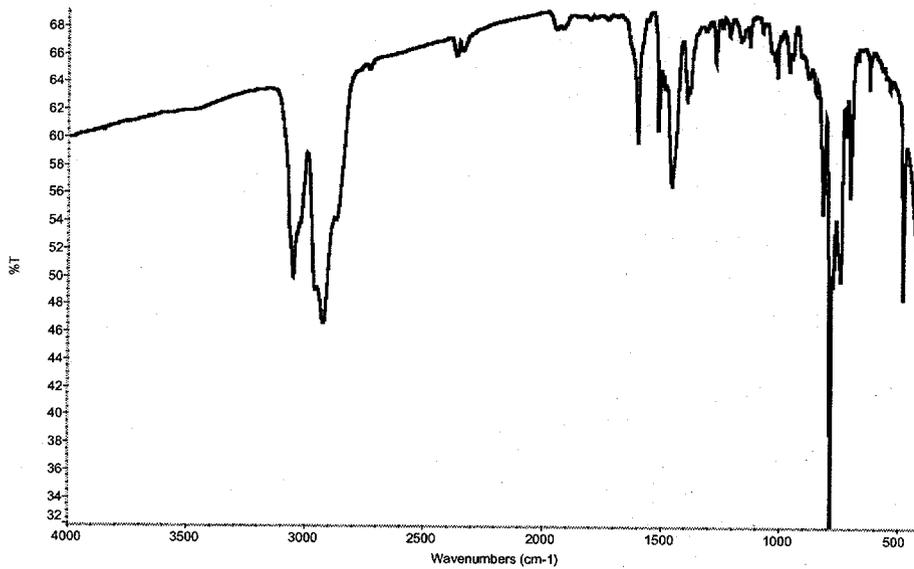


Figure B-4

Final

Final Characterization Heavy Pyrolysis Fuel Oil

Analysis Date: 5 Feb 04



%T = % Transmittance

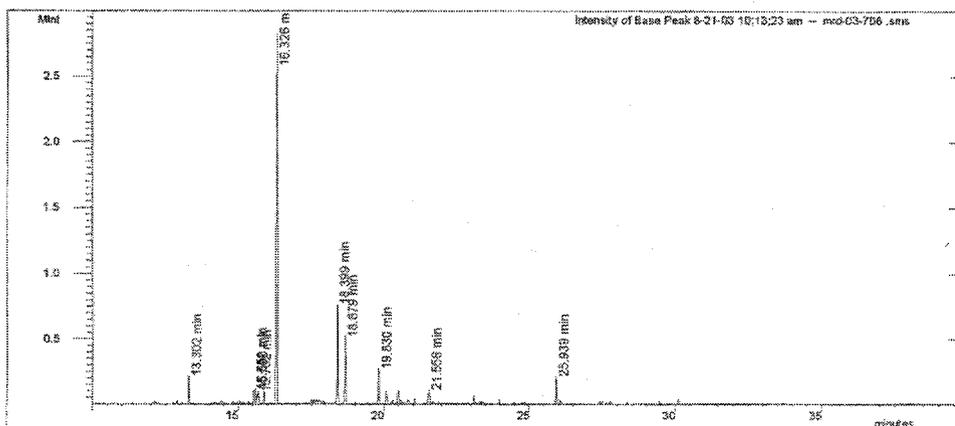
APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

INITIAL TOTAL ION CHROMATOGRAM

Figure B-5

AREA PERCENT REPORT

Data File Name:	c:\saturnws\8-21-03 10:13:23 am -- mrd-03-768 .sms	Acquisition Date:	8/21/03 10:13:24 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) initial characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.302	BB	1.9	1213022	4.86
15.552	BV	2.1	1037451	4.16
15.586	VV	2.2	895935	3.59
16.326	VV	2.7	1011392	4.05
16.326	VB	1.9	9296601	37.27
18.399	VB	1.7	4306416	17.26
18.679	BB	1.9	3339778	13.39
19.830	BB	1.7	1375559	5.51
21.556	VV	2.2	1524481	6.11
25.939	BB	1.8	942704	3.78

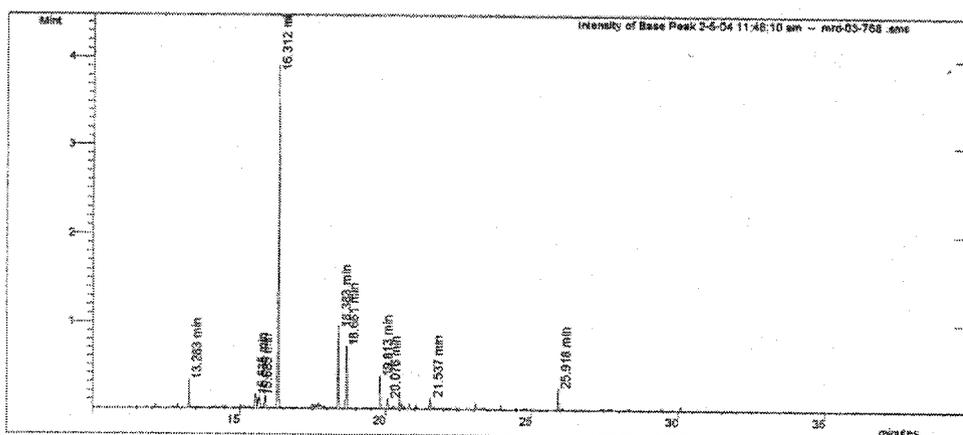
**APPENDIX B- TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**FINAL TOTAL ION CHROMATOGRAM**

**Figure B-6**

**AREA PERCENT REPORT**

Data File Name:	c:\saturnws\2-5-04 11:48:10 am -- mrd-03-768 .sms	Acquisition Date:	2/5/04 11:48:11 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) final characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.283	BB	1.8	1973177	5.02
15.535	BB	2.0	3045234	7.75
15.685	TF	0.0	1653164	4.21
16.312	TF	0.0	13855800	35.25
18.383	VV	0.0	6504165	16.55
18.661	VV	0.0	5061242	12.88
19.813	TF	0.0	2145104	5.46
20.076	VB	0.0	1399923	3.56
21.537	TF	0.0	2322773	5.91
25.918	MV	0.0	1346340	3.43

## APPENDIX C - PROTOCOL

### PROTOCOL

#### OLF-105.0-HPV10-EMBSI

*Study Title:* Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil

*EMBSI Study Number:* 176858

*Test Substance:* MRD-03-768

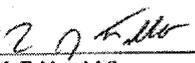
*Date:* August 11, 2003

*Room Number:* LE -337

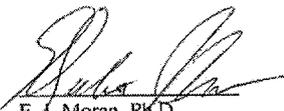
*Proposed Key Dates:*

Experimental Start .....	20-Oct-03
Experimental Termination .....	24-Oct-03
Draft Report Completion .....	21-Nov-03
Final Report Completion .....	5-Mar-04

*Approved By:*

  
\_\_\_\_\_  
E. J. Febbo, M.S.  
Study Director  
ExxonMobil Biomedical Sciences, Inc.  
1545 Route 22 East, P.O. Box 971  
Annandale, New Jersey 08801-0971

15 Sep 03  
Date

  
\_\_\_\_\_  
E. J. Moran, Ph.D.  
Sponsor Representative

9/9/03  
Date

SAFETY FIRST

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
176858; MRD-03-768

PAGE 2

### INTRODUCTION

#### *Objective*

This study will be conducted for the Sponsor to evaluate the acute toxicity of the water accommodated fractions (WAFs) of MRD-03-768 to the rainbow trout, *Oncorhynchus mykiss* in a 96-hour semi-static test.

#### *Sponsor*

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, VA 22209

#### *Testing Facility*

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

#### *Compliance*

This test will be conducted in general agreement with OECD<sup>1</sup> guidelines, and will be performed in compliance with OECD<sup>2</sup> and USEPA<sup>3</sup> GLP standards.

#### *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 C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
176858; MRD-03-768

PAGE 3

### MATERIALS and METHODS

#### *Test Substance Identification*

EMBSI Code: MRD-03-768

Industry Stream Name: Heavy Pyrolysis Fuel Oil

CAS Number	CAS Inventory Name
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum

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

#### *Characterization of Test Substance*

The neat test substance will be characterized as described in SOP A.3.4.10 using the following analyses: Ultra-violet/Visible and Infrared Spectrophotometry and Gas Chromatography with Mass Selective Detection; density will also be determined. The test substance will be used in a number of studies at the testing facility. Characterization of the test substance will be performed at the testing facility prior to its use in the first of these studies. Stability assessment will span the duration of all studies. The results of the characterization and stability assessment will be appended to the final report. Characterization and stability documentation will be maintained at the testing facility.

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, will be considered the "pure" substance for dosing purposes.

#### *Analysis of Mixtures*

Samples will be taken from each 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. The method of analysis will be automated static headspace gas chromatography with flame ionization detection (HS GC-FID). Samples will be analyzed using a Perkin-Elmer HS 40 Headspace Sampler connected to a Perkin Elmer AutoSystem XL Gas Chromatograph with flame ionization detector. The gas chromatograph will be equipped with a 30m x 0.53mm id DB-5 capillary column with 1.5µm film thickness (or equivalent). Analytical standards will be prepared and analyzed at concentrations bracketing the sample concentrations except in the case of those samples below the method's limit of quantification.

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
176858; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *Vehicle/Dilution Water*

Reconstituted water<sup>2</sup>: Vehicle/dilution water will be aerated prior to use.

#### *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 (13-15°C), continuously aerated to maintain the dissolved oxygen level at  $\geq 80\%$  of the saturation value. Fish will also be held under 12 to 16 hours of illumination daily. 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.

Fish are held under static conditions using biological and mechanical filtration and are typically fed at least five days per week with Finfish Starter and/or Tetramin®.

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

#### *Number and Sex*

Number: 72      Sex: Not Applicable

#### *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 rate, replicate, and chamber number.

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
176858; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *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 24 hours prior to, or during the study.

#### *Contaminants*

There are no known contaminants in the feed used for acclimation or the vehicle/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 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. 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 C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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### EXPERIMENTAL PROCEDURE

#### *Equilibrium Test*

An equilibrium study will be performed prior to testing to determine the most appropriate mixing duration. Specific analytical (e.g. GC analysis) will be used to detect soluble components of the substance. Individual WAFs at 1 mg/L and 10 mg/L will be prepared and sampled after 24 and 48 hours of mixing. The vortex will be set at  $\leq 10\%$  of the static liquid depth. All mixing vessels will be closed using foil covered stoppers during mixing. The equilibrium phase will not extend beyond 48 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. This phase of the study will not be subject to GLP standards.

#### *Range Finding Test*

A 48-hour range finding test will be performed to determine definitive test loading rates. WAFs at the following loading rates will be tested; 0.1 mg/L, 1 mg/L, 10 mg/L, 100 mg/L and a control. The WAFs will be prepared by adding the appropriate amount of test substance to dilution water in glass vessels. The vessels will be closed using foil covered stoppers, and will mix on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by equilibrium testing ( $\pm 1$  hour). The vortex will be set at  $\leq 10\%$  of the static liquid depth. The treatments will be allowed to settle and chill to test temperature for 1 to 2 hours after mixing. The range finding test may be extended up to 96 hours if necessary. One replicate at each loading rate will be prepared containing 5 fish. An approximately 80% to 90% renewal of the test solutions will be performed at 24 hours. New WAFs will be prepared for the renewal. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

#### *Definitive Test Design*

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	12 (4 per 3 replicates)
2	TBD	12
3	TBD	12
4	TBD	12
5	TBD	12
6	TBD	12

TBD = To Be Determined

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Preparation and Administration of Test Substance*

Individual WAFs will be prepared for each loading rate by adding the appropriate amount of the test substance to the vehicle/dilution water in glass aspirator bottles. The vessels will be closed using foil covered stoppers. The solutions will be mixed with Teflon® coated stirbars on magnetic stirplates. The vortex will be set at  $\leq 10\%$  of the static liquid depth. The solutions will mix for the appropriate time as determined by equilibrium testing ( $\pm 1$  hour) at room temperature ( $22^{\circ} \pm 2^{\circ} \text{C}$ ). At the end of mixing, the solutions will be allowed to settle and cool for approximately 1 to 2 hours in a waterbath. 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 three replicate chambers. An approximately 80% to 90% renewal of the test solutions will be performed at approximately 24-hour intervals. New WAFs will be prepared daily for the renewals.

#### *Test Chamber and Volume of Solution*

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

#### *Exposure Duration*

96 hours ( $\pm 1$  hour)

#### *Environmental Conditions*

Acceptable test water temperatures:  $14^{\circ} \pm 1^{\circ} \text{C}$ . Diurnal light: 16 hours light : 8 hours dark.

An environmental condition study will be activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

#### *Experimental Evaluation*

Observations for mortality will be performed and recorded at 3, 6, 24, 48, 72 and 96 hours ( $\pm 1$  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.

After completion of the study, the monitoring of environmental conditions will be discontinued. All remaining fish will be euthanized using a 2 g/L tricaine methane sulphate (MS 222) solution, prepared in laboratory dilution water. Control fish will be individually weighed and their total lengths measured.

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
176858; MRD-03-768

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### 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 on the "old" solutions (composite of the replicates) 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%.

#### *Loading*

Loading will not exceed 1.0g 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). 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 have resulted in oxygen depletion in treatment solutions. The loading rate (g of fish per liter of solution) with regards to oxygen depletion will be assessed during the range finder. A loading between 0.2 - 0.6 g of fish per liter of solution will be the target for the range finder.

#### *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.

#### *Calculations*

Test results are used to derive the LC/LL<sub>50</sub>, (or other appropriate statistical value) defined as the concentration and/or loading rate of the test substance estimated to kill 50% of the test organisms within a specified period of exposure. The statistical method used to calculate the LC/LL<sub>50</sub> values and their associated 95% confidence limits will be either a maximum likelihood analysis based on D. J. Finney, 1971<sup>7</sup>, a Trimmed Spearman-Kärber Method<sup>6</sup>, a Binomial Method<sup>7</sup> or a graphical method<sup>8</sup>.

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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### REPORT

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

Test substance:

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

Test fish:

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

Test conditions:

- test procedure used, equilibration test results
- vehicle/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 rates/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:

- maximum loading rate/concentration causing no mortality
- minimum loading rate/concentration causing 100% mortality
- percent of organisms that were dead per treatment
- individual daily observations, including daily and cumulative mortality, survival and abnormal responses of the fish
- $LL_{50}$  and/or  $LC_{50}$  with 95% confidence limit at each observation interval, if possible.
- statistical procedures followed
- graph of the loading rate/concentration-response curve at the end of the test, if applicable
- methods used and results obtained from chemical analysis

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

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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### 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 and the appropriate guidelines and Good Laboratory Practice Regulations.

### GUIDELINE EXCEPTIONS

Due to the limited solubility of the test substance the following exceptions to the guidelines will apply for this study:

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

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

Contaminant analysis of the feed and water are not performed in a GLP compliant manner. This is not believed to affect the results of the analysis.

## APPENDIX C - PROTOCOL (CONT'D)

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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### REFERENCES

1. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals. Section 2: Effects on Biotic Systems, Guideline 203: Fish, Acute Toxicity Test. Adopted 17 July 1992.
2. OECD. Principles of Good Laboratory Practice (GLP), C(97)186 (Final), 1997.
3. United States Environmental Protection Agency (USEPA). Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
4. American Public Health Association, American Water Works Association and Water Environment Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18<sup>th</sup> ed. American Public Health Association, Washington, D.C. Method 8010F (Table 8010-I).
5. Finney, D.J., 1971. Probit Analysis, 3rd Edition, London: Cambridge University Press.
6. Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. *Environmental Science and Technology*, Vol. 11, No. 7, p.714-719.
7. Stephan, C. E., Methods for Calculating an LC<sub>50</sub>, *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.
8. Weber, C.J., 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.

**APPENDIX C - PROTOCOL (CONT'D)**

Fish, Acute Toxicity Test on Heavy Pyrolysis Fuel Oil:  
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**DISTRIBUTION**

Study Director .....	E. J. Febbo
Sponsor Representative .....	E. J. Moran
Section Head, Laboratory Operations .....	R. L. Rucker
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.....	P. N. Unanka
.....	J. Yarusinsky
Analytical Chemistry .....	D. J. Letinski
Quality Assurance .....	Staff
Contract Administrator .....	B. J. Foster

**FISH, ACUTE TOXICITY TEST on HEAVY PYROLYSIS FUEL OIL**  
**Study No. 176858; MRD-03-768**

**APPENDIX C - PROTOCOL (CONT'D)**

PROTOCOL CHANGE RECORD  
 FISH, ACUTE TOXICITY TEST on HEAVY PYROLYSIS FUEL OIL

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 Number: 176858

Revision Number: 1

Date: 11-Nov-03

**Pg. 6/ Definitive Test Design**

*Previous Statement:*

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	12 (4 per 3 replicates)
2	TBD	12
3	TBD	12
4	TBD	12
5	TBD	12
6	TBD	12

TBD = To Be Determined

*Revised Statement:*

GROUP	LOADING RATE (mg/L)	NUMBER OF ORGANISMS
1 (Control)	0	12 (4 per 3 replicates)
2	0.63	12
3	1.3	12
4	2.5	12
5	5	12
6	10	12

*Justification:* addition of definitive loading rates

Required signatures:

*E. J. Moran by [Signature]*  
 E. J. Moran  
 Sponsor Representative

11-24-03  
 Date

*[Signature]*  
 E. J. Febbo  
 Study Director

18 Nov 03  
 Date

**Robust Summary  
Fish, Acute Toxicity**

<b>Test Substance:</b>	<p>Industry Stream Name (acronym): Heavy Pyrolysis Fuel Oil</p> <table border="0"> <tr> <td><u>CAS Number:</u></td> <td><u>CAS Inventory Name:</u></td> </tr> <tr> <td>68513-69-9</td> <td>Residues, petroleum, steam-cracked light</td> </tr> <tr> <td>64741-62-4</td> <td>Clarified oils, petroleum, catalytic cracked</td> </tr> <tr> <td>69013-21-4</td> <td>Fuel oil, pyrolysis</td> </tr> <tr> <td>8002-05-9</td> <td>Petroleum</td> </tr> </table> <p>In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.</p>	<u>CAS Number:</u>	<u>CAS Inventory Name:</u>	68513-69-9	Residues, petroleum, steam-cracked light	64741-62-4	Clarified oils, petroleum, catalytic cracked	69013-21-4	Fuel oil, pyrolysis	8002-05-9	Petroleum
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69013-21-4	Fuel oil, pyrolysis										
8002-05-9	Petroleum										
<b>Method/Guideline:</b>	OECD Guideline 203										
<b>Year (guideline):</b>	1992										
<b>Type (test type):</b>	Fish Acute Toxicity Test										
<b>GLP (Y/N):</b>	Yes										
<b>Year (study performed):</b>	2003										
<b>Species:</b>	<i>Oncorhynchus mykiss</i>										
<b>Analytical Monitoring:</b>	Yes										
<b>Exposure Period:</b>	96 hours										
<b>Statistical Method:</b>	<p>The 24 - 96 hour LL<sub>50</sub> and LC<sub>50</sub> values were determined using a Trimmed Spearman-Kärber Method (Hamilton et al., 1977).</p> <p>Hamilton, M., R. Russo, R. Thurston, 1977. Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. <i>Environmental Science and Technology</i>, Vol. 11, No. 7, p.714-719.</p>										
<p><b>Test Conditions:</b></p> <ul style="list-style-type: none"> <li><b>Note: Concentration preparation, vessel type, volume, replication, environmental conditions, organisms supplier, loading, deviations from guideline or protocol.</b></li> </ul>	<p>Individual Water Accommodated Fractions (WAF's) were prepared for each treatment. The test substance was added to 18 L of reconstituted water in glass aspirator bottles (capacity 22 L). The solutions were mixed for 24 hours using a 3% vortex (of the static liquid depth). The test solutions were removed through the outlet at the bottom of each mixing vessel into three replicates of approximately 4.5 L in 4 L size aspirator bottles (no headspace). Four fish were added to each replicate and the replicates were closed with foil covered neoprene stoppers. Daily renewals were performed by removing ~90% of the test solution through the outlet at the bottom of the aspirator bottle and refilling with fresh solution. The fish were received from Thomas Fish Company, Anderson, CA. The fish were not fed during the study. They were held for 12 days in study dilution water prior to use and were 36 days old at the start of the study. Fish mean weight = 0.194 g, mean total length = 3.1 cm, test loading = 0.172 g of fish/L.</p>										

	<p>Mean test temperature: 13.6°C (S.D. = 0.1), diurnal light: approximately 16 hours light and 8 hours dark with 644 to 653 Lux during full daylight periods. Dissolved oxygen ranged from 6.7 to 8.5 mg/L and pH ranged from 6.5 to 8.0 during the study. Water hardness was 98 mg/L as CaCO<sub>3</sub>.</p> <p>Due to the complex nature and limited water solubility of the test substance, the following exceptions to the guideline apply for this study:</p> <p>The concentration of the test substance in solution was not determined prior to use. It was deemed more appropriate to prepare individual treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution.</p> <p>The protocol required that the fish would be held at test temperature (13-15°C) for at least 7 days prior to use in the test. The fish were held at 12.8°C for the 7 days prior to use in the study. This deviation is not believed to have affected the outcome or integrity of the study.</p>																																																																							
<p><b>Results:</b></p> <p><b>Units/Value:</b></p> <p><b>Note: Analytical method, biological observations, control survival.</b></p>	<p>The maximum actual loading rate causing no mortality after 96-hours was 2.6 mg/L. The maximum measured concentration causing no mortality after 96-hours was 2.5 mg/L. The minimum actual loading rate causing 100% mortality after 96-hours was 11 mg/L. The minimum measured concentration causing 100% mortality after 96-hours was 9.1 mg/L. The method of analysis was gas chromatography with flame ionization detection (GC-FID).</p> <p>Lethal Loading (LL<sub>50</sub>) / Lethal Concentration (LC<sub>50</sub>) Values (mg/L)</p> <table border="1" data-bbox="738 1039 1437 1186"> <thead> <tr> <th></th> <th>LL<sub>50</sub></th> <th>LC<sub>50</sub></th> </tr> </thead> <tbody> <tr> <td>3 &amp; 6 hours</td> <td>&gt;11*</td> <td>&gt;9.1*</td> </tr> <tr> <td>24 hours</td> <td>7.5 (6.7-8.4)</td> <td>5.8 (5.2-6.4)</td> </tr> <tr> <td>48 hours</td> <td>5.9 (4.8-7.3)</td> <td>4.7 (3.9-5.6)</td> </tr> <tr> <td>72 &amp; 96 hours</td> <td>5.6 (4.5-6.9)</td> <td>4.4 (3.7-5.3)</td> </tr> </tbody> </table> <p>* Not a calculated value, no mortality was observed in the highest loading rate/concentration at 3 hours, 42% mortality was observed in the highest loading rate/concentration at 6 hours therefore the EL/EC<sub>50</sub> is greater than the highest loading rate/concentration tested.</p> <p>Values in parentheses are 95% confidence intervals</p> <p>Summary of In-Life observations - % Mortality</p> <table border="1" data-bbox="738 1375 1485 1585"> <thead> <tr> <th></th> <th>Control</th> <th>0.63</th> <th>1.4</th> <th>2.6</th> <th>5.8</th> <th>11</th> </tr> </thead> <tbody> <tr> <td>Loading Rate (mg/L)</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>Meas. Conc. (mg/L)</td> <td>0</td> <td>0.30</td> <td>1.2</td> <td>2.5</td> <td>4.1</td> <td>9.1</td> </tr> <tr> <td>3 hours</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>6 hours</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>42</td> </tr> <tr> <td>24 hours</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>8</td> <td>100</td> </tr> <tr> <td>48 hours</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>42</td> <td>100</td> </tr> <tr> <td>72 &amp; 96 hours</td> <td>0</td> <td>0</td> <td>0</td> <td>0</td> <td>58</td> <td>100</td> </tr> </tbody> </table>		LL <sub>50</sub>	LC <sub>50</sub>	3 & 6 hours	>11*	>9.1*	24 hours	7.5 (6.7-8.4)	5.8 (5.2-6.4)	48 hours	5.9 (4.8-7.3)	4.7 (3.9-5.6)	72 & 96 hours	5.6 (4.5-6.9)	4.4 (3.7-5.3)		Control	0.63	1.4	2.6	5.8	11	Loading Rate (mg/L)							Meas. Conc. (mg/L)	0	0.30	1.2	2.5	4.1	9.1	3 hours	0	0	0	0	0	0	6 hours	0	0	0	0	0	42	24 hours	0	0	0	0	8	100	48 hours	0	0	0	0	42	100	72 & 96 hours	0	0	0	0	58	100
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<p><b>Conclusion:</b></p>	<p>After <i>Oncorhynchus mykiss</i> were exposed to WAFs prepared from Heavy Pyrolysis Fuel Oil for 96-hours, the LL<sub>50</sub> was 5.6 mg/L and the LC<sub>50</sub> was 4.4 mg/L.</p>																																																																							
<p><b>Reliability:</b></p>	<p>1-Reliable without restrictions.</p>																																																																							
<p><b>Reference:</b></p>	<p>ExxonMobil Biomedical Sciences, Inc. 2004. FISH, ACUTE TOXICITY TEST on HEAVY PYROLYSIS FUEL OIL. Study # 176858</p>																																																																							
<p><b>Other (source):</b></p>	<p>Olefins Panel, American Chemistry Council</p>																																																																							

# **ExxonMobil** BIOMEDICAL SCIENCES, INC.

**OLF-105.0-HPV10-EMBSI**

**ALGA, GROWTH INHIBITION TEST on  
HEAVY PYROLYSIS FUEL OIL**

**FINAL REPORT**

**STUDY NUMBER: 176867**

**TEST SUBSTANCE: MRD-03-768**

**PERFORMED FOR:**

**American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209**

**PERFORMED AT:**

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

**COMPLETION DATE: October 29, 2004**

**04TP 117**

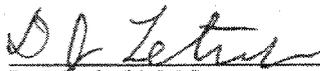
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APPROVAL SIGNATURES

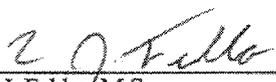
  
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J. J. Freeman, Ph.D., D.A.B.T.  
Acting Section Head, Laboratory Operations

13 Oct 04  
Date

  
\_\_\_\_\_  
D. J. Letinski, M.S.  
Supervisor, Environmental Chemistry

15 Oct 04  
Date

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<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards except as outlined on page 11-13.

  
\_\_\_\_\_  
E. J. Febbo, M.S.  
Study Director  
ExxonMobil Biomedical Sciences, Inc.  
1545 Route 22 East, P.O. Box 971  
Annandale, New Jersey 08801-0971

29 OCT 04  
Date

The final report was accepted by the Sponsor.

  
\_\_\_\_\_  
E. J. Moran, Ph.D.  
Sponsor's Representative

10/21/04  
Date

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QUALITY ASSURANCE STATEMENT

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STUDY NUMBER: 176867

TEST SUBSTANCE: MRD-03-768

STUDY SPONSOR: American Chemistry Council

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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.

<u>Study Phase Inspected</u>	<u>Date(s) of Inspection</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
Protocol	07 Aug 03	07 Aug 03	13,14 Aug 03
48-Hour Observations	10 Dec 03	10 Dec 03	11,15 Dec 03
Final Report	27-29 Apr 04	29 Apr 04	10,14 May 04
Second Review of Final Report	05 May 04	05 May 04	10,14 May 04

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

  
\_\_\_\_\_  
W. James Boyer, Ph.D.  
Data Integrity & Quality Assurance / Archives  
Section Head

12 Oct 04  
Date

**PERSONNEL**

Study Director:	E. J. Febbo, M.S.
Sponsor Representative:	E. J. Moran, Ph.D.
Acting Section Head, Laboratory Operations:	J. J. Freeman, Ph.D., D.A.B.T.  R. L. Rucker, Ph.D. (Prior to December 9, 2003)
Data Integrity & Quality Assurance / Archives Section Head:	W. J. Bover, Ph.D.
Supervisor, Environmental Toxicology & Compound Preparation:	E. J. Febbo, M.S.
Supervisor, Environmental Chemistry:	D. J. Letinski, M.S.

### SUMMARY

This study was conducted for the Sponsor to evaluate the effects of the water-accommodated fractions (WAFs) of MRD-03-768 (Heavy Pyrolysis Fuel Oil) on the growth of the alga, *Pseudokirchneriella subcapitata*, in a 96-hour static test, conducted according to OECD Guideline 201.

Individual treatments were prepared by adding the appropriate amount of test substance to 2.0 L of algal nutrient media in glass aspirator bottles (capacity 2.3 L) and stirring on magnetic stirplates using an approximately 7% (of the static liquid depth) vortex for 24.5 hours. After approximately one hour without stirring, the aqueous portions (WAFs) were removed for testing. The following table defines the target loading rates, actual loading rates and measured concentrations.

TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	MEASURED CONCENTRATION† (mg/L)
0	0	0
0.18	0.20	0.07‡
0.44	0.39	0.42
1.1	1.1	1.1
2.8	2.6	2.1
7.0	7.2	6.4

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

† Concentration based on mean (Day 0 and Day 4) measured concentrations.

‡ Based on Day 0 only, since the Day 4 sample was below detection limits.

Twelve replicate chambers were established for each treatment and the control. The test chambers were completely filled (no headspace) with the appropriate WAF and were sealed with ground glass stoppers. Each chamber contained two 14 mm glass spheres to facilitate mixing. Test chambers were placed on a shaker table and oscillated at 100 rpm to keep the algae in suspension. The study was performed under continuous light conditions (approximately 8497 Lux) at approximately 24°C. The pH in the test solutions ranged from 7.4-7.6 at the beginning of the test and from 7.5-8.7 at the end of the test. Three replicates from each loading rate were sacrificed daily for cell density determinations.

**SUMMARY (CONT'D)**

Acute toxicity results are expressed as the Effect Loading / Effect Concentration 50 (EL/EC<sub>50</sub>); that is, the loading rate or concentration of test substance in dilution medium which is calculated to result in a 50% reduction in growth derived from either the average specific growth rate (r) or the area under the growth curves (b) relative to the control for the specified time of exposure. The No Observed Effect Loading Rate / No Observed Effect Concentration (NOELR/NOEC) is the highest loading rate or concentration which does not exhibit a statistical difference from the control.

Hours	E <sub>r</sub> L50 mg/L	E <sub>r</sub> C50 mg/L	E <sub>b</sub> L50 mg/L	E <sub>b</sub> C50 mg/L
72	2.3 (CNC)	2.0 (CNC)	1.5 (1.3-1.6)	1.3 (1.2-1.4)
96	2.1 (CNC)	1.8 (CNC)	1.4 (1.3-1.6)	1.3 (1.2-1.4)

Values in parentheses ( ) are 95% confidence intervals.  
 CNC = Could Not Calculate

Hours	average specific growth rate		area under the growth curves	
	NOELR	NOEC	NOELR	NOEC
72	0.39 mg/L	0.42 mg/L	0.20 mg/L	0.07 mg/L
96	0.39 mg/L	0.42 mg/L	0.39 mg/L	0.42 mg/L

## INTRODUCTION

### *Objective*

This study was conducted for the Sponsor to evaluate the effects of the water-accommodated fractions (WAFs) of the test substance on the growth of the alga, *Pseudokirchneriella subcapitata*, in a 96-hour static test.

### *Sponsor*

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, Virginia 22209

### *Testing Facility*

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

### *Study Initiation Date*

September 15, 2003

### *In-life Test Period*

December 8, 2003 to December 12, 2003

### *Experimental Termination Date*

December 16, 2003

### *Compliance*

This test was conducted in compliance with OECD<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards with the exceptions listed on page 11-13 and was performed in agreement with OECD<sup>3</sup> Guideline 201 with the exceptions listed on page 20.

## MATERIALS and METHODS

### *Test Substance Identification*

EMBSI Identification:	MRD-03-768
Industry Stream Name (acronym):	Heavy Pyrolysis Fuel Oil
<u>CAS Number:</u>	<u>CAS Inventory Name:</u>
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum
Supplier:	Dow Chemical Company Freeport, TX
Date Received:	July 29, 2003
Expiration Date:	July 2008
Description:	Dark Amber liquid

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

### *Stream Derivation*

In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.

### *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.

### *Vehicle / Dilution Water*

Algal Nutrient Media<sup>4</sup> - filtered through a sterile 0.45  $\mu\text{m}$  filter (referenced as acceptable medium in OECD 201 guideline), with 400 mg of  $\text{NaHCO}_3$  per liter, added as a carbon source in a no headspace environment. The algal medium meets the following limits of essential constituents:  $\text{P} \leq 0.7 \text{ mg/L}$ ,  $\text{N} \leq 10 \text{ mg/L}$ , chelators  $\leq 10^{-3} \text{ mmol/L}$  and hardness ( $\text{Ca} + \text{Mg}$ )  $\leq 0.6 \text{ mmol/L}$ . See Table 1, page 22 for composition of the algal media. The initial pH of the media was 7.9 - 8.0. The pH was adjusted to 7.4 - 7.6 prior to treatment preparation using 1.0M HCl.

## MATERIALS and METHODS (CONT'D)

### *Analysis of Mixtures*

Samples of each treatment WAF and the control were taken on Day 0 prior to the addition of algae and at termination (composite of replicates) with no headspace. The samples were stored under refrigeration until analyzed. The method of analysis was gas chromatography with flame ionization detection (GC-FID). Samples were analyzed using a Perkin Elmer Autosystem XL Gas Chromatograph. Analytical standards were prepared and analyzed at concentrations bracketing the sample concentrations. The results of the analyses are included in Appendix A starting on page 25.

### *Characterization of the Test Substance*

The neat test substance was characterized and the stability determined by the testing facility using the following analyses: UltraViolet/Visible and Infrared Spectrophotometry, Gas Chromatography with Mass Selective Detection and Density. The test substance was used in a number of studies at the testing facility. Characterization of the test substance was performed at the testing facility prior to its use in the first of these studies and after completion of the final study. Documentation of characterization and stability assessment is maintained at the testing facility (see Appendix B, page 27).

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, was considered the "pure" substance.

### *Test System*

*Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*)

Culture date: December 3, 2003

### *Justification for Selection of Test System*

*Pseudokirchneriella subcapitata* has been used in safety evaluations and is a common test species for freshwater toxicity studies.

### *Supplier*

Cultured at the Environmental Toxicology Laboratory of the testing facility. Initial strain (#1648) provided by UTEX, The Culture Collection of Algae MCDB, School of Biological Sciences, The University of Texas at Austin, Austin, TX 78712. Lot # 20 (slant 20B) received by the laboratory on 13-Feb-01.

## MATERIALS and METHODS (CONT'D)

### *Culture Methods*

Algae are cultured and tested in approximately 300 mL of nutrient media (same as vehicle/dilution water with the exception of additional  $\text{NaHCO}_3$ ) prepared with deionized water and reagent grade chemicals. Cell counts are performed weekly to ensure that the cells are in log phase of growth and to verify that the culture is axenic. A new culture is started weekly using inoculum from the previous culture. Cultures of *P. subcapitata* are held at 22 - 25°C under continuous illumination (8000 Lux  $\pm$  20%) provided by cool-white fluorescent bulbs.

### *Number*

Initial concentration of algae was approximately  $1.0 \times 10^4$  cells/mL in each replicate chamber.

### *Age at Initiation of Exposure*

Algae were taken from 5-day old stock cultures in log phase of growth.

### *Test System Identification*

Test organisms were not individually identified. All test chambers were labeled to show study number, loading rate, replicate, observation day and chamber number.

### *Selection*

Replicates 1 through 12 of each loading rate were inoculated with algae and were placed on a shaker table for the duration of the study. Chamber positions were randomly assigned using a computer generated randomization schedule.

### *Contaminants*

There are no known contaminants in the nutrient medium believed to be at levels high enough to interfere with this study. The nutrient medium is prepared from reagent grade chemicals and UV-sterilized, deionized well water that is treated and distributed throughout the testing facility via PVC and stainless-steel pipes. The feed water supplying the deionized water system is analyzed periodically for priority pollutants, un-ionized ammonia, total suspended solids, and for bacterial properties by Accutest<sup>®</sup>, 2235 Route 130, Dayton, NJ 08810. Contaminant analysis of the water was not performed in a GLP compliant manner. This is not believed to affect the results of the analysis. Documentation is maintained at the testing facility.

## EXPERIMENTAL PROCEDURE

### *Equilibrium Test*

An equilibrium study was performed prior to testing to determine the most appropriate mixing duration. Gas chromatography with flame ionization detection (GC-FID) was used to detect the test substance. Two sets of individual WAFs at approximately 1 mg/L and 10 mg/L were prepared and sampled after approximately 24 and 48 hours. The solutions were stirred using a  $\leq 10\%$  (of the static liquid depth) vortex. All mixing vessels were closed using foil covered rubber stoppers during mixing. Three replicates were analyzed at each sampling interval. The equilibrium phase did not extend beyond 48-hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test (to be used for the fish study). The goal was to achieve equilibrium of the soluble components without compromising a loss of the lighter components (it is the lighter components that usually contribute to toxicity). The results indicate that a mixing duration of 24 hours was sufficient for the soluble components of the test substance to achieve equilibrium. This phase of the study was not subject to GLP standards.

Summary of Equilibrium Test Results		
Loading Rate (mg/L)	24 Hour Stirring* (mg/L)	48 Hour Stirring* (mg/L)
1	0.455 (SD = 0.013)	0.435 (SD = 0.018)
10	6.79 (SD = 0.15)	6.70 (SD = 0.049)

1 mg /L PQL (Practical Quantitation Limit) = 0.1 mg/L

10 mg /L PQL (Practical Quantitation Limit) = 0.7 mg/L

\* Average of three replicates.

### *Range Finding Test*

A 72-hour static range finding test was performed for the Sponsor to determine the loading rates of Heavy Pyrolysis Fuel Oil (MRD-03-768) for the definitive Alga, Growth Inhibition Test.

Water-accommodated fractions (WAFs) were prepared at nominal loading rates of 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L. The actual loading rates were determined to be 0.11 mg/L, 1.1 mg/L, 10 mg/L, and 110 mg/L. A control treatment consisting only of algal nutrient media and sodium bicarbonate (added as a carbon source in a no headspace environment) also was prepared. WAFs were prepared by adding the appropriate amount of test substance, via tuberculin syringes, to algal nutrient media and sodium bicarbonate in glass aspirator bottles (mixing vessels) containing Teflon<sup>®</sup> coated stir bars.

## EXPERIMENTAL PROCEDURE (CONT'D)

### Range Finding Test (Cont'd)

The syringes were weighed with and without test substance to determine the actual loading rates. The mixing vessels were closed with foil covered rubber stoppers and the treatments were stirred using a 7% (of the static liquid depth) vortex at room temperature (approximately 21.6°C) on magnetic stirplates for approximately 24 hours. As stirring initiated and after stirring, all treatments appeared clear and colorless with test substance evident on the surface. Test substance was also observed on the bottom of the mixing vessel of the 110 mg/L treatment after stirring. The WAFs were removed through the outlet at the bottom of the stirring vessels approximately one hour after cessation of stirring.

Nine replicate test chambers were established for each loading rate and the control. Test chambers consisted of autoclaved glass 125 mL Erlenmeyer flasks, conditioned by rinsing with the appropriate WAF, containing two 14 mm glass spheres to facilitate mixing. Approximately 140 mL of the aqueous portion of each treatment (WAF) was transferred through the outlet at the bottom of the mixing vessels into the test chambers. Each test chamber was filled (no headspace), inoculated with the algae *Pseudokirchneriella subcapitata* (approximately  $1.0 \times 10^4$  cells per mL), and then sealed with ground glass stoppers. Test chambers were placed in an environmentally controlled chamber, and continuously oscillated on a shaker table at 100 rpm to keep the algae in suspension. The test was performed under continuous lighting conditions of approximately 8249 Lux at a mean temperature of 23.7°C (sd=0.2). The pH of the WAFs at the beginning of the test ranged from 7.8 to 7.9, and ranged from 7.8 to 9.0 at the end of the test. Each day, three test chambers from each treatment were sacrificed for hemacytometer cell counts. The table below summarizes the % inhibition based on rounded mean cell count values. This phase of the study was not subject to GLP standards.

Heavy Pyrolysis Fuel Oil, 72 hour Range Finding Results			
Loading Rate (mg/L)	24 hour % Inhibition*	48 hour % Inhibition*	72 hour % Inhibition*
0.11	2.2	0	6.0
1.1	0	17	21
10	76	93	99
110	90	98	100

\* % inhibition compared to the Control group

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Definitive Test Design*

GROUP	TARGET LOADING RATE* (mg/L)	ACTUAL LOADING RATE** (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	0	$1.0 \times 10^4$ (per 12 replicates)
2	0.18	0.20	$1.0 \times 10^4$
3	0.44	0.39	$1.0 \times 10^4$
4	1.1	1.1	$1.0 \times 10^4$
5	2.8	2.6	$1.0 \times 10^4$
6	7.0	7.2	$1.0 \times 10^4$

\* Target loading rate as per protocol.

\*\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

### *Preparation and Administration of Test Substance*

Individual treatments were prepared for each loading rate by adding the appropriate amount of test substance to 2.0 L of algal nutrient media and sodium bicarbonate (added as a carbon source in a no headspace environment) in glass aspirator bottles (capacity 2.3 L). The test substance was added to the aspirator bottles using tuberculin syringes. The syringes were weighed with and without the test substance to determine the actual loading rate. The mixing vessels were closed with foil covered rubber stoppers. The mixtures were stirred using an approximately 7% (of the static liquid depth) vortex for 24.5 hours on magnetic stirplates with Teflon<sup>®</sup> coated stirbars at room temperature (23.0 - 23.3°C). As stirring initiated and after stirring, all treatments appeared clear/colorless with the test substance floating at the surface. The WAFs equilibrated to test temperature and were removed through the outlet at the bottom of the stirring vessels 1 hour and 15 minutes after cessation of stirring.

Twelve replicates were prepared for each treatment by filling the test chambers with the appropriate WAF. Twelve replicates of the control were prepared in the same manner using algal nutrient media and sodium bicarbonate.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Test Chamber / Volume of Solution*

Test chambers were 125 mL size autoclaved glass Erlenmeyer flasks sealed with ground glass stoppers to prevent contamination, evaporation and/or volatilization, each containing two 14 mm glass spheres to facilitate mixing. The chambers were filled with approximately 140 mL of the appropriate WAF (no headspace). Test chambers were conditioned with the test solutions prior to the test. The test chambers were placed on a shaker table (100 rpm) to keep the algae in suspension.

### *Exposure Duration*

96 hours ( $\pm$  1 hour)

### *Exposure Conditions*

Mean test temperature: 24.2°C (sd = 0.5). The temperature of the test solutions at the start of the study was 24.2 to 24.3°C and at termination was 23.4 to 23.6°C.

Continuous light: intensity was 8431 to 8595 Lux.

An environmental condition study was activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

Oscillation Rate: 100 rpm (verified daily).

### *Experimental Evaluation*

Cell density was determined for each test and control chamber using a hemacytometer and microscope at 24, 48, 72 and 96 hours ( $\pm$  1 hour) after the beginning of the test. Cell density determinations were performed on three replicates at each observation interval and the replicates were then discarded. No undissolved test substance was observed in the test chambers during the study. The pH of each treatment was measured on Day 0 and daily after cell density determinations (composite of the three replicates).

### EXPERIMENTAL PROCEDURE (CONT'D)

#### Calculations

To determine the test substance loading rate/concentration effect relationship two approaches were used: the area under the growth curve and the growth rate slope.

The area under the growth curve of cell density vs time was approximated by the following:

$$A_c = \frac{N_{1,c} - N_{0,c}}{2} \times t_1 + \frac{N_{1,c} + N_{2,c} - 2N_{0,c}}{2} \times [t_2 - t_1] + \dots + \frac{N_{n-1,c} + N_{n,c} - 2N_{0,c}}{2} \times [t_n - t_{n-1}] \quad \{1\}$$

where  $c$  = loading rate or concentration  
 $A_c$  = Area under the growth curve at test concentration  $c$   
 $N_{0,c}$  = nominal number of cells/mL at time zero and test concentration  $c$   
 $N_{n,c}$  = nominal number of cells/mL at time  $n$  and test concentration  $c$   
 $t_n$  = time of  $n^{\text{th}}$  measurement after start of testing

The percent inhibition of the cell growth rate at each loading rate/concentration was calculated as:

$$I_c = \frac{A_0 - A_c}{A_0} \times 100 \quad \{2\}$$

where  $c$  = loading rate or concentration  
 $I_c$  = percent inhibition at loading rate/concentration  $c$   
 $A_0$  = area under the control growth curve  
 $A_c$  = area under the growth curve at  $c$ , based on equation {1}

The growth rate slope at concentration  $c$  was determined from the regression equation of cell count on time:

$$\ln(N_{t,c}) = \alpha_c + \mu_c \times t \quad \{3\}$$

where  $N_{t,c}$  = measured number of cells/mL at concentration  $c$  and time  $t$   
 $\alpha_c$  = intercept term (not used in further estimation)  
 $\mu_c$  = growth rate slope at concentration  $c$

In the report the percent inhibition is reported as  $I_c$  based on equation {2}.

The median effect concentration ( $E_bC_{50}$ ) and confidence intervals for inhibition of growth were determined by a probit regression calculation of the probit of the growth inhibition ( $I_c$  - eqn. 2) vs the log of the concentration and associated confidence intervals based on the methods of Finney<sup>5</sup>. Calculations were based on the PROC PROBIT procedure in SAS<sup>6</sup>.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Calculations (Cont'd)*

The NOEC for the  $E_bC_{50}$  was based on Duncan's Multiple Range test<sup>7</sup> and Dunnett's test<sup>8</sup> determined from the GLM procedure of SAS<sup>6</sup> with percent inhibition ( $I_c$  - eqn. 2) as the dependent variable and concentration ( $c$ ) as the independent variable. The Shapiro-Wilk test<sup>9</sup> for normality was used to test if the assumption of normality of the residuals was met; since the residuals were normally distributed the NOEC was based on the estimated values.

In the report the growth rate slope at concentration  $c$  is reported as  $\mu_c$  based on equation {3}.

The median effect concentration ( $E_rC_{50}$ ) and confidence intervals for growth rate slope were determined by a probit regression calculation of the probit of the growth rate slope ( $\mu_c$  - eqn. 3) vs the log of the concentration and associated confidence intervals based on the methods of Finney<sup>5</sup>. Calculations were based on the PROC PROBIT procedure in SAS<sup>6</sup>.

The NOEC for the  $E_rC_{50}$  was based on Duncan's Multiple Range test<sup>7</sup> and Dunnett's test<sup>8</sup> determined from the GLM procedure of SAS<sup>6</sup> with growth rate slope ( $\mu_c$  - eqn. 3) as the dependent variable and concentration ( $c$ ) as the independent variable. The Shapiro-Wilk test<sup>9</sup> for normality was used to test if the assumption of normality of the residuals was met; since the residuals were normally distributed the NOEC was based on the estimated values.

### RESULTS

Acute toxicity results are expressed as the Effect Loading / Effect Concentration 50 (EL/EC<sub>50</sub>); that is, the loading rate or concentration of test substance in dilution medium which is calculated to result in a 50% reduction in growth derived from either the average specific growth rate (r) or the area under the growth curves (b) relative to the control for the specified time of exposure. The No Observed Effect Loading Rate / No Observed Effect Concentration (NOELR/NOEC) is the highest loading rate or concentration which does not exhibit a statistical difference from the control.

Hours	E <sub>r</sub> L50 mg/L	E <sub>r</sub> C50 mg/L	E <sub>b</sub> L50 mg/L	E <sub>b</sub> C50 mg/L
72	2.3 (CNC)	2.0 (CNC)	1.5 (1.3-1.6)	1.3 (1.2-1.4)
96	2.1 (CNC)	1.8 (CNC)	1.4 (1.3-1.6)	1.3 (1.2-1.4)

Values in parentheses ( ) are 95% confidence intervals.  
 CNC = Could Not Calculate

Hours	average specific growth rate		area under the growth curves	
	NOELR	NOEC	NOELR	NOEC
72	0.39 mg/L	0.42 mg/L	0.20 mg/L	0.07 mg/L
96	0.39 mg/L	0.42 mg/L	0.39 mg/L	0.42 mg/L

### RESULTS (CONT'D)

The percent inhibition compared to the control is shown in the table below.

Loading Rate* (Concentration)** mg/L	Based on the Slopes of the Growth Rates		Based on the Areas Under the Growth Curves	
	72 hours	96 hours	72 hours	96 hours
0.20 (0.07)	-2.0	-1.7	-2.1	-1.9
0.39 (0.42)	0	-2.2	7.6	1.3
1.1 (1.1)	11	7.1	34	31
2.6 (2.1)	83	86	92	97
7.2 (6.4)	97	98	99	100

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and Day 4) measured concentrations.

Negative (-) value indicates a stimulatory effect.

Table 2 presents the cell concentrations per flask. Table 3 presents the mean cell concentrations per treatment during the test and pH. The growth curves are shown in Figure 1. Appendix A presents the analytical methods and results.

The test was considered acceptable since cell density in the Control increased by a factor of  $\geq 16$  within three days.

### CONCLUSIONS

After 72 and 96 hours of exposure to WAFs prepared from Heavy Pyrolysis Fuel Oil the following conclusions were made:

Effects on growth rate (r) based upon actual loading rates:

72 hr ErL50 = 2.3 mg/L

96 hr ErL50 = 2.1 mg/L

Effects on biomass (b) based upon actual loading rates:

72 hr EbL50 = 1.5 mg/L

96 hr EbL50 = 1.4 mg/L

Effects on growth rate (r) based upon measured concentrations:

72 hr ErC50 = 2.0 mg/L

96 hr ErC50 = 1.8 mg/L

Effects on biomass (b) based upon measured concentrations:

72 and 96 hr EbC50 = 1.3 mg/L

### **GUIDELINE EXCEPTIONS**

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

The concentration of the test substance in solution was not determined prior to use. Test substance analysis was performed on samples of the WAFs prior to the start of the test and at termination. The initial concentration of the test substance was not maintained at 80% in the three lower loading rates throughout the test (this may be due to biological activity or physical processes in the test chambers).

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

The test duration was 96 hours, instead of 72 hours. Both 72 and 96-hour endpoints have been determined. The extended duration was at the sponsor's request.

### **PROTOCOL DEVIATIONS**

No protocol deviations occurred for this study.

### **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 include 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.

## REFERENCES

1. Organization for Economic Cooperation and Development (OECD), Principles of Good Laboratory Practice, C(97)186 (Final), 1997.
2. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
3. Organization for Economic Cooperation and Development (OECD), Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, Guideline 201: Alga, Growth Inhibition Test. 1984.
4. USEPA, The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. July 1978.
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**TABLE 1**  
**COMPOSITION OF ALGAL NUTRIENT MEDIUM**

<u>COMPOUND</u>	<u>CONCENTRATION</u> (mg/L)	<u>ELEMENT</u>	<u>CONCENTRATION</u> (mg/L)
NaNO <sub>3</sub>	25.500	N	4.200
MgCl <sub>2</sub> ·6H <sub>2</sub> O	12.164	Mg	2.904
CaCl <sub>2</sub> ·2H <sub>2</sub> O	4.410	Ca	1.202
MgSO <sub>4</sub> ·7H <sub>2</sub> O	14.700	S	1.911
K <sub>2</sub> HPO <sub>4</sub>	1.044	P	0.186
NaHCO <sub>3</sub> *	15.000	Na	11.001
		K	0.469
		C	2.143

\* An additional 400 mg of NaHCO<sub>3</sub>/L, added as a carbon source in a no headspace environment.

<u>COMPOUND</u>	<u>CONCENTRATION</u> (mg/L)	<u>ELEMENT</u>	<u>CONCENTRATION</u> (mg/L)
H <sub>3</sub> BO <sub>3</sub>	185.520	B	32.460
MnCl <sub>2</sub> ·4H <sub>2</sub> O	415.610	Mn	115.374
ZnCl <sub>2</sub>	3.271	Zn	1.570
CoCl <sub>2</sub> ·6H <sub>2</sub> O	1.428	Co	0.354
CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.012	Cu	0.004
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	7.260	Mo	2.878
FeCl <sub>3</sub> ·6H <sub>2</sub> O	160.000	Fe	33.051
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	300.00		

**TABLE 2**  
**CELL CONCENTRATIONS PER FLASK (cells/mL)**

Loading Rate* (Concentration**) mg/L	Day 0	Rep.	Day 1	Rep.	Day 2	Rep.	Day 3	Rep.	Day 4
Control (0)	1.0 x 10 <sup>4</sup>	1	6.0 x 10 <sup>4</sup>	4	1.5 x 10 <sup>5</sup>	7	6.9 x 10 <sup>5</sup>	10	1.9 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	2	5.3 x 10 <sup>4</sup>	5	7.0 x 10 <sup>4</sup>	8	7.5 x 10 <sup>5</sup>	11	1.1 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	3	4.9 x 10 <sup>4</sup>	6	1.2 x 10 <sup>5</sup>	9	7.2 x 10 <sup>5</sup>	12	1.6 x 10 <sup>6</sup>
0.20 (0.07)	1.0 x 10 <sup>4</sup>	1	4.1 x 10 <sup>4</sup>	4	1.3 x 10 <sup>5</sup>	7	7.0 x 10 <sup>5</sup>	10	1.8 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	2	5.1 x 10 <sup>4</sup>	5	1.1 x 10 <sup>5</sup>	8	7.2 x 10 <sup>5</sup>	11	1.4 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	3	5.1 x 10 <sup>4</sup>	6	1.9 x 10 <sup>5</sup>	9	6.6 x 10 <sup>5</sup>	12	1.6 x 10 <sup>6</sup>
0.39 (0.42)	1.0 x 10 <sup>4</sup>	1	5.1 x 10 <sup>4</sup>	4	1.2 x 10 <sup>5</sup>	7	6.8 x 10 <sup>5</sup>	10	1.3 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	2	3.4 x 10 <sup>4</sup>	5	1.5 x 10 <sup>5</sup>	8	6.5 x 10 <sup>5</sup>	11	1.9 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	3	4.3 x 10 <sup>4</sup>	6	1.4 x 10 <sup>5</sup>	9	5.3 x 10 <sup>5</sup>	12	1.8 x 10 <sup>6</sup>
1.1 (1.1)	1.0 x 10 <sup>4</sup>	1	5.0 x 10 <sup>4</sup>	4	9.8 x 10 <sup>4</sup>	7	4.2 x 10 <sup>5</sup>	10	1.3 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	2	3.8 x 10 <sup>4</sup>	5	8.6 x 10 <sup>4</sup>	8	4.1 x 10 <sup>5</sup>	11	1.3 x 10 <sup>6</sup>
	1.0 x 10 <sup>4</sup>	3	4.8 x 10 <sup>4</sup>	6	1.0 x 10 <sup>5</sup>	9	4.7 x 10 <sup>5</sup>	12	8.5 x 10 <sup>5</sup>
2.6 (2.1)	1.0 x 10 <sup>4</sup>	1	3.3 x 10 <sup>4</sup>	4	2.3 x 10 <sup>4</sup>	7	1.8 x 10 <sup>4</sup>	10	1.8 x 10 <sup>4</sup>
	1.0 x 10 <sup>4</sup>	2	2.0 x 10 <sup>4</sup>	5	2.0 x 10 <sup>4</sup>	8	3.1 x 10 <sup>4</sup>	11	3.5 x 10 <sup>4</sup>
	1.0 x 10 <sup>4</sup>	3	3.9 x 10 <sup>4</sup>	6	1.4 x 10 <sup>4</sup>	9	3.0 x 10 <sup>4</sup>	12	2.9 x 10 <sup>4</sup>
7.2 (6.4)	1.0 x 10 <sup>4</sup>	1	5.0 x 10 <sup>3</sup>	4	7.5 x 10 <sup>3</sup>	7	1.1 x 10 <sup>4</sup>	10	5.0 x 10 <sup>3</sup>
	1.0 x 10 <sup>4</sup>	2	1.5 x 10 <sup>4</sup>	5	6.3 x 10 <sup>3</sup>	8	1.4 x 10 <sup>4</sup>	11	1.4 x 10 <sup>4</sup>
	1.0 x 10 <sup>4</sup>	3	1.4 x 10 <sup>4</sup>	6	1.0 x 10 <sup>4</sup>	9	1.3 x 10 <sup>4</sup>	12	1.8 x 10 <sup>3</sup>

Rep. = Replicate

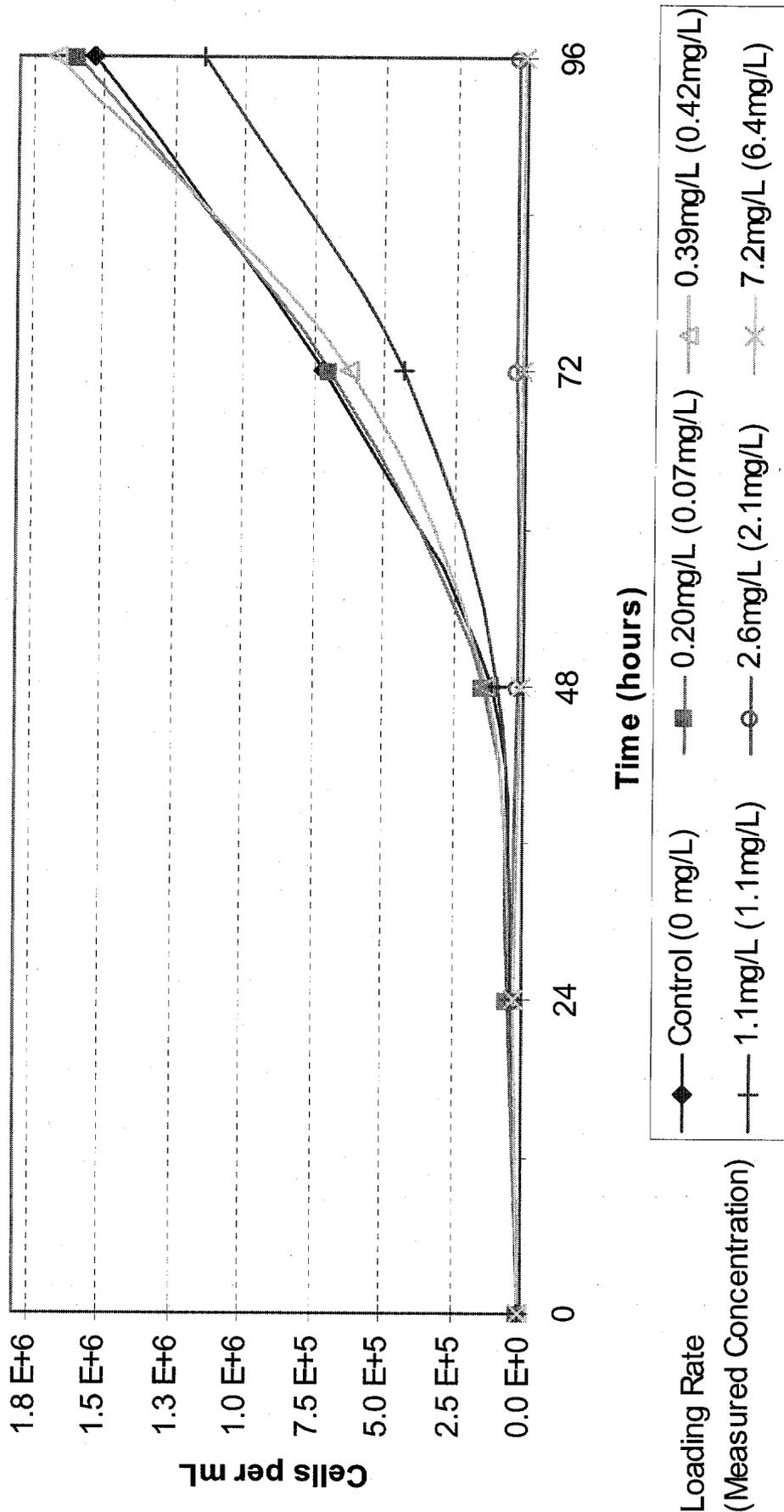
**TABLE 3**  
**MEAN CELL CONCENTRATIONS (cells/mL) and pH**

Loading Rate* (Concentration**) mg/L	Day 0	pH	Day 1	pH	Day 2	pH	Day 3	pH	Day 4	pH
Control (0)	1.0 x 10 <sup>4</sup>	7.6	5.4 x 10 <sup>4</sup>	8.0	1.1 x 10 <sup>5</sup>	8.4	7.2 x 10 <sup>5</sup>	8.5	1.5 x 10 <sup>6</sup>	8.4
0.20 (0.07)	1.0 x 10 <sup>4</sup>	7.6	4.8 x 10 <sup>4</sup>	7.9	1.4 x 10 <sup>5</sup>	8.5	6.9 x 10 <sup>5</sup>	8.5	1.6 x 10 <sup>6</sup>	8.5
0.39 (0.42)	1.0 x 10 <sup>4</sup>	7.5	4.3 x 10 <sup>4</sup>	7.8	1.4 x 10 <sup>5</sup>	8.5	6.2 x 10 <sup>5</sup>	8.6	1.7 x 10 <sup>6</sup>	8.6
1.1 (1.1)	1.0 x 10 <sup>4</sup>	7.6	4.5 x 10 <sup>4</sup>	7.8	9.5 x 10 <sup>4</sup>	8.4	4.3 x 10 <sup>5</sup>	8.4	1.2 x 10 <sup>6</sup>	8.7
2.6 (2.1)	1.0 x 10 <sup>4</sup>	7.4	3.1 x 10 <sup>4</sup>	7.8	1.9 x 10 <sup>4</sup>	8.2	2.6 x 10 <sup>4</sup>	7.7	2.7 x 10 <sup>4</sup>	7.5
7.2 (6.4)	1.0 x 10 <sup>4</sup>	7.6	1.1 x 10 <sup>4</sup>	7.7	7.9 x 10 <sup>3</sup>	8.0	1.3 x 10 <sup>4</sup>	7.5	9.3 x 10 <sup>3</sup>	7.5

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

\*\* Concentration based on mean (Day 0 and Day 4) measured concentrations.

FIGURE 1  
 GROWTH CURVES



## APPENDIX A - ANALYTICAL METHODS and RESULTS

Heavy Pyrolysis Fuel Oil WAF samples (ca. 125 mL) were collected with no headspace in amber glass bottles with Teflon<sup>®</sup> lined caps. Samples were refrigerated pending extraction with hexane and analysis by gas chromatography with flame ionization detection (GC-FID).

Samples of WAFs prepared at each loading level were taken on Day 0 from the stirring vessels and from the "old" WAFs of each treatment (composite of replicates) on Day 4 at termination.

Samples were allowed to come to room temperature prior to extraction. Depending on the nominal sample concentration, approximately 25, 50 or 125 mL sample volumes were transferred to glass extraction bottles and 4.0 or 5.0 mL of hexane was added as extraction solvent. The exact amount of water extracted was determined by using tared extraction bottles and re-weighing the bottles after the appropriate volume of sample was dispensed.

After the addition of hexane, the extraction bottles were crimp sealed with Teflon<sup>®</sup> faced septum caps and extracted by hand for approximately one minute followed by 60 minutes on a mechanical shaker. The contents were then allowed to settle for at least 30 minutes before the hexane (upper) layer was transferred to small (ca. 4 mL) glass vials. An aliquot was removed from the 4 mL vial to a GC autosampler vial for analysis. Hexane extracts were stored in a freezer pending analysis.

Heavy Pyrolysis Fuel Oil standards in hexane and hexane extracts were analyzed on a Perkin Elmer Autosystem XL Gas Chromatograph with a 15m x 0.53mm id Rtx-5 capillary column with 1.5  $\mu$ m film thickness (Restek) and a 5m Integra guard column. The carrier gas was helium at 15 mL/min. The oven temperature was programmed from 55°C for 6 minutes up to 250°C at 30°C/minute. Automated large volume injections of 30 $\mu$ L were made in the programmable split/splitless (PSS) mode. The FID temperature was 275°C and the detector attenuation setting was -5.

Heavy Pyrolysis Fuel Oil standards were analyzed at concentrations of 3.84, 12.8, and 64  $\mu$ g/mL. The extraction method provided quantitative recoveries and was validated by spiking Heavy Pyrolysis Fuel Oil in water at 0.961  $\mu$ g/mL. Five replicate spikes were extracted and the mean recovery (standard deviation) was 108% (6.7%).

Data were acquired and processed with Perkin Elmer Totalchrom Workstation software. Heavy Pyrolysis Fuel Oil eluted as a complex mixture at approximate retention time of 8.5 minutes under the instrumental conditions employed.

The Practical Quantitation Limit (PQL) for water samples was approximately 0.12  $\mu$ g/mL taking into account the concentration of the lowest analyzed standard (3.84  $\mu$ g/mL), the maximum sample volume (approximately 125 mL) and the minimum extract solvent volume (4.0 mL).

**APPENDIX A - ANALYTICAL METHODS and RESULTS (CONT'D)**

**TABLE A-1**

Loading Rate* (mg/L)	Measured Concentration (mg/L)		
	Day 0	Day 4	Average
0 (Control)	ND	ND	ND
0.20	0.0724 (below PQL)	ND	0.07**
0.39	0.486	0.348	0.42
1.1	1.18	0.931	1.1
2.6	2.29	1.87	2.1
7.2	6.87	5.92	6.4

\* Actual loading rate (weight) of test substance added to the vehicle/dilution water.

ND = Not Detected

\*\* Since the Day 4 sample was below detection limits, The Day 0 value was used for statistical analysis.

PQL (Practical Quantitation Limit) = ~0.12 mg/L

## APPENDIX B - TEST SUBSTANCE CHARACTERIZATION

The test substance was initially characterized on August 19 and 21, 2003. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and GC-MS analysis. Stability of the neat test substance was confirmed by repeating these same analyses on February 5, 2004 after the completion of this study.

UV-VIS spectra are presented in Figures B-1 and B-2 representing, the initial and final spectrum at concentrations of 10 and 12 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 B-3 and B-4 representing the initial and final spectra. FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of  $4\text{ cm}^{-1}$ , gain of 1 and scan number of 32.

The test substance was also characterized by GC-MS using a Varian Saturn 2000 GC-MS system with a Varian 3800 gas chromatograph. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, Heavy Pyrolysis Fuel Oil was analyzed against an ASTM D2887 calibration mixture. Figures B-5 and B-6 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components detected between retention times 13 and 26 minutes. This corresponds to bracketing by the standard hydrocarbons n-decane and n-octadecane under the analytical conditions employed. The single most abundant component eluted at approximately 16.3 minutes.

The test substance's initial and final density was measured at 20°C using an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 1.068 g/mL and the final density was 1.069 g/mL. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water, methanol and hexane. All test substance solutions were prepared with methylene chloride as the solvent.

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.

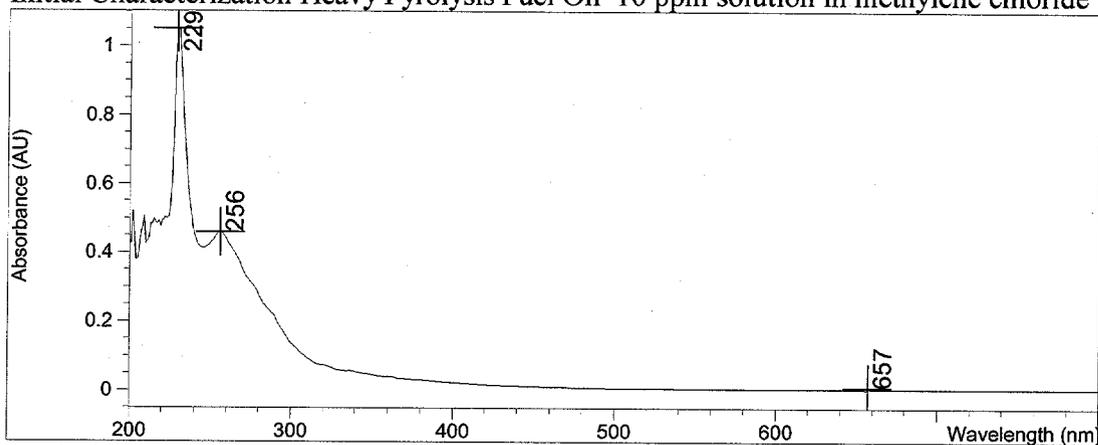
**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**UV-VIS SPECTRA**

**Figure B-1**

**Initial**

Initial Characterization Heavy Pyrolysis Fuel Oil 10 ppm solution in methylene chloride



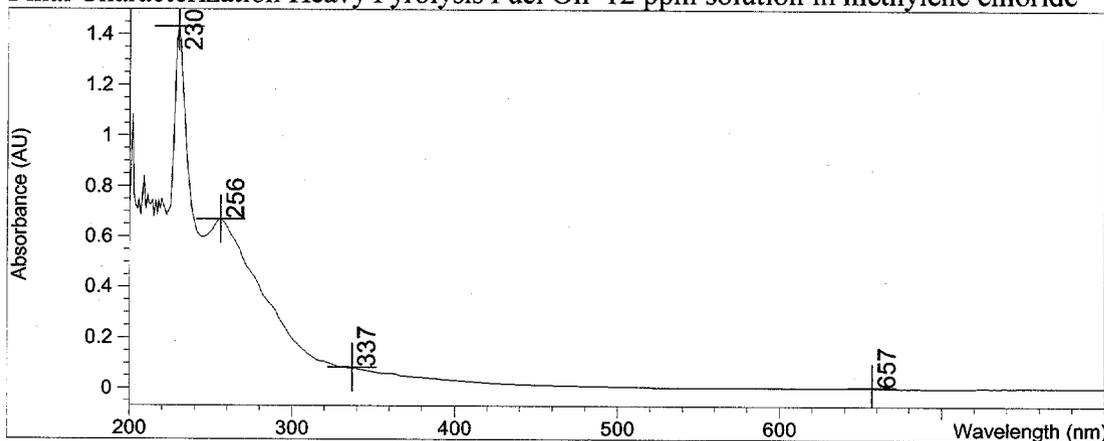
Analysis Date: 19 August 03

Peak 229nm Absorbance = 1.051  
Peak 256nm Absorbance = 0.4597  
Peak 657nm Absorbance = 0.0095

**Figure B-2**

**Final**

Final Characterization Heavy Pyrolysis Fuel Oil 12 ppm solution in methylene chloride



Analysis Date: 5 February 04

Peak 230nm Absorbance = 1.4298  
Peak 256nm Absorbance = 0.6677  
Peak 337nm Absorbance = 0.0832  
Peak 657nm Absorbance = 0.0068

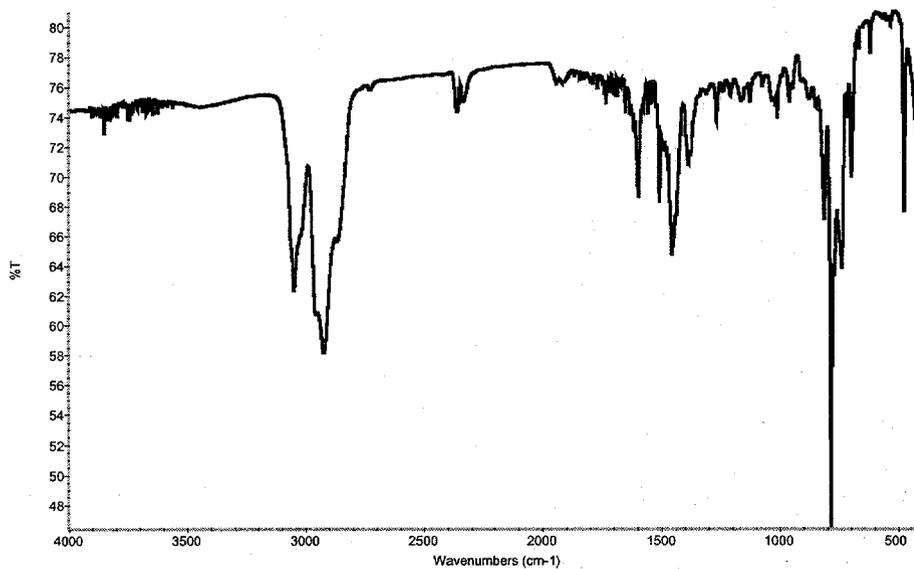
**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**FT-IR SPECTRA**

**Figure B-3**

**Initial**

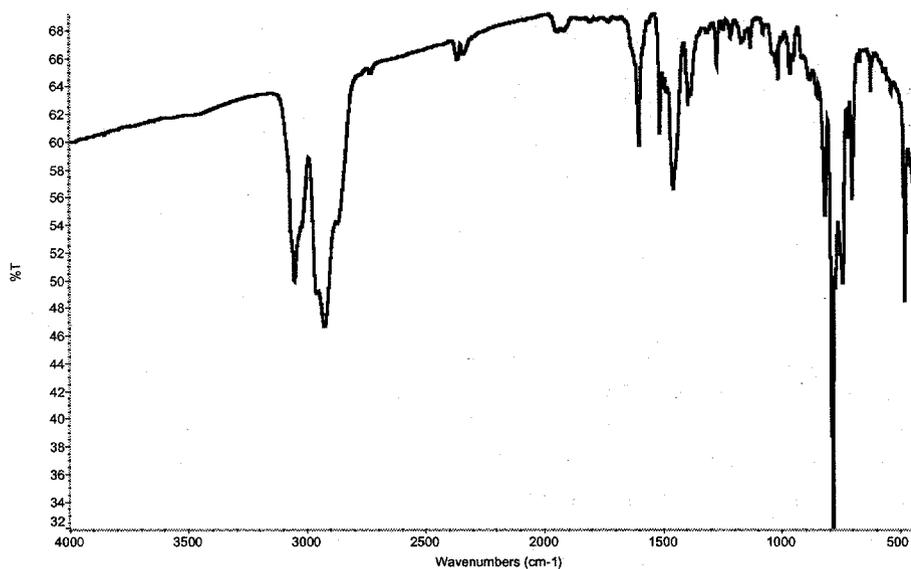
Initial Characterization Heavy Pyrolysis Fuel Oil      Analysis Date: 19 Aug 03



**Figure B-4**

**Final**

Final Characterization Heavy Pyrolysis Fuel Oil      Analysis Date: 5 Feb 04



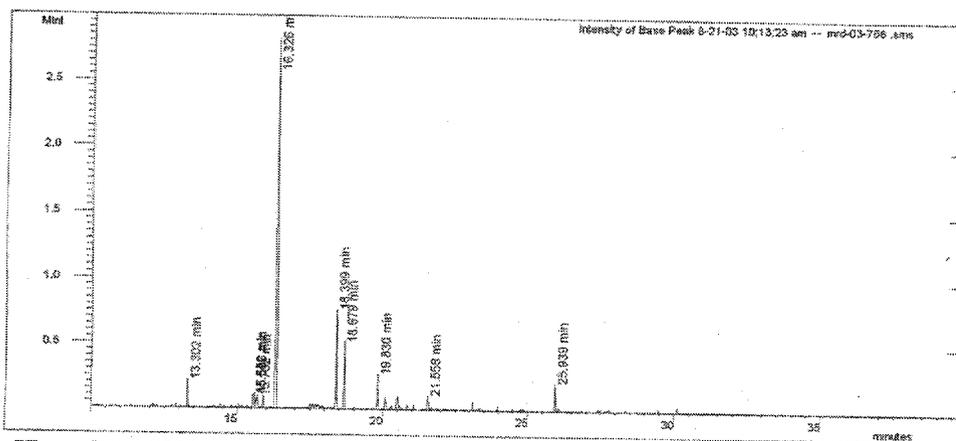
%T = % Transmittance

**APPENDIX B - TEST SUBSTANCE CHARACTERIZATION (CONT'D)**

**INITIAL TOTAL ION CHROMATOGRAM**  
**Figure B-5**

**AREA PERCENT REPORT**

<b>Data File Name:</b>	c:\learnws\8-21-03 10:13:23 am -- mrd-03-768 .sms	<b>Acquisition Date:</b>	8/21/03 10:13:24 AM
<b>Inst. Method:</b>	C:\SaturnWS\Crude Oil Charac.mth	<b>Instrument ID:</b>	Saturn GC/MS #1
<b>Sample Name:</b>	MRD-03-768	<b>Inj. Notes:</b>	MRD-03-768 (heavy fuel oil) initial characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.302	BB	1.9	1213022	4.86
15.552	BV	2.1	1037451	4.16
15.586	VV	2.2	895935	3.59
15.702	VV	2.7	1011392	4.05
16.326	VB	1.9	9296601	37.27
18.399	VB	1.7	4306416	17.26
18.679	BB	1.9	3339778	13.39
19.830	BB	1.7	1375559	5.51
21.556	VV	2.2	1524481	6.11
25.939	BB	1.8	942704	3.78

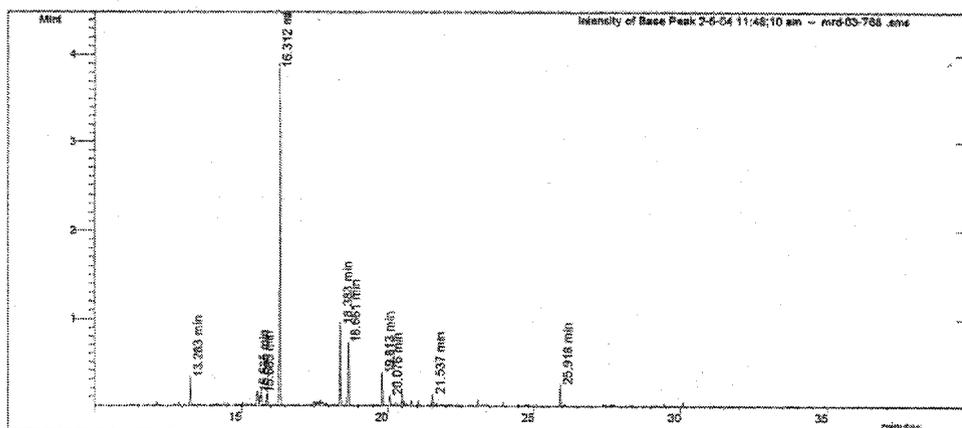
APPENDIX B- TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FINAL TOTAL ION CHROMATOGRAM

Figure B-6

AREA PERCENT REPORT

Data File Name:	c:\saturnws\2-5-04 11:48:10 am -- mrd-03-768 .sms	Acquisition Date:	2/5/04 11:48:11 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) final characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.283	BB	1.8	1973177	5.02
15.535	BB	2.0	3045234	7.75
15.685	TF	0.0	1653164	4.21
16.312	TF	0.0	13855800	35.25
18.383	VV	0.0	6504165	16.55
18.661	VV	0.0	5061242	12.88
19.813	TF	0.0	2145104	5.46
20.076	VB	0.0	1399923	3.58
21.537	TF	0.0	2322773	5.91
25.918	MV	0.0	1346340	3.43

## APPENDIX C - PROTOCOL

### PROTOCOL

OLF-105.0-HPV10-EMBSI

**Study Title:** Alga, Growth Inhibition Test on  
Heavy Pyrolysis Fuel Oil

**EMBSI Study Number:** 176867

**Test Substance:** MRD-03-768

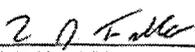
**Date:** August 11, 2003

**Room Number:** LE-337/343

**Proposed Key Dates:**

Experimental Start .....	13-Oct-03
Experimental Termination .....	17-Oct-03
Draft Report Completion .....	14-Nov-03
Final Report Completion .....	27-Feb-04

**Approved By:**

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

15 Sep 03  
Date

  
E. J. Moran, Ph.D.  
Sponsor Representative

9/9/03  
Date

**SAFETY FIRST**

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

PAGE 2

### INTRODUCTION

#### *Objective*

This study will be conducted for the Sponsor to evaluate the effects on growth of the water accommodated fractions (WAFs) of MRD-03-768 to the alga, *Pseudokirchneriella subcapitata* in a 96-hour static test.

#### *Sponsor*

American Chemistry Council  
1300 Wilson Blvd.  
Arlington, VA 22209

#### *Testing Facility*

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

#### *Compliance*

This test will be conducted in general agreement with the OECD<sup>1</sup> guidelines, and will be conducted in compliance with OECD<sup>2</sup> and USEPA<sup>3</sup> GLP standards.

#### *Justification for Selection of Test System*

*Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) has been used in safety evaluations 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 C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### MATERIALS and METHODS

#### *Test Substance Identification*

EMBST Code: MRD-03-768

Industry Stream Name: Heavy Pyrolysis Fuel Oil

CAS Number	CAS Inventory Name
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum

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

#### *Characterization of Test Substance*

The neat test substance will be characterized as described in SOP A.3.4.10 using the following analyses: Ultra-violet/Visible and Infrared Spectrophotometry and Gas Chromatography with Mass Selective Detection; density will also be determined. The test substance will be used in a number of studies at the testing facility. Characterization of the test substance will be performed at the testing facility prior to its use in the first of these studies. Stability assessment will span the duration of all studies. The results of the characterization and stability assessment will be appended to the final report. Characterization and stability documentation will be maintained at the testing facility.

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, will be considered the "pure" substance for dosing purposes.

#### *Analysis of Mixtures*

Samples will be taken from each treatment WAF and control solution on Day 0 prior to the addition of algae and at termination (composite of the remaining three replicates). The samples will be taken with no headspace. The method of analysis will be automated static headspace gas chromatography with flame ionization detection (HS GC-FID). Samples will be analyzed using a Perkin-Elmer HS 40 Headspace Sampler connected to a Perkin Elmer AutoSystem XL Gas Chromatograph with flame ionization detector. The gas chromatograph will be equipped with a 30m x 0.53mm id DB-5 capillary column with 1.5um film thickness (or equivalent). Analytical standards will be prepared and analyzed at concentrations bracketing the sample concentrations except in the case of those samples below the method's limit of quantification.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *Vehicle/Dilution Water*

Algal Nutrient Media<sup>4</sup> - filtered through a sterile 0.45µm filter (referenced as acceptable medium in OECD 201 guideline), with 400mg of NaHCO<sub>3</sub> per liter, added as a carbon source in a no headspace environment. The algal medium meets the following limits of essential constituents: P ≤ 0.7 mg/L, N ≤ 10 mg/L, chelators ≤ 10<sup>-3</sup> mmol/L and hardness (Ca + Mg) ≤ 0.6 mmol/L.

#### *Test System*

*Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*)

#### *Supplier*

Cultured at the Environmental Toxicology Laboratory of the testing facility. Initial strain (#1648) provided by UTEX, The Culture Collection of Algae MCDB, School of Biological Sciences, The University of Texas at Austin, Austin, TX 78712.

#### *Culture Methods*

Algae are cultured in approximately 300 mL of nutrient media (same as vehicle/dilution water with the exception of additional NaHCO<sub>3</sub>) prepared with deionized water and reagent grade chemicals. Cell counts are performed weekly to ensure that the cells are in log phase of growth and to verify that the culture is axenic. A new culture is started weekly using inoculum from the previous culture. Cultures of *P. subcapitata* are held at 22 - 25°C under continuous illumination (8000 Lux ± 20%) provided by cool-white fluorescent bulbs.

#### *Number*

Initial concentration of algae will be  $\sim 1.0 \times 10^4$  cells per mL in each replicate chamber.

#### *Age At Initiation of Exposure*

Algae will be taken from stock cultures in log phase of growth (4-10 days).

#### *Test System Identification*

All test chambers will be labeled to show study number, loading rate, replicate, observation day and chamber number.

#### *Selection*

Replicates 1 through 12 of each treatment will be inoculated with algae. All test flasks will then be placed on a shaker table for the duration of the study. Chamber positions on the shaker table will be randomly assigned using a computer generated randomization schedule. A printout of the randomization schedule will be included in the raw data.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### MATERIALS and METHODS (CONT'D)

#### *Contaminants*

There are no known contaminants in the vehicle/dilution water (algal nutrient media) believed to be at levels high enough to interfere with this study. The media is prepared from reagent grade chemicals and 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 analysis of the water is not performed in a GLP compliant manner. This is not believed to affect the results of the analysis. Contaminant analysis results are maintained at the testing facility.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### EXPERIMENTAL PROCEDURE

#### *Equilibrium Test*

An equilibrium study will be performed prior to testing to determine the most appropriate mixing duration. Specific analytical (e.g. GC analysis) will be used to detect soluble components of the substance. Individual WAFs at 1 mg/L and 10 mg/L will be prepared and sampled after 24 and 48 hours of mixing. The vortex will be set at  $\leq 10\%$  of the static liquid depth. All mixing vessels will be closed using foil covered stoppers during mixing. The equilibrium phase will not extend beyond 48 hours due to a potential loss of volatile components of the test substance in the mixing vessels and logistical constraints associated with a semi-static renewal type test. This phase of the study will not be subject to GLP standards.

#### *Range Finding Test*

A 72-hour range finding test will be performed to determine the loading rates for the definitive test. WAFs prepared at the following loading rates will be tested, 0.1 mg/L, 1.0 mg/L, 10 mg/L, 100 mg/L and a control of nutrient media. The WAFs will be prepared by adding the appropriate amount of test substance to algal nutrient media in glass vessels. The vessels will be closed using foil covered rubber/neoprene stoppers and will mix on magnetic stirplates with Teflon® coated stirbars for the appropriate time as determined by equilibrium testing ( $\pm 1$  hour). The vortex will be set at  $\leq 10\%$  of the static liquid depth. The treatments will be allowed to settle and equilibrate to test temperature for 1 hour ( $\pm 15$  mins.) after mixing. Test flasks will be conditioned by rinsing with the appropriate solution. Nine replicates at each loading will be prepared. Each test flask will be inoculated with  $\sim 1.0 \times 10^4$  cells per mL. The procedures followed for the range finding study will be documented in the raw data. This phase of the study will not be subject to GLP standards.

#### *Definitive Test Design*

GROUP	LOADING RATE (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	$\sim 1.0 \times 10^4$ (per 12 replicates)
2	TBD	$\sim 1.0 \times 10^4$
3	TBD	$\sim 1.0 \times 10^4$
4	TBD	$\sim 1.0 \times 10^4$
5	TBD	$\sim 1.0 \times 10^4$
6	TBD	$\sim 1.0 \times 10^4$

TBD = To Be Determined

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### EXPERIMENTAL PROCEDURE (CONT'D)

#### *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 algal nutrient media in glass aspirator bottles. The vessels will be closed using foil covered rubber/neoprene stoppers. The solutions will be mixed with Teflon® coated stirbars on magnetic stirplates. The vortex will be set at  $\leq 10\%$  of the static liquid depth. The solutions will mix for the appropriate time as determined by equilibrium testing ( $\pm 1$  hour) at room temperature ( $22^{\circ} \pm 2^{\circ}\text{C}$ ). At the end of mixing, the solutions will be allowed to settle and equilibrate to test temperature for 1 hour ( $\pm 15$  minutes). At the end of the settling period the solutions will be removed from the mixing vessels through the outlet at the bottom of the vessels. Test flasks will be conditioned by rinsing with the appropriate solution. Twelve replicates at each loading will be prepared. Each test flask will be inoculated with  $\sim 1.0 \times 10^6$  cells per mL.

#### *Test Chamber and Volume of Solution*

Test chambers will be autoclaved glass 125mL Erlenmeyer flasks sealed with ground glass stoppers to prevent contamination, evaporation and/or volatilization. Each chamber will contain  $\sim 140\text{mL}$  of test solution (no headspace) and two 14mm glass spheres to facilitate mixing.

#### *Exposure Duration*

96 hours ( $\pm 1$  hour)

#### *Environmental Conditions*

Range of acceptable test temperatures:  $23^{\circ} \pm 2^{\circ}\text{C}$ .  
Continuous light at 8000 Lux  $\pm 20\%$ .  
Oscillation Rate: 100rpm  $\pm 10\%$ .  
Oscillation rate and lighting will be verified daily.

An environmental condition study will be activated on the laboratory computer system (MicroVAX 3100-20E running validated, customized acquisition software) at the start of the study to provide a record of the continuous measurements for temperature and lighting in the test area.

#### *Experimental Evaluation*

Cell density is determined for each test and control chamber using a hemacytometer and microscope at 24, 48, 72 and 96 hours ( $\pm 1$  hour) after the beginning of the test. Cell density determinations will be performed on three replicates at each observation interval and the replicates will then be discarded. Any test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) will be recorded at that time. The pH of each treatment will be measured on Day 0 and daily after cell density determinations (composite of three replicates). The pH is expected to vary by more than one unit during the test due to the no headspace environment of the study design.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
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### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Test Acceptability*

A test may not be acceptable if cell density in the Control does not increase by a factor of  $\geq 16$  within three days.

#### *Calculations*

Test results are used to derive Effect Loading/Concentration 50 (EL/EC<sub>50</sub>) values (or other appropriate statistical values), defined as the loading rate or concentration of the test substance in the vehicle/dilution water which results in a 50% reduction in growth, as determined by either the area under the growth curve or the average specific growth rate (relative to the Control) for the specified time of exposure. The specific growth rate for each loading rate/concentration will be determined by calculating the slope of the regression line of the ln (cell density) versus time using the PROC REGRESSION procedure from SAS<sup>5</sup>. The areas under the growth curves and average specific growth rate will be calculated in accordance with the formulas listed in the OECD guideline<sup>1</sup>. The method used to calculate the 72 and 96-hour EL/EC<sub>50</sub> values will be either the maximum likelihood analysis based on D. J. Finney, 1971<sup>6</sup>, the inverse interpolation method of Snedecor and Cochran<sup>7</sup>, or other appropriate statistical methods where appropriate. The 72 and 96-hour No Observed Effect Loading/Concentration (NOEL/NOECC) will be determined using the ANOVA procedure<sup>8</sup> of SAS.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### 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 algae: origin, lab culture, strain, method of cultivation

Test conditions:

- date of the start and end of the test
- test procedure used, equilibration test results
- composition of the medium
- temperature and pH values of the test solutions at the start and end of the test
- methods of preparation of test solutions
- loading rates/concentrations used
- information on concentrations of the test substance in the test solutions
- light intensity and quality
- description of the test chambers, volume of solution
- culturing apparatus

Results

- cell density for each flask at each measuring point and method for measuring cell density
- mean values of cell density
- graphical presentation of the concentration effect relationship, growth curves
- EC or EL values and method of calculation
- NOEC or NOEL
- other observed effects

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

## APPENDIX C - PROTOCOL (CONT'D)

Alga. Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### 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 and the appropriate guidelines and Good Laboratory Practice Regulations.

### GUIDELINE EXCEPTIONS

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

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

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

The test duration will be 96 hours, instead of 72 hours. Both 72 and 96-hour endpoints will be determined. The extended duration is at the sponsor's request.

## APPENDIX C - PROTOCOL (CONT'D)

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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### REFERENCES

1. Organization for Economic Cooperation and Development (OECD). Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, Guideline 201: Alga, Growth Inhibition Test. 1984.
2. OECD, Principles of Good Laboratory Practice, C(97)186 (Final), 1997.
3. United States Environmental Protection Agency (USEPA), Toxic Substance Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792, 1989.
4. USEPA. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test. EPA-600/9-78-018. July 1978.
5. SAS User's Guide: Statistics, Version 5.18 Edition. SAS Institute, Inc., Cary, NC. 1985.
6. Finney, D.J. 1971. Probit Analysis, 3rd Edition, London: Cambridge University Press.
7. Snedecor, G.W. and W.G. Cochran, *Statistical Methods*, 8<sup>th</sup> Edition. 1989. Iowa State University Press / Ames.
8. Duncan, D.B. 1975. *t-Tests and Intervals for Comparisons Suggested by the Data*, *Biometrics*, 31, 339-359.

**APPENDIX C - PROTOCOL (CONT'D)**

Alga, Growth Inhibition Test on Heavy Pyrolysis Fuel Oil:  
176867; MRD-03-768

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**DISTRIBUTION**

Study Director .....	E. J. Febbo
Sponsor Representative .....	E. J. Moran
Section Head, Laboratory Operations .....	R. L. Rucker
Study Technicians .....	R. F. Blattenberger
.....	P. N. Unanka
.....	J. Yarusinsky
Analytical Chemistry .....	D. J. Letinski
Compound Prep .....	Staff
Quality Assurance .....	Staff
Contract Administrator .....	B. J. Foster

**APPENDIX C - PROTOCOL (CONT'D)**

PROTOCOL CHANGE RECORD  
 ALGA, GROWTH INHIBITION TEST on HEAVY PYROLYSIS FUEL OIL

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 Number: 176867

Revision Number: 1

Date: 4-Nov-03

**Pg. 6 / Definitive Test Design**

*Previous Statement:*

GROUP	LOADING RATE (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	$-1.0 \times 10^4$ (per 12 replicates)
2	TBD	$-1.0 \times 10^4$
3	TBD	$-1.0 \times 10^4$
4	TBD	$-1.0 \times 10^4$
5	TBD	$-1.0 \times 10^4$
6	TBD	$-1.0 \times 10^4$

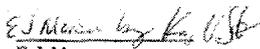
TBD = To Be Determined

*Revised Statement:*

GROUP	LOADING RATE (mg/L)	NUMBER OF CELLS PER mL
1 (Control)	0	$-1.0 \times 10^4$ (per 12 replicates)
2	0.18	$-1.0 \times 10^4$
3	0.44	$-1.0 \times 10^4$
4	1.1	$-1.0 \times 10^4$
5	2.8	$-1.0 \times 10^4$
6	7.0	$-1.0 \times 10^4$

*Justification:* addition of definitive loading rates

Required signatures:

  
 E. J. Moran  
 Sponsor Representative

11-24-03  
 Date

  
 E. J. Febbo  
 Study Director

18-Nov-03  
 Date

**Robust Summary  
Alga Toxicity**

<b>Test Substance:</b>	<p>Industry Stream Name (acronym): Heavy Pyrolysis Fuel Oil</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"><u>CAS Number:</u></td> <td style="width: 50%; border: none;"><u>CAS Inventory Name:</u></td> </tr> <tr> <td style="border: none;">68513-69-9</td> <td style="border: none;">Residues, petroleum, steam-cracked light</td> </tr> <tr> <td style="border: none;">64741-62-4</td> <td style="border: none;">Clarified oils, petroleum, catalytic cracked</td> </tr> <tr> <td style="border: none;">69013-21-4</td> <td style="border: none;">Fuel oil, pyrolysis</td> </tr> <tr> <td style="border: none;">8002-05-9</td> <td style="border: none;">Petroleum</td> </tr> </table> <p>In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.</p>	<u>CAS Number:</u>	<u>CAS Inventory Name:</u>	68513-69-9	Residues, petroleum, steam-cracked light	64741-62-4	Clarified oils, petroleum, catalytic cracked	69013-21-4	Fuel oil, pyrolysis	8002-05-9	Petroleum
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68513-69-9	Residues, petroleum, steam-cracked light										
64741-62-4	Clarified oils, petroleum, catalytic cracked										
69013-21-4	Fuel oil, pyrolysis										
8002-05-9	Petroleum										
<b>Method/Guideline:</b>	OECD Guideline 201										
<b>Year (guideline):</b>	1984										
<b>Type (test type):</b>	Alga Toxicity Test										
<b>GLP (Y/N):</b>	Yes										
<b>Year (study performed):</b>	2003										
<b>Species:</b>	<i>Pseudokirchneriella subcapitata</i>										
<b>Analytical Monitoring:</b>	Yes										
<b>Exposure Period:</b>	96 hours										
<b>Statistical Method:</b>	<p>The <math>E_bC_{50}</math>, <math>E_rC_{50}</math> and confidence intervals for inhibition of growth/growth rate slope were determined by a probit regression calculation of the probit of the growth inhibition/growth rate slope vs the log of the concentration and associated confidence intervals based on the methods of D. J. Finney (Finney, 1971). Calculations were based on the PROC PROBIT procedure of SAS (SAS, 2002). The NOEC for the <math>E_bC_{50}</math> and <math>E_rC_{50}</math> was based on Multiple Range tests (Duncan, 1975) and (Dunnett, 1964), determined from the GLM procedure of SAS (SAS, 2002). The Shapiro-Wilk (Shapiro-Wilk, 1965) test for normality was used to test if the assumption of normality of the residuals was met; since the residuals were normally distributed the NOEC was based on the estimated values.</p> <p>Finney, D.J. 1971. Probit Analysis, 3rd Edition, London: Cambridge University Press.</p> <p>SAS Version 8, SAS Institute, Inc., Cary, NC. 2002.</p> <p>Duncan, D.B. 1975, "t-Tests and Intervals for Comparisons Suggested by the Data", Biometrics, 31, 339-359.</p> <p>Dunnett, C. 1964, "New Tables for Multiple Comparisons With A Control", Biometrics, Vol 20, No. 3, pg 482-491.</p> <p>Shapiro, S.S. and Wilk, M.B. 1965, "n analysis of variance test for normality (complete samples)" Biometrika, 52, pg 591-611.</p>										

<p><b>Test Conditions:</b></p> <ul style="list-style-type: none"> <li><b>Note: Concentration preparation, vessel type, volume, replication, environmental conditions, organisms supplier, loading, deviations from guideline or protocol.</b></li> </ul>	<p>Individual Water Accommodated Fractions (WAF's) were prepared for each treatment. The test substance was added to 2.0 L of algal nutrient medium augmented with sodium bicarbonate in glass aspirator bottles (capacity 2.3 L). The solutions were mixed for 24.5 hours using an 7% vortex (of the static liquid depth). The test solutions were removed through the outlet at the bottom of each mixing vessel into 12 replicates of approximately 140 mL in 125 mL Erlenmeyer flasks (no headspace) containing two 14 mm glass spheres to facilitate mixing. The test chambers were inoculated with algae (<math>1.0 \times 10^4</math> cells/mL) and were sealed with ground glass stoppers. Three replicates were sacrificed daily for cell density determination. The test chambers were placed on a shaker table (100 rpm) to keep the algae in suspension. The test was performed under static conditions with no aeration. The algae was cultured in-house from 5 day old stock cultures in log phase growth.</p> <p>Mean test temperature: 24.2°C (sd = 0.5). Continuous light: intensity was 8431 to 8595 Lux. The pH ranged from 7.4 to 7.6 in the test solutions at test initiation and ranged from 7.0 to 8.7 at test termination.</p> <p>Due to the complex nature and limited water solubility of the test substance, the following exceptions to the guideline apply for this study: The concentration of the test substance in solution was not determined prior to use. Test substance analysis was performed on samples of the WAFs at the start of the test (day 0) and at termination (day 4). The initial concentration of the test substance was not maintained at 80% in the three lower loading rates throughout the test (this may be due to biological activity or physical processes in the test chambers). It was appropriate to prepare individual treatment solutions by adding the test substance to dilution water and removing the WAF of each mixture for testing than to prepare dilutions of a stock solution. The test duration was 96 hours, instead of 72 hours. However, both 72 and 96-hour endpoints were determined.</p> <p>None of the above exceptions are believed to have affected the outcome, integrity, or quality of the study.</p>
<p><b>Results:</b></p> <p><b>Units/Value:</b></p> <p><b>Note: Analytical method, biological observations, control survival.</b></p>	<p>Effects on growth rate (r) based upon actual loading rates:</p> <p>72 hr ErL50 = 2.3 mg/L (CNC)  96 hr ErL50 = 2.1 mg/L (CNC)  72 and 96 hr NOELR = 0.39 mg/L</p> <p>Effects on biomass (b) based upon actual loading rates:</p> <p>72 hr EbL50 = 1.5 mg/L (1.3-1.6 mg/L)  96 hr EbL50 = 1.4 mg/L (1.3-1.6 mg/L)  72 hr NOELR = 0.20 mg/L  96 hr NOELR = 0.39 mg/L</p> <p>Effects on growth rate (r) based upon measured concentrations:</p> <p>72 hr ErC50 = 2.0 mg/L (CNC)  96 hr ErC50 = 1.8 mg/L (CNC)  72 and 96 hr NOEC = 0.42 mg/L</p> <p>Effects on biomass (b) based upon measured concentrations:</p> <p>72 and 96 hr EbC50 = 1.3 mg/L (1.2-1.4 mg/L)  72 hr NOEC = 0.07 mg/L  96 hr NOEC = 0.42 mg/L</p> <p>Values in parentheses are 95% confidence intervals.  CNC = Could Not Calculate</p>

	<p>The analytical method used was static headspace gas chromatography with flame ionization detection.</p> <p>Summary of In-Life observations - % Inhibition</p> <table border="1"> <tr> <td>Loading Rate* (mg/L) Control</td> <td>0.20</td> <td>0.39</td> <td>1.1</td> <td>2.6</td> <td>7.2</td> </tr> <tr> <td>Meas. Conc.† (mg/L)</td> <td>0</td> <td>0.07‡</td> <td>0.42</td> <td>1.1</td> <td>2.1</td> <td>6.4</td> </tr> </table> <p>Based on Growth Rate</p> <table border="1"> <tr> <td>72 hours</td> <td>n/a</td> <td>-2.0</td> <td>0</td> <td>11</td> <td>83</td> <td>97</td> </tr> <tr> <td>96 hours</td> <td>n/a</td> <td>-1.7</td> <td>-2.2</td> <td>7.1</td> <td>86</td> <td>98</td> </tr> </table> <p>Based on Biomass</p> <table border="1"> <tr> <td>72 hours</td> <td>n/a</td> <td>-2.1</td> <td>7.6</td> <td>34</td> <td>92</td> <td>99</td> </tr> <tr> <td>96 hours</td> <td>n/a</td> <td>-1.9</td> <td>1.3</td> <td>31</td> <td>97</td> <td>100</td> </tr> </table> <p>* Actual loading rate (weight) of test substance added to the vehicle/dilution water.  † Concentration based on mean (Day 0 and Day 4) measured concentrations.  ‡ Based on Day 0 only, since the Day 4 sample was below detection limits.  Negative(-) value indicates a stimulatory effect.</p>	Loading Rate* (mg/L) Control	0.20	0.39	1.1	2.6	7.2	Meas. Conc.† (mg/L)	0	0.07‡	0.42	1.1	2.1	6.4	72 hours	n/a	-2.0	0	11	83	97	96 hours	n/a	-1.7	-2.2	7.1	86	98	72 hours	n/a	-2.1	7.6	34	92	99	96 hours	n/a	-1.9	1.3	31	97	100
Loading Rate* (mg/L) Control	0.20	0.39	1.1	2.6	7.2																																					
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96 hours	n/a	-1.7	-2.2	7.1	86	98																																				
72 hours	n/a	-2.1	7.6	34	92	99																																				
96 hours	n/a	-1.9	1.3	31	97	100																																				
<b>Conclusions:</b>	<p>Effects on growth rate (r) based upon actual loading rates:  72 hr ErL50 = 2.3 mg/L  96 hr ErL50 = 2.1 mg/L</p> <p>Effects on biomass (b) based upon actual loading rates:  72 hr EbL50 = 1.5 mg/L  96 hr EbL50 = 1.4 mg/L</p> <p>Effects on growth rate (r) based upon measured concentrations:  72 hr ErC50 = 2.0 mg/L  96 hr ErC50 = 1.8 mg/L</p> <p>Effects on biomass (b) based upon measured concentrations:  72 and 96 hr EbC50 = 1.3 mg/L</p>																																									
<b>Reliability:</b>	(1)-Reliable without restriction																																									
<b>Reference:</b>	ExxonMobil Biomedical Sciences, Inc. 2004. ALGA, GROWTH INHIBITION TEST on HEAVY PYROLYSIS FUEL OIL. Study # 176867.																																									
<b>Other (source):</b>	Olefins Panel, American Chemistry Council																																									

# **ExxonMobil** BIOMEDICAL SCIENCES, INC.

**OLF-105.0-HPV10-EMBSI**

**FINAL REPORT**

**STUDY NUMBER: 176894A**

**TEST SUBSTANCE: MRD-03-768**

**READY BIODEGRADABILITY: OECD 301F  
MANOMETRIC RESPIROMETRY TEST ON  
HEAVY PYROLYSIS FUEL OIL**

**PERFORMED FOR:**

**American Chemistry Council  
1300 Wilson Boulevard  
Arlington, VA 22209**

**PERFORMED AT:**

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

**COMPLETION DATE: October 28, 2004**

**04TP 118**

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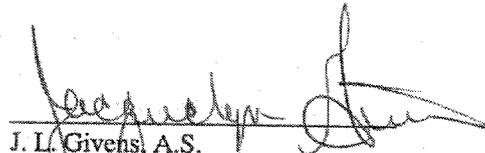
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APPROVAL SIGNATURES

  
\_\_\_\_\_  
J. J. Freeman, Ph.D., D.A.B.T  
Acting Section Head, Laboratory Operations

13 Oct 04  
Date

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<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards with the exceptions outlined on pages 9 and 10.

  
\_\_\_\_\_  
J. L. Givens, A.S.  
Study Director  
ExxonMobil Biomedical Sciences, Inc.  
1545 Route 22 East, P.O. Box 971  
Annandale, New Jersey 08801-0971

28 Oct 04  
Date

The final report was accepted by the Sponsor.

  
\_\_\_\_\_  
E. J. Moran, Ph.D.  
Sponsor's Representative

10/28/04  
Date

**READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768**

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QUALITY ASSURANCE STATEMENT

---

STUDY NUMBER: 176894A

TEST SUBSTANCE: MRD-03-768

STUDY SPONSOR: American Chemistry Council

---

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.

<u>Study Phase Inspected</u>	<u>Date(s) of Inspection</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
Protocol	15 Aug 03	15 Aug 03	20,21 Aug 03
TSS Determination	22,23 Sep 03	23 Sep 03	25,26 Sep 03
Final Report	04-06 Feb 04	06 Feb 04	11,13 May 04
Second Review of Final Report	10,11 May 04	11 May 04	07,15 Jun 04

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

  
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W. James Bover, Ph.D.  
Data Integrity & Quality Assurance/Archives  
Section Head

12 Oct 04  
Date

**READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768**

**PERSONNEL**

Study Director:	J. L. Givens, A.S.
Sponsor Representative:	E. J. Moran, Ph.D.
Primary Study Technician:	B. A. Kelley, A.S.
Acting Section Head, Laboratory Operations:	J. J. Freeman, Ph.D., D.A.B.T.  R. L. Rucker, Ph.D. (Prior to December 9, 2003)
Section Head, Data Integrity & Quality Assurance/Archives:	W. J. Bover, Ph.D.
Supervisor, Environmental Toxicology & Compound Preparation:	E. J. Febbo, M.S.

**SUMMARY**

The biodegradability potential of the test substance and a positive control substance was studied in aerobic, aqueous test systems. This study was performed in general agreement with the procedure outlined in the OECD 301F<sup>3</sup> guideline. Biodegradability of the substances was determined by measuring oxygen consumption in a test medium containing trace nutrients and inoculated with activated sludge supernatant. The test substance, Heavy Pyrolysis Fuel Oil, was evaluated at a mean concentration of 50 mg/L. The positive control substance, sodium benzoate, was evaluated at a concentration of 51 mg/L.

The mean percent biodegradation of triplicate test systems of Heavy Pyrolysis Fuel Oil was determined to be 29% over a 28 day testing period. The OECD guideline criteria for classification of a chemical as readily biodegradable states: 1) percent biodegradation must reach 60% within 28 days, and 2) the 60% degradation must be attained within 10 days of exceeding 10% biodegradation. Heavy Pyrolysis Fuel Oil did not achieve 60% biodegradation and therefore, based on the OECD guideline, can not be considered readily biodegradable.

The OECD guideline validity requirement states that the difference of extremes of replicate biodegradation values should be less than 20% on day 28 and the positive control should achieve >60% of the theoretical oxygen demand (ThOD) by day 14. Further, the oxygen uptake of the inoculum blank should not exceed 60 mg/L on day 28. Sodium benzoate biodegraded to >60% by day 3 and the average of the cumulative oxygen consumed in the blank systems was 21 mg/L. The difference of extremes for the test substance replicates exceeded 20%. It is believed the greater difference was due to the poor solubility of the test substance and the low biodegradation achieved. Based on the study data and the passing of the validity requirements, with the exception of the difference of extremes, the test is considered valid.

Daily percent biodegradation and mg oxygen uptake results for the test substance and sodium benzoate are reported in Tables 1 and 2, respectively. The table below summarizes day 28 data.

Test System	Replicate #	% Biodegradation	Mean (SD)	Final pH
Sodium Benzoate	1	91.31	88.93 (2.21)	6.75
	2	86.93		6.75
	3	88.56		6.74
MRD-03-768	1	33.24	29.03 (5.75)	6.59
	2	31.36		6.54
	3	22.48		6.58

## INTRODUCTION

### *Objective*

This study was conducted for the sponsor in order to evaluate the potential of the test substance to biodegrade in an aerobic, aqueous environment for use in environmental hazard assessment.

### *Sponsor*

American Chemistry Council  
1300 Wilson Boulevard  
Arlington, VA 22209

### *Testing Facilities*

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

Quantitative Technologies Inc. (QTI) (Elemental Analysis Only)  
P.O. Box 470  
Salem Industrial Park, Bldg 5  
Whitehouse, New Jersey 08888

### *Study Initiation Date*

September 15, 2003

### *Experimental Start*

September 23, 2003

### *Experimental Completion*

October 22, 2003

## INTRODUCTION (CONT'D)

### *Compliance*

This study was performed in general agreement with the test method described in OECD 301F<sup>3</sup> with the exception of the inoculum preparation which was performed in general agreement with ASTM D5864<sup>4</sup> guideline. Additional exceptions are listed on pages 13 and 14.

The study was conducted in compliance with OECD<sup>1</sup> and USEPA<sup>2</sup> Good Laboratory Practice (GLP) standards with the exceptions outlined pages 9 and 10.

### *Justification for Selection of Test System*

Selection of the aerobic aquatic biodegradation test is based upon the OECD 301F<sup>3</sup> guideline. This test method is used to determine biodegradability by measuring oxygen consumption in a test system consisting of an activated sludge supernatant, test or positive control substance and a nutrient source. Further, activated sludge has historically been used to evaluate the persistence of chemicals in the environment.

### *Justification of Dosing Route*

The test substance could possibly be found in aqueous solution in a wastewater treatment facility.

## MATERIALS AND METHODS

### *Test Substance Identification*

EMBSI Identification:	MRD-03-768
Industry Stream Name (acronym):	Heavy Pyrolysis Fuel Oil
<u>CAS Number:</u>	<u>CAS Inventory Name:</u>
68513-69-9	Residues, petroleum, steam-cracked light
64741-62-4	Clarified oils, petroleum, catalytic cracked
69013-21-4	Fuel oil, pyrolysis
8002-05-9	Petroleum
Supplier:	Dow Chemical Company
Date Received:	Freeport, TX
Expiration Date:	July 29, 2003
Description:	July 2008
	Dark Amber liquid

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

### *Stream Derivation*

In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.

### *Characterization of Test Substance*

The neat test substance was characterized and the stability determined by the testing facility using the following analysis: UltraViolet/Visible and Infrared Spectrophotometry, Gas Chromatography with Mass Selective Detection and Density. The test substance was used in a number of studies at the testing facility. Characterization of the test substance was performed at the testing facility prior to its use in the first of these studies and after completion of the final study. Documentation of characterization and stability assessment is maintained at the testing facility (see Appendix A).

## MATERIALS AND METHODS (CONT'D)

### *Characterization of Test Substance (cont'd)*

An aliquot of the test substance was sent to Quantitative Technologies Inc. (QTI) for elemental analysis, specifically carbon, hydrogen, nitrogen and oxygen. Percent carbon, hydrogen, nitrogen, and oxygen was determined using a Perkin-Elmer 2400 CHN Elemental Analyzer equipped with an oxygen accessory kit. For CHN the analyzer used combustion to convert the sample elements to simple gases. Upon entering the analyzer, the sample was combusted in a pure oxygen environment. The product gases were separated under steady state conditions, and measured as a function of thermal conductivity. For oxygen, pyrolysis was used to convert the oxygen to carbon monoxide which was separated from the other pyrolozates under steady state conditions and measured as a function of thermal conductivity. QTI is not a GLP compliant facility and therefore may not have performed the analysis in a GLP compliant manner. However, a standard (Canola Oil) was employed to monitor the quality of the data. The values were within the standard limits.

Documentation outlining the methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, was considered the "pure" substance.

### *Sample Retention*

A sample of the neat test substance has been retained in the testing facility archives.

### *Positive Control Substance*

Substance Identification: Sodium benzoate, 99% - Manufacturer: Aldrich Chemical Company, Lot No 09519HA

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

Characterization: The documentation of the stability, identity, purity, strength, and composition or other characteristics, which appropriately identify the control substance, was provided by the manufacturer. This documentation is identified as the Certificate of Analysis which can be found in the raw data and Appendix B.

## MATERIALS AND METHODS (CONT'D)

### *Carrier*

Glass distilled water (gdH<sub>2</sub>O) was used as the carrier. The glass distilled 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 feed water for the deionized water system is analyzed by Accutest®, 2235 Route 130, Dayton, New Jersey 08810. Results of the water analyses are maintained at the testing facility. 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 not performed in a GLP compliant manner. This is not believed to have an adverse effect on the study results.

### *Inoculum*

Fresh activated sludge was used as the inoculum. The activated sludge was obtained from the Clinton Sanitary Wastewater Treatment Plant, Annandale, New Jersey on September 22, 2003. This treatment facility was selected because it deals predominantly with domestic sewage as specified in the guideline. There were no known contaminants in the fresh activated sludge believed to be present at levels high enough to interfere with this study. A complete description of the inoculum preparation is provided in Appendix D.

### *Solutions*

Mineral salt solutions:

- Phosphate buffer - pH 7.2 (VWR, Lot# 2224)
- Ferric chloride - 0.025% (VWR, Lot# 2315)
- Magnesium sulfate - 2.25% (VWR, Lot# 2343)
- Calcium chloride - 2.75% (VWR, Lot# 2308)

There were no known contaminants in the solutions believed to be present at levels that may interfere with the study. All solutions were refrigerated when not in use.

Test medium: The test medium was prepared one day before the test began. A total of 20 liters of glass distilled water was collected in a carboy. The glass distilled water in the carboy was then amended with mineral salt solutions and inoculum. A complete description of the test medium preparation is provided in Appendix C.

## MATERIALS AND METHODS (CONT'D)

### *Solutions (cont'd)*

Positive control substance stock solution: A stock solution of sodium benzoate at a concentration of approximately 10,000 mg/L was prepared in glass distilled water. An O.I. Analytical Model 1010 Total Organic Carbon (TOC) Analyzer determined the actual carbon content of the sodium benzoate stock solution. The instrument employs a wet oxidation technique with a nondispersive infrared (NDIR) detector. TOC results are contained in Appendix D. The stock solution was refrigerated when not in use.

### *Test System*

The test system was considered as any combination of the following in a flask:

- Test Substance
- Positive Control Substance
- Mineral Salt Solutions
- Activated Sludge Supernatant
- Glass Distilled Water

All test containers used in this study were uniquely identified as to appropriate composition, i.e., Blank -1, 2, 3; MRD-03-768 - 1, 2, 3, etc. All glassware was washed with Chemsolve® and then rinsed with glass-distilled water to remove any residual organic carbon. All glassware was inspected to ensure cleanliness. The manometric cells were rinsed with two portions of acetone, filled with soapy water and allowed to stand for a few hours. The cells were then rinsed with glass distilled water followed by acetone then finally air dried.

## EXPERIMENTAL PROCEDURE

### *Procedure Summary*

The test procedure evaluated the ready biodegradability of the test and positive control substance by microorganisms in water. The consumption of oxygen was determined by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved carbon dioxide was absorbed in a solution of 10N sodium hydroxide (NaOH). The amount of oxygen taken up by the microbial population during biodegradation of the test or positive control substance (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of theoretical oxygen demand (ThOD). The ThOD calculation is found in Appendix D. The test was performed in general agreement with the OECD 301F<sup>3</sup> guideline with the following clarifications/exceptions:

### *Clarification*

1. The apparatus is an electrolytic respirometer, manufactured by Co-ordinated Environmental Service (CES), Ltd. (Kent, England). The system is based on a proven oxygen generating process coupled to a sensitive manometric cell. The sample was placed in a sample flask, which was then sealed by a manometric cell/CO<sub>2</sub> trap and immersed in a temperature stabilized water bath. For the duration of the experiment, the sample was stirred by a magnetically coupled stirrer. As the biodegradation process progressed, the microorganisms consumed O<sub>2</sub> converting it to CO<sub>2</sub> during aerobic respiration. The CO<sub>2</sub> produced was absorbed by a solution of 10N NaOH in the CO<sub>2</sub> trap, which caused a net reduction in gas pressure within the sample flask. This pressure reduction was detected by the manometric cell and triggered the electrolytic process. The electrolytic process generated oxygen, which restored the pressure in the sample flask. The magnitude and duration of the electrolyzing current is proportional to the amount of oxygen supplied to the microorganisms.

## EXPERIMENTAL PROCEDURE (CONT'D)

### *Clarification (Cont'd)*

2. The test and positive control substances were tested at concentrations of approximately 50 mg/L. Sodium benzoate was administered to the respective test systems as an aliquot of an aqueous stock solution. The test substance was administered by direct addition on glass fiber filters. No aqueous stock solutions were prepared for the test substance because of its poor solubility in water. Also no concentration verification was performed since the test substance is poorly soluble in water.
3. The blank, positive control, and test substance test systems were tested in triplicate.
4. Bias was minimized by preparing the test medium on a large volume basis. In addition, the test medium was aerated for approximately 24 hours to improve homogeneity and ensure random distribution of test organisms to all test systems. The pH of the test medium was 7.25. The initial pH of individual systems was not determined due to the poor solubility of the test substance in water.
5. Dissolved organic carbon (DOC) analysis was not performed due to the poor solubility of the test substance.
6. The test was terminated after 28 days.

### *Exceptions*

1. No toxicity or abiotic sterile control systems were tested.
2. Test medium was prepared on a large volume basis, aerated and aliquoted into each test container, instead of preparation in the individual test systems.
3. The commercial phosphate buffer, used in preparation of the test medium, has a pH value of 7.2 rather than 7.4. The phosphate buffer, purchased from VWR, has been approved by the American Public Health Association (APHA) for use in the Biological Oxygen Demand (BOD) analysis. The BOD buffer has the same composition as the buffer stated in the OECD 301F<sup>3</sup> and ASTM 5864<sup>4</sup> guidelines.
4. The inoculum was mixed for 2 minutes in a blender at low speed, instead of medium speed.

## RESULTS AND DISCUSSION

Testing was conducted to assess the biodegradability potential of the test substance, Heavy Pyrolysis Fuel Oil, in triplicate test systems at an mean concentration of 50 mg/L. The biodegradation of the positive control substance, sodium benzoate, was also measured in triplicate test systems at an average concentration of 51 mg/L. Blank test systems, which did not contain the test or positive control substance, were run concurrently in triplicate. This study was conducted at a temperature range  $22 \pm 1^\circ\text{C}$  for twenty-eight days in general agreement with the OECD 301F<sup>3</sup> guideline.

The percent biodegradation results and mg oxygen consumed for each test system are reported in Tables 1 and 2, respectively. The oxygen consumed by the test systems was corrected for oxygen consumption occurring in the blank test systems. The mean percent biodegradation for Heavy Pyrolysis Fuel Oil was determined to be 29% over a 28 day test period.

This test is considered valid since it met the OECD guideline validity requirements as follows: 1) sodium benzoate biodegraded to >60% of the ThOD by day 3 and 2) the average of the cumulative oxygen consumed in the blank systems was 21 mg/L. A graphical illustration representing the mean percent biodegradation and the mg oxygen consumed by the test systems is reported in Figures 1 and 2, respectively.

The inoculum was prepared using activated sludge from the Clinton Sanitary Wastewater Treatment Plant, Annandale, New Jersey. The Easicult® TTC dip slide results indicated an average bacterial population of  $10^5$  colony forming units (CFU)/mL. Biodegradation results for the test and positive control substances were calculated as the net amount of oxygen (mg) consumed by the test system multiplied by a constant. The net amount of oxygen was calculated as the difference between the test system oxygen consumption (mg) and the average oxygen (mg) consumed by the blanks. The amount of oxygen consumed was recorded at each hourly interval for each test system using the CES aerobic respirometer. The constant is defined as the inverse of the product of the ThOD and the mg of the test or positive control substance added to that system multiplied by 100. The ThOD was calculated based on the empirical formula of the appropriate substance. The actual amount of substance added to each system is reported in Appendix A. The ThOD and methods used to determine percent biodegradation are described in Appendix C. The mean percent biodegradation and ThOD calculations were written in Microsoft® Excel 97 therefore, some rounding differences may be noted. Although the Excel program reports rounded values, internal rounding was not performed to calculate the final results.

## CONCLUSION

The test substance, Heavy Pyrolysis Fuel Oil, achieved a mean percent biodegradation of 29% and cannot be considered readily biodegradable.

## RECORDS

All appropriate materials, methods and experimental measurements required by the protocol have been 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 have been 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 study documentation and a non-study specific sample of the test substance will be maintained in the archives of the testing facility for 10 years, after which time the records will be offered to the sponsor prior to disposal.

## REFERENCES

1. Organization for Economic Cooperation and Development, Principles of Good Laboratory Practice, C(97) 186/Final, 1997.
2. USEPA Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards, 40 CFR Part 792, 1989.
3. Organization for Economic Cooperation and Development, Guidelines for the Testing of Chemicals, Ready Biodegradability, 301F Manometric Respirometry Test (1992).
4. ASTM D5864 Standard Test Method for Determining the Aerobic Aquatic Biodegradation of Lubricants and their Components (1995)

**TABLE 1**  
**PERCENT BIODEGRADATION RESULTS**

DAY OF TEST	SODIUM BENZOATE					MRD-03-768				
	Rep 1	Rep 2	Rep 3	Mean	SD	Rep 1	Rep 2	Rep 3	Mean	SD
1	20.28	20.08	10.63	17.00	5.51	0.00	0.03	0.00	0.01	0.02
2	59.88	58.90	58.90	59.23	0.57	0.00	0.00	0.00	0.00	0.00
3	68.43	67.19	64.07	66.56	2.25	10.05	9.91	8.58	9.51	0.81
4	79.03	77.28	76.82	77.71	1.17	12.78	12.40	11.08	12.09	0.89
5	83.06	81.70	81.67	82.14	0.79	11.93	11.42	10.13	11.16	0.93
6	84.75	84.12	83.90	84.26	0.44	10.83	9.09	8.86	9.59	1.08
7	84.73	81.98	83.59	83.43	1.38	9.98	9.48	8.83	9.43	0.58
8	86.12	82.98	84.66	84.59	1.57	11.07	10.73	9.92	10.57	0.59
9	87.19	84.42	86.05	85.89	1.39	11.77	11.84	10.65	11.42	0.67
10	88.59	85.37	87.15	87.04	1.61	13.15	13.34	11.93	12.81	0.77
11	88.18	84.52	86.35	86.35	1.83	15.29	15.98	14.63	15.30	0.68
12	88.33	84.58	86.50	86.47	1.88	19.42	19.77	19.35	19.51	0.23
13	88.74	85.12	86.89	86.92	1.81	24.71	24.47	22.04	23.74	1.48
14	89.24	85.51	87.19	87.31	1.87	25.91	25.21	22.31	24.48	1.91
15	90.12	86.00	87.78	87.97	2.07	26.60	25.83	22.48	24.97	2.19
16	90.41	86.22	87.97	88.20	2.10	27.15	26.20	22.48	25.28	2.47
17	90.70	86.46	88.17	88.44	2.13	27.70	26.59	22.48	25.59	2.75
18	91.02	86.68	88.36	88.69	2.19	28.26	26.86	22.48	25.87	3.02
19	91.31	86.93	88.56	88.93	2.21	28.67	27.26	22.48	26.14	3.24
20	91.31	86.93	88.56	88.93	2.21	29.64	28.06	22.48	26.73	3.76
21	91.31	86.93	88.56	88.93	2.21	30.05	28.33	22.48	26.95	3.97
22	91.31	86.93	88.56	88.93	2.21	30.47	28.75	22.48	27.23	4.21
23	91.31	86.93	88.56	88.93	2.21	30.88	29.16	22.48	27.51	4.44
24	91.31	86.93	88.56	88.93	2.21	31.02	29.30	22.48	27.60	4.52
25	91.31	86.93	88.56	88.93	2.21	31.58	29.85	22.48	27.97	4.83
26	91.31	86.93	88.56	88.93	2.21	32.13	30.26	22.48	28.29	5.12
27	91.31	86.93	88.56	88.93	2.21	32.82	30.95	22.48	28.75	5.51
28	91.31	86.93	88.56	88.93	2.21	33.24	31.36	22.48	29.03	5.75

Difference of extremes = ((Highest replicate value - Lowest Replicate Value) / Mean of High and Low values) x 100

MRD-03-768      Difference of extremes =  $\frac{(33.24) - (22.48)}{27.86} \times 100 =$

39 %

READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
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**TABLE 2**  
**MG OXYGEN CONSUMED RESULTS**

DAY OF TEST	BLANK					SODIUM BENZOATE					MRD-03-768				
	Rep 1	Rep 2	Rep 3	Mean	SD	Rep 1	Rep 2	Rep 3	Mean	SD	Rep 1	Rep 2	Rep 3	Mean	SD
1	0.54	0.42	0.02	<b>0.33</b>	<b>0.27</b>	17.66	17.49	9.41	<b>14.85</b>	<b>4.71</b>	0.19	0.37	0.00	<b>0.19</b>	<b>0.19</b>
2	0.83	0.62	0.35	<b>0.60</b>	<b>0.24</b>	51.79	50.96	50.96	<b>51.24</b>	<b>0.48</b>	0.19	0.37	0.00	<b>0.19</b>	<b>0.19</b>
3	1.69	1.46	0.98	<b>1.38</b>	<b>0.36</b>	59.87	58.81	56.14	<b>58.27</b>	<b>1.92</b>	16.51	16.39	14.47	<b>15.79</b>	<b>1.14</b>
4	3.21	2.92	2.23	<b>2.79</b>	<b>0.50</b>	70.35	68.85	68.45	<b>69.22</b>	<b>1.00</b>	22.03	21.57	19.70	<b>21.10</b>	<b>1.23</b>
5	6.54	6.04	4.62	<b>5.73</b>	<b>1.00</b>	76.74	75.57	75.55	<b>75.95</b>	<b>0.68</b>	23.70	23.03	21.20	<b>22.64</b>	<b>1.29</b>
6	12.52	11.66	8.62	<b>10.93</b>	<b>2.05</b>	83.38	82.84	82.65	<b>82.96</b>	<b>0.38</b>	27.24	24.70	24.47	<b>25.47</b>	<b>1.54</b>
7	17.26	16.24	13.77	<b>15.76</b>	<b>1.79</b>	88.19	85.84	87.22	<b>87.08</b>	<b>1.18</b>	30.78	30.11	29.24	<b>30.04</b>	<b>0.77</b>
8	18.20	17.10	15.72	<b>17.01</b>	<b>1.24</b>	90.63	87.94	89.38	<b>89.32</b>	<b>1.35</b>	33.67	33.26	32.15	<b>33.03</b>	<b>0.79</b>
9	19.10	17.93	16.43	<b>17.82</b>	<b>1.34</b>	92.36	89.98	91.38	<b>91.24</b>	<b>1.20</b>	35.55	35.76	34.09	<b>35.13</b>	<b>0.91</b>
10	20.43	18.78	17.41	<b>18.87</b>	<b>1.51</b>	94.61	91.86	93.38	<b>93.28</b>	<b>1.38</b>	38.67	39.09	37.09	<b>38.28</b>	<b>1.05</b>
11	21.93	20.24	18.51	<b>20.23</b>	<b>1.71</b>	95.61	92.48	94.05	<b>94.05</b>	<b>1.57</b>	43.25	44.44	42.57	<b>43.42</b>	<b>0.95</b>
12	22.10	20.68	19.01	<b>20.60</b>	<b>1.55</b>	96.11	92.90	94.55	<b>94.52</b>	<b>1.61</b>	49.83	50.54	50.15	<b>50.17</b>	<b>0.36</b>
13	22.26	20.89	19.14	<b>20.76</b>	<b>1.56</b>	96.63	93.53	95.05	<b>95.07</b>	<b>1.55</b>	57.98	57.83	54.42	<b>56.74</b>	<b>2.01</b>
14	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	97.13	93.94	95.38	<b>95.48</b>	<b>1.60</b>	59.85	59.04	54.92	<b>57.94</b>	<b>2.64</b>
15	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	97.88	94.36	95.88	<b>96.04</b>	<b>1.77</b>	60.89	59.98	55.17	<b>58.68</b>	<b>3.07</b>
16	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.13	94.55	96.04	<b>96.24</b>	<b>1.80</b>	61.73	60.54	55.17	<b>59.15</b>	<b>3.49</b>
17	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.38	94.75	96.21	<b>96.45</b>	<b>1.83</b>	62.56	61.12	55.17	<b>59.62</b>	<b>3.92</b>
18	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.65	94.94	96.38	<b>96.66</b>	<b>1.87</b>	63.39	61.54	55.17	<b>60.03</b>	<b>4.31</b>
19	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	64.02	62.14	55.17	<b>60.44</b>	<b>4.66</b>
20	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	65.47	63.35	55.17	<b>61.33</b>	<b>5.44</b>
21	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	66.10	63.77	55.17	<b>61.68</b>	<b>5.76</b>
22	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	66.72	64.39	55.17	<b>62.09</b>	<b>6.11</b>
23	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	67.35	65.02	55.17	<b>62.51</b>	<b>6.47</b>
24	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	67.56	65.22	55.17	<b>62.65</b>	<b>6.58</b>
25	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	68.39	66.06	55.17	<b>63.21</b>	<b>7.06</b>
26	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	69.22	66.68	55.17	<b>63.69</b>	<b>7.49</b>
27	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	70.26	67.72	55.17	<b>64.38</b>	<b>8.08</b>
28	22.26	21.12	19.14	<b>20.84</b>	<b>1.58</b>	98.90	95.15	96.54	<b>96.86</b>	<b>1.90</b>	70.89	68.35	55.17	<b>64.80</b>	<b>8.44</b>

FIGURE 1  
PERCENT BIODEGRADATION

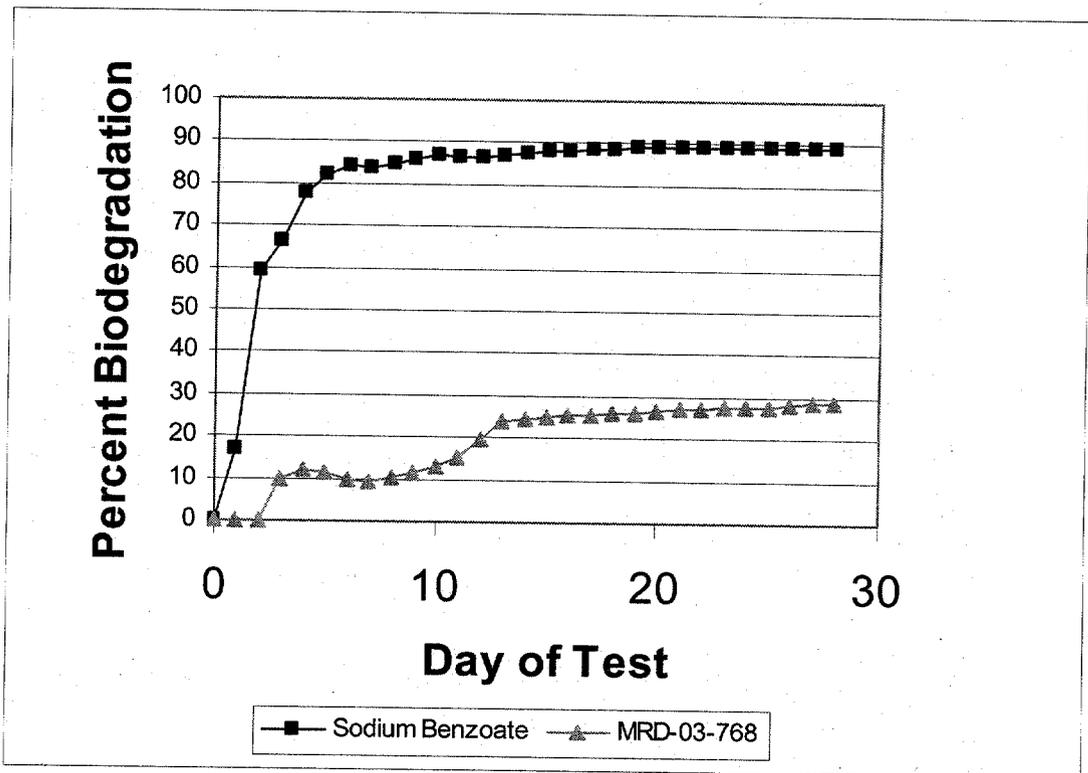
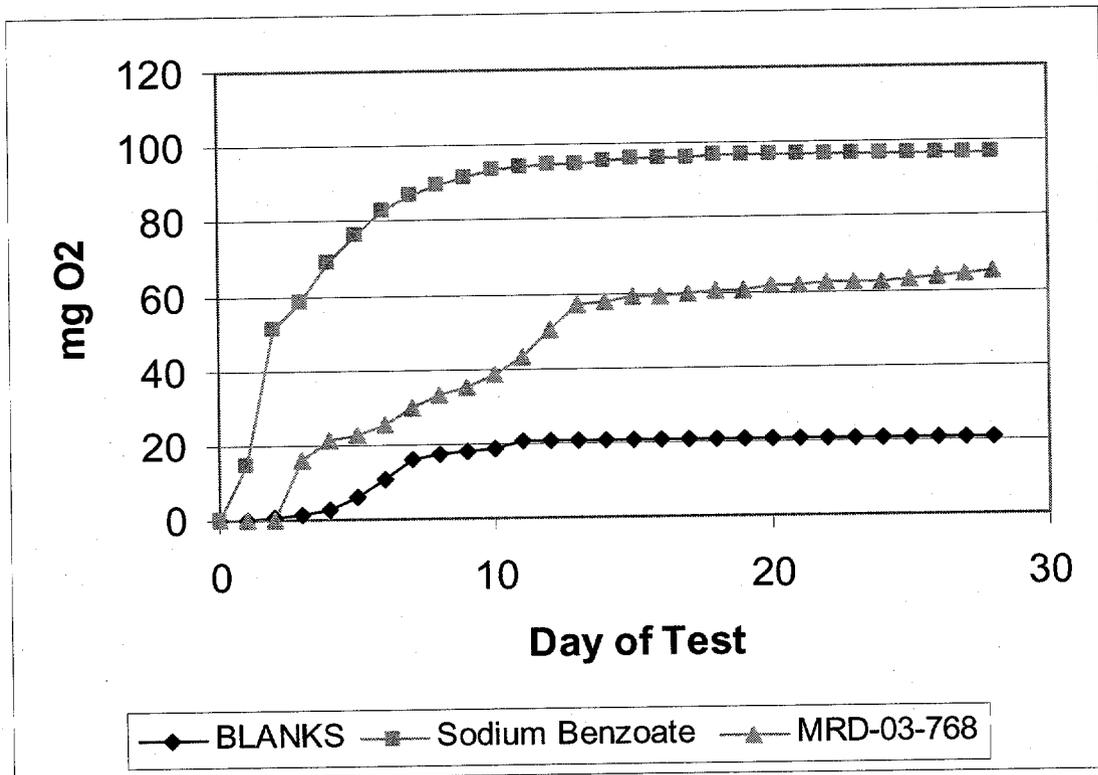


FIGURE 2  
MG OXYGEN CONSUMED



## APPENDIX A - TEST SUBSTANCE CHARACTERIZATION

The test substance was initially characterized on August 19 and 21, 2003. Analyses included Ultraviolet-Visible (UV-VIS) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy, density and GC-MS analysis. Stability of the neat test substance was confirmed by repeating these same analyses on February 5, 2004 after the completion of this study.

UV-VIS spectra are presented in Figures A-1 and A-2 representing, the initial and final spectrum at concentrations of 10 and 12 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 A-3 and A-4 representing the initial and final spectra. FT-IR spectra were acquired on a Thermo Nicolet Avatar 360 FT-IR spectrometer with a KBr plate. The spectra were obtained with the following settings: resolution of  $4\text{ cm}^{-1}$ , gain of 1, and scan number of 32.

The test substance was also characterized by GC-MS using a Varian Saturn 2000 GC-MS system with a Varian 3800 gas chromatograph. For comparison of relative retention times to a series of known hydrocarbons under the analytical conditions employed, Heavy Pyrolysis Fuel Oil was analyzed against an ASTM D2887 calibration mixture. Figures A-5 and A-6 represent the initial and final GC-MS total ion chromatograms, respectively. The test substance eluted as a complex mixture with numerous chromatographic components detected between retention times 13 and 26 minutes. This corresponds to bracketing by the standard hydrocarbons n-decane and n-octadecane under the analytical conditions employed. The single most abundant component eluted at approximately 16.3 minutes.

The test substance initial and final density was measured at  $20^{\circ}\text{C}$  using an Anton Paar DMA 4500 Density/Specific gravity/Concentration meter. The initial density was measured as 1.068 g/mL and the final density was 1.069 g/mL. The test substance was observed to be a liquid under ambient laboratory conditions and immiscible in water, methanol and hexane. All test substance solutions were prepared with methylene chloride as the solvent.

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.

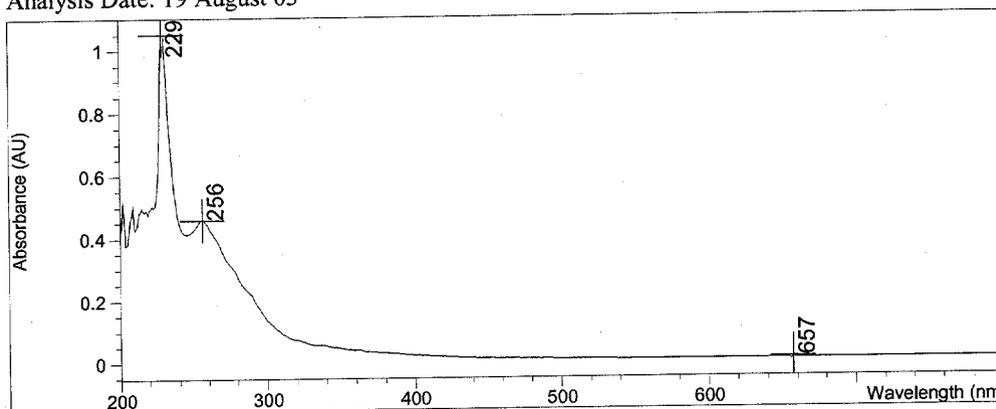
APPENDIX A - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

UV-VIS SPECTRA

Figure A-1

Initial

Initial Characterization Heavy Pyrolysis Fuel Oil 10 ppm solution in methylene chloride  
Analysis Date: 19 August 03

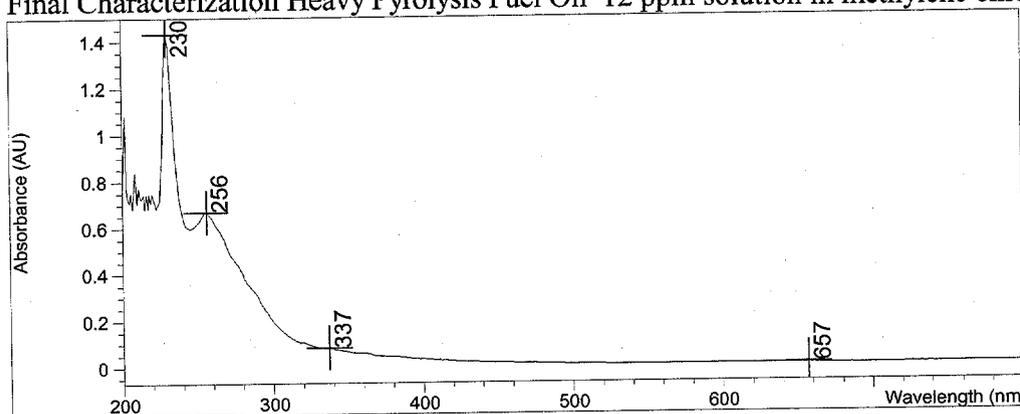


Peak 229nm Absorbance = 1.051  
Peak 256nm Absorbance = 0.4597  
Peak 657nm Absorbance = 0.0095

Figure A-2

Final

Final Characterization Heavy Pyrolysis Fuel Oil 12 ppm solution in methylene chloride



Analysis Date: 5 February 04  
Peak 230nm Absorbance = 1.4298  
Peak 256nm Absorbance = 0.6677  
Peak 337nm Absorbance = 0.0832  
Peak 657nm Absorbance = 0.0068

APPENDIX A - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FT-IR SPECTRA

Figure A-3

Initial

Initial Characterization Heavy Pyrolysis Fuel Oil Analysis Date: 19 Aug 03

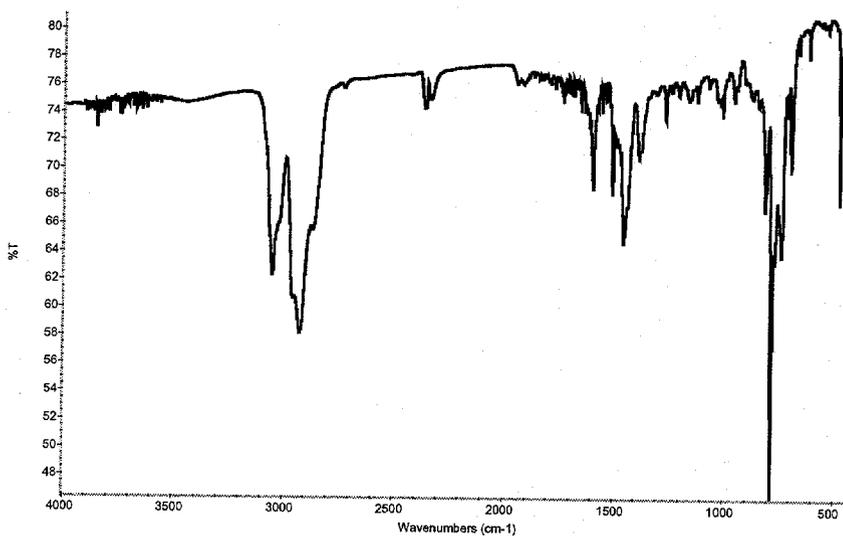
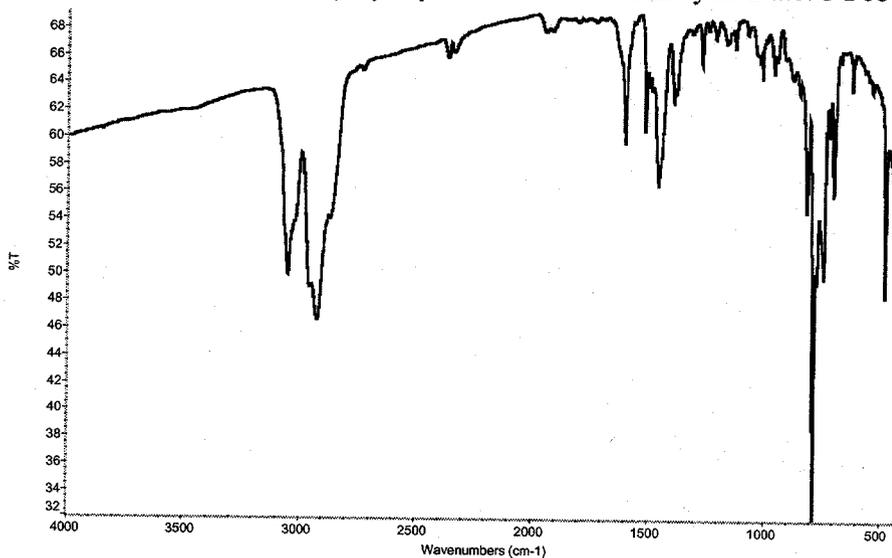


Figure A-4

Final

Final Characterization Heavy Pyrolysis Fuel Oil Analysis Date: 5 Feb 04



%T = % Transmittance

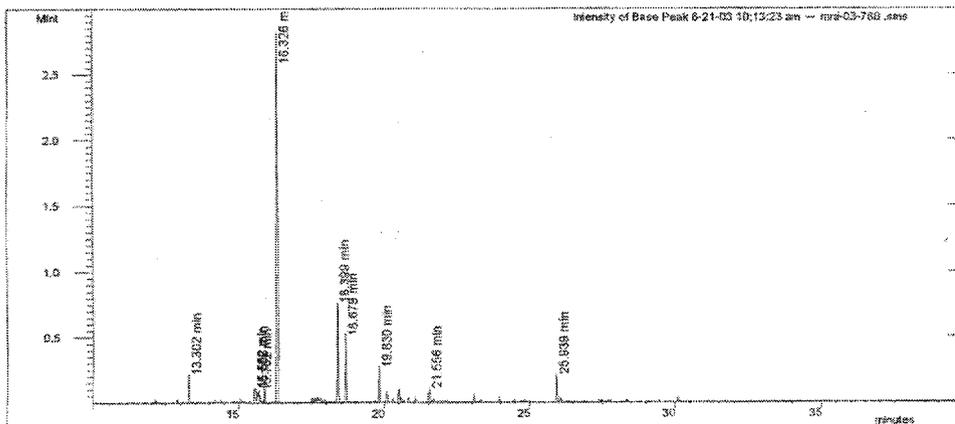
APPENDIX A - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768

INITIAL TOTAL ION CHROMATOGRAM  
Figure A-5

AREA PERCENT REPORT

Data File Name:	c:\saturnws\8-21-03 10:13:23 am -- mrd-03-768 .sms	Acquisition Date:	8/21/03 10:13:24 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) initial characterization (10% in CS2) 0.5uL injection



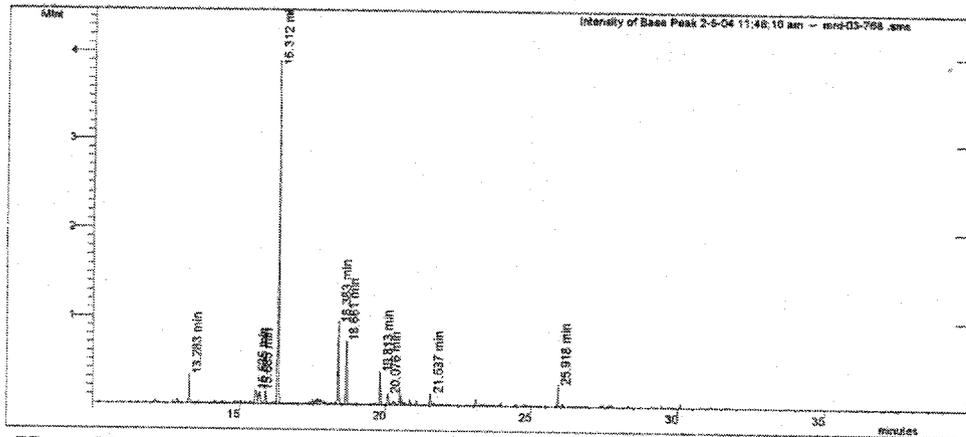
RT	BC	Width	Area	Area %
13.302	BB	1.9	1213022	4.86
15.552	BV	2.1	1037451	4.16
15.586	VV	2.2	895935	3.59
15.702	VV	2.7	1011392	4.05
16.326	VB	1.9	9296601	37.27
18.399	VB	1.7	4306416	17.26
18.679	BB	1.9	3339778	13.39
19.830	BB	1.7	1375559	5.51
21.556	VV	2.2	1524481	6.11
25.939	BB	1.8	942704	3.78

APPENDIX A - TEST SUBSTANCE CHARACTERIZATION (CONT'D)

FINAL TOTAL ION CHROMATOGRAM  
 Figure A-6

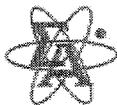
AREA PERCENT REPORT

Data File Name:	c:\saturnws\2-5-04 11:48:10 am -- mrd-03-768 .sms	Acquisition Date:	2/5/04 11:48:11 AM
Inst. Method:	C:\SaturnWS\Crude Oil Charac.mth	Instrument ID:	Saturn GC/MS #1
Sample Name:	MRD-03-768	Inj. Notes:	MRD-03-768 (heavy fuel oil) final characterization (10% in CS2) 0.5uL injection



RT	BC	Width	Area	Area %
13.283	BB	1.8	1973177	5.02
15.535	BB	2.0	3045234	7.75
15.685	TF	0.0	1653164	4.21
16.312	TF	0.0	13855800	35.25
16.383	VV	0.0	6504165	16.55
18.661	VV	0.0	5061242	12.88
19.813	TF	0.0	2145104	5.46
20.076	VB	0.0	1399923	3.56
21.537	TF	0.0	2322773	5.91
25.918	MV	0.0	1346340	3.43

APPENDIX B - SODIUM BENZOATE CERTIFICATE OF ANALYSIS



SIGMA-ALDRICH

**Certificate of Analysis**

Product Name Sodium benzoate  
Product Number 10,916-9  
Product Brand ALDRICH  
CAS Number 532-32-1  
Molecular Formula  $C_7H_5NaO_2$   
Molecular Weight 144.10

TEST	SPECIFICATION	LOT 09519HA RESULTS
APPEARANCE	WHITE POWDER OR CRYSTALS	WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD
TITRATION	98.5% - 101.5% (WITH HClO <sub>4</sub> )	99.1% (WITH HClO <sub>4</sub> )
HIGH PRESSURE LIQUID CHROMATOGRAPHY	98.5% (MINIMUM)	99.5%
QUALITY CONTROL ACCEPTANCE DATE		JULY 2002

Ronnie J Martin, Supervisor  
Quality Control

## APPENDIX C - MANOMETRIC RESPIROMETRY TEST PROCEDURE

### *Inoculum Preparation*

Fresh activated sludge was obtained on day -1 of the test. Duplicate 10 mL aliquots of the activated sludge were filtered through pre weighed Whatman 934-AH filter pads in a Buchner funnel and vacuum flask set up. The filter pads were placed in an aluminum pan and dried in an oven for sixty five minutes at 103°C. After cooling the filters were reweighed and the mean total suspended solids concentration was determined to be 3.32 g/L. The activated sludge was aerated with carbon dioxide free air prepared by passing through an Ascarite II scrubber for four hours. A 500 mL aliquot of the sludge was then homogenized in a blender for two minutes at low speed. The homogenized sample was allowed to settle for forty minutes, after which the supernatant was decanted (avoiding carry-over of sludge solids). An aliquot of the supernatant was used to determine microbial activity. The microbial activity was determined using an Easicult®-TTC dip slide, Lot No. 123901. The agar stick was removed from the culturing tube and the agar dipped into the supernatant aliquot. Excess supernatant was blotted off with a clean paper towel, and the agar stick was then placed back into the culture tube. The whole unit was placed into a dark environmental chamber for 48 hours at  $20 \pm 1^\circ\text{C}$  monitored by Aquatic Toxicology Data Systems (ATDS). Based on comparison of the density of colonies growing on the agar with the model density chart provided by the supplier, the microbial activity was determined to be  $10^5$  CFU/mL. The remaining decanted sludge supernatant was used for final preparation of the test medium on day -1 (see test medium preparation).

### *Test Medium Preparation*

Twenty liters of glass distilled water was collected in a carboy. A quantity of 460 mL of glass distilled water was removed from the carboy to obtain a final volume of twenty liters after addition of the mineral salt solutions and activated sludge supernatant. The following quantities of mineral salt solutions and inoculum were added to the carboy:

- 1 mL of magnesium sulfate solution per liter of glass distilled water
- 1 mL of calcium chloride solution per liter of glass distilled water
- 10 mL of phosphate buffer solution per liter of glass distilled water
- 1 mL of ferric chloride solution per liter of glass distilled water
- 10 mL of activated sludge supernatant per liter of glass distilled water

The test medium was aerated with carbon dioxide free air for approximately 24 hours before use.

**APPENDIX C - MANOMETRIC RESPIROMETRY TEST PROCEDURE  
(CONT'D)**

*Preparation of the Test Systems*

Test systems were prepared as follows:

Test System 1L Respirometer Flask	Amount of Neat Test Substance MRD-03-768 Added (mg)	Amount of 10237 mg/L Sodium Benzoate Stock Solution Added (mL)	Test Medium (Liter)
Blank	Rep 1	---	1.0
	Rep 2	---	1.0
	Rep 3	---	1.0
Sodium Benzoate	Rep 1	5.0	1.0
	Rep 2	5.0	1.0
	Rep 3	5.0	1.0
MRD-03-768	Rep 1	49.7	1.0
	Rep 2	50.0	1.0
	Rep 3	50.4	1.0

The test substance test systems were dosed by weighing onto a glass fiber filter which was added into a test flask containing one liter of test medium. Each test system was sealed immediately after addition of the test substance to minimize loss due to volatilization. The sodium benzoate systems were dosed by adding 5.0 mL of 10237 mg/L stock solution to the respective test systems. After assembly of the test systems, stirring was initiated, the equipment was checked to ensure no leaks were present, and the oxygen uptake measurements were begun. No further attention was required other than printing the respirometer data and making daily checks during normal working hours to see that the correct temperature ( $22 \pm 1^\circ\text{C}$ ) and adequate stirring were maintained. At the end of 28 days, the pH of the contents in each flask was measured using a Hanna ® pH checker.

## APPENDIX D - CALCULATIONS

### *Theoretical Oxygen Demand (ThOD)*

The empirical formula and the theoretical oxygen demand (ThOD) of the test substance were calculated from elemental analysis data (assuming 100 gram test substance). Sodium benzoate ThOD was calculated using the empirical formula and was determined to be 1.67 mg O<sub>2</sub>/mg sodium benzoate. The ThOD calculation of the test and positive control substance was based on Annex IV of OECD 301F<sup>3</sup> guideline.

TEST SUBSTANCE	% CARBON	% HYDROGEN	% OXYGEN	MOLE OF CARBON	MOLE OF HYDROGEN	MOLE OF OXYGEN	MOL. WT.	ThOD
MRD-03-768	92.16	7.23	0.52	7.67	7.17	0.03	99.83	3.03

Elemental Analysis performed by QTI

The test substance %N value will not be included in ThOD because it is less than 0.05% and will not affect the final ThOD value

MOLE = % ELEMENT (IN GRAMS)/ATOMIC WT OF ELEMENT  
AT. WT OF C = 12.011 G/MOLE  
AT. WT OF H = 1.0079 G/MOLE  
AT. WT OF O = 15.999 G/MOLE

MOL. WT. = (MOLE OF CARBON x AT. WT.)+(MOLE OF HYDROGEN x AT. WT.)+(MOLE OF OXYGEN x AT. WT.)

$$\text{ThOD} = \frac{16 \times ((2 \times \text{mole C}) + (0.5 \times \text{mole H}) - (\text{mole O}))}{\text{Molecular weight}} = \text{mg O}_2/\text{mg test substance}$$

### *Sodium Benzoate Concentration*

The sodium benzoate stock solution concentration was determined to be 10237 mg sodium benzoate/L by dividing the solution total organic carbon (TOC) content (5938 mg carbon /L) by the percent carbon of sodium benzoate (58%). A 5 mL aliquot of the solution added to each test system contained 51.2 mg of sodium benzoate. The pH of the stock solution was 7.23.

#### APPENDIX D - CALCULATIONS (CONT'D)

Percent biodegradation values were calculated by the respirometer software. The following parameters for each system were entered into the software: the ThOD, and the mass of positive control or test substance added. The software incorporates the hourly logged oxygen uptake value along with the entered parameters into the following calculations to derive the percent biodegradation.

$$\text{Constant} = 100 \frac{1}{\text{ThOD} \times \text{mg test substance in vessel}}$$

$$\% \text{Percent Biodegradation} = (\text{mg } O_2 \text{ uptake by test substance} - \bar{X} \text{ mg } O_2 \text{ uptake by blank}) \times \text{constant}$$

The mg of oxygen consumed by the blank represents the average oxygen consumption for the triplicate test systems.

APPENDIX E - PROTOCOL

- PROTOCOL -

OLF-105.0-HPV10-EMBSI

**Study Title:** READY BIODEGRADABILITY: OECD 301F  
Manometric Respirometry Test on Heavy Pyrolysis Fuel Oil

**EMBSI Study Number:** 176894A

**Test Substance:** MRD-03-768

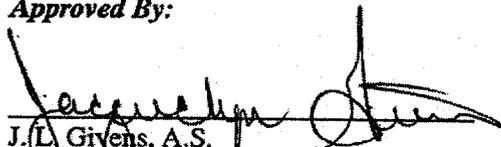
**Date:** August 12, 2003

**Room Number:** LG361

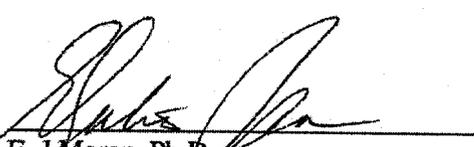
**Proposed Key Dates:**

Experimental Start .....	27 Aug 03
Experimental Termination.....	24 Sep 03
Draft Report Completion.....	29 Oct 03
Final Report Completion .....	28 Jan 04

**Approved By:**

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

15 Sep 03  
Date

  
E. J. Moran, Ph.D.  
Sponsor Representative

9/9/03  
Date

SAFETY FIRST

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### INTRODUCTION

#### *Objective*

This study will be conducted for the Sponsor in order to evaluate the potential of the test substance to biodegrade in an aerobic, aqueous environment for use in environmental hazard assessment.

#### *Sponsor*

American Chemistry Council  
1300 Wilson Boulevard  
Arlington, VA 22209

#### *Testing Facilities*

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

Quantitative Technologies Inc. (QTI)(Elemental Analysis Only)  
P.O. Box 470  
Salem Industrial Park, Bldg 5  
Whitehouse, New Jersey 08888

#### *Compliance*

This study will be performed in general agreement with the test method described in the reference section of the protocol (Reference 1) with the exception of the inoculum preparation which will be performed in general agreement with the inoculum preparation section of Reference 2. Additional exceptions are listed on page 9.

The study will be conducted in compliance with the OECD and USEPA Good Laboratory Practice (GLP) standards (References 3 and 4).

#### *Justification for Selection of Test System*

Selection of the aerobic aquatic biodegradation test is based upon the OECD Guideline (Reference 1). The test method determines biodegradability by measuring oxygen consumption in a test system consisting of an activated sludge supernatant, test substance and a nutrient source. Further, activated sludge has historically been used to evaluate the persistence of chemicals in the environment.

READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### INTRODUCTION (CONT'D)

#### *Justification of Dosing Route*

The test substance could possibly be found in aqueous solution in a wastewater treatment facility.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### MATERIALS AND METHODS

#### *Test Substance Identification*

EMBSI code  
MRD-03-768

Sponsor's Identification  
Industry Stream Name: Heavy Pyrolysis Fuel Oil

CAS Number  
68513-69-9  
64741-62-4  
69013-21-4  
8002-05-9

CAS Inventory Name  
Residues, petroleum, steam-cracked light  
Clarified oils, petroleum, catalytic cracked  
Fuel oil, pyrolysis  
Petroleum

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

#### *Characterization of Test Substance*

The neat test substance will be characterized and the stability determined by the testing facility as described in SOP A.3.4.10 using the following analysis: UltraViolet/Visible and Infrared Spectrophotometry, Gas Chromatography with Mass Selective Detection and Density. The test substance will be used in a number of studies at the testing facility. Characterization of the test substance will be performed at the testing facility prior to its use in the first of these studies. Stability assessment will span the duration of all studies. Documentation of characterization and stability assessment will be maintained at the testing facility and the results appended to the final report.

An aliquot of the test substance will be sent to Quantitative Technologies Inc. (QTI) for elemental analysis, specifically carbon, hydrogen, nitrogen and oxygen. The Carbon, Hydrogen, Nitrogen, and Oxygen will be determined using a Perkin-Elmer 2400 CHN Elemental Analyzer equipped with an oxygen accessory kit. For CHN the analyzer will use combustion to convert the sample elements to simple gases. Upon entering the analyzer, the sample will be combusted in a pure oxygen environment. The product gases will be separated under steady state conditions, and measured as a function of thermal conductivity. For oxygen, pyrolysis will be used to convert the oxygen to carbon monoxide which will be separated from the other pyrolozates under steady state conditions and measured as a function of thermal conductivity. This laboratory is not a GLP compliant facility and therefore may not have performed the analysis in a GLP compliant manner. However, a standard (Canola Oil) will be employed to monitor the quality of the data. If values are outside of the standard limits, the analysis will be repeated.

The methods of synthesis, fabrication, and/or derivation of the test substance has been provided by the sponsor and is maintained at the testing facility. The test substance, as received, will be considered the "pure" substance.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### MATERIALS AND METHODS (CONT'D)

#### *Positive Control Substance*

Substance Identification: Sodium Benzoate  
Manufacturer - Aldrich Chemical Company  
Lot # 09519HA

Storage Conditions: Neat substance will be stored at room temperature.

Documents which detail the stability, identity, solubility, strength, purity and composition or other characteristics which appropriately identify the positive control substance were provided by the manufacturer. The document is a certificate of analysis. Copies of the document will be included in the raw data and final report.

#### *Carrier*

Glass distilled water will be used as the carrier. The glass distilled 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 feed water for the deionized water system is analyzed by Accutest®, 2235 Route 130, Dayton, New Jersey 08810. Results of the water analyses are maintained at the testing facility. There are no known contaminants in the water believed to be present at levels that may interfere with this study. Contaminant analysis of the water is not performed in a GLP compliant manner. This is not believed to have an adverse affect on the study results.

#### *Inoculum*

The inoculum will be fresh activated sludge obtained from the Clinton Sanitary Wastewater Treatment Plant, Annandale, New Jersey. This source has been selected since the treatment facility deals predominantly with domestic sewage as specified in the guideline. There are no known contaminants in the fresh activated sludge believed to be present at levels that may interfere with this study. Fresh activated sludge will be obtained on day -1 of the Manometric Respirometry Test. The total suspended solids (TSS) concentration of the sludge will be confirmed and adjusted, if necessary, prior to use (3-5 g/L). A 10 mL aliquot of the mixed sludge will be filtered through a pre-weighed Whatman 934-AH filter pad in a Buchner filter and vacuum flask set up. The filter pad will be placed in an aluminum pan and dried in an oven set at  $105^{\circ}\text{C} \pm 5^{\circ}\text{C}$  for at least one hour. After cooling, the filter will be reweighed and the TSS determined. This procedure will be performed in duplicate and the average value will be reported.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### MATERIALS AND METHODS (CONT'D)

#### *Inoculum (cont'd)*

The sludge will be aerated for 4 hours with carbon dioxide free air prepared by passing through an Ascarite II scrubber. An aliquot of 500 mL of the mixed liquor will be homogenized for 2 minutes in a blender at low speed. The homogenized sample will be allowed to settle for at least 30 minutes, after which the supernatant will be decanted (avoiding carry-over of sludge solids). Settling time will be dictated by the clarity of the supernatant. An aliquot of the supernatant will be used for final preparation of the test medium (see test medium preparation). An aliquot of the remaining supernatant will be used to determine microbial activity with an Easicult®-TTC dip slide. This will be accomplished by removing the agar stick from the culturing tube, and dipping the agar into the supernatant aliquot. Excess supernatant will be blotted off with a clean paper towel, and the agar stick will then be placed back into the culture tube. The whole unit will be placed into a dark Environmental Chamber for 48 hours at  $20 \pm 1^\circ\text{C}$ . After 48 hours, the whole unit will be observed, and the density of the colonies growing on the medium will be compared to the model density chart provided by the supplier.

#### *Solutions*

Mineral Salt Solutions:	Phosphate buffer pH 7.2
	Ferric chloride (0.025%)
	Magnesium sulfate (2.25%)
	Calcium chloride (2.75%)

The manufacturer and lot number for each solution will be recorded in both the raw data and the report. There are no known contaminants in the solutions believed to be present at levels that may interfere with this study. All solutions will be refrigerated when not in use.

#### *Test Medium*

The test medium will be prepared at least one day before the test begins. One or two carboys (glass or nalgene) will each be filled with sufficient volume of glass distilled water depending on the volume required for the number of test systems being prepared. The following additions of mineral salts will be made to each carboy:

- 1 mL of magnesium sulfate solution per liter of glass distilled water
- 1 mL of calcium chloride solution per liter of glass distilled water
- 10 mL of phosphate buffer solution per liter of glass distilled water
- 1 mL of ferric chloride solution per liter of glass distilled water

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### MATERIALS AND METHODS (CONT'D)

#### *Test Medium (cont'd)*

Final preparation of the test medium is achieved on day -1 by adding 10 mL of the activated sludge supernatant (see preparation as described in the inoculum section) per liter of glass distilled water to each carboy.

**Sodium Benzoate Stock Solution:** A stock solution of sodium benzoate will be prepared in glass distilled water. The stock solution will be refrigerated when not in use. Stock solution concentration will be confirmed by Total Organic Carbon (TOC) analysis as per SOP G.2.8.28. The pH of the stock will be measured, and adjusted to  $7.4 \pm 0.2$  if necessary.

#### *Test System*

The test system will be considered as any combination of the following in a test container:

- Test Substance
- Positive Control Substance
- Mineral Salt Solutions
- Activated Sludge Supernatant
- Glass Distilled Water

All test containers used in this study will be uniquely identified as to appropriate composition, i.e., Blank - 1, 2, 3; Test Substance - 1, 2, 3; etc. More than one test substance may be tested concurrently using the same set of blanks and positive control substance test systems. All glassware will be washed with Chemsolve® glassware cleaner, then rinsed with glass distilled H<sub>2</sub>O to remove any residual organic carbon prior to use. The glassware will be inspected for cleanliness before use. The manometric cells will be rinsed with two portions of acetone, then filled with soapy water and allowed to stand for a few hours. The cells will then be rinsed with glass distilled water and rinsed one more time with acetone then finally air dried.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### EXPERIMENTAL PROCEDURE

#### *Procedure Summary*

The test procedure evaluates the ready biodegradability of a test substance by microorganisms in water. The consumption of oxygen is determined by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved carbon dioxide is absorbed in a solution of potassium hydroxide or another suitable absorbent. The amount of oxygen taken up by the microbial population during biodegradation of the test substance (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of theoretical oxygen demand (ThOD). ThOD will be calculated based on the chemical structure supplied by the sponsor or elemental analysis, performed by QTI, and using the equation found in Reference 1. This test is performed in general agreement with the guideline from Reference 1 with the following clarifications/exceptions:

#### *Clarification*

1. The apparatus is an electrolytic respirometer, manufactured by Co-ordinated Environmental Service, Ltd. (Kent, England). The system is based on a proven oxygen generating process coupled to a sensitive manometric cell. The sample is placed in a sample flask, which is then sealed by a manometric cell/CO<sub>2</sub> trap and immersed in a temperature stabilized water bath. For the duration of the experiment, the sample is stirred by a magnetically coupled stirrer. As the biodegradation process progresses, the microorganisms convert O<sub>2</sub> to CO<sub>2</sub>. The CO<sub>2</sub> is absorbed by the alkali CO<sub>2</sub> trap and causes a net reduction in gas pressure within the sample flask. This pressure reduction is detected by the manometric cell and triggers the electrolytic process. This generates oxygen and restores the pressure in the sample flask. The magnitude of the electrolyzing current and the duration of the current is proportional to the amount of oxygen supplied to the microorganisms.
2. The test and positive control substance will be tested at concentrations of approximately 50 mg/L. Sodium Benzoate will be administered to the respective test systems as an aliquot of an aqueous stock solution. The test substance will be administered by direct addition in a manner that will minimize loss due to volatilization. An aqueous stock solution will not be prepared for the test substance because of the low water solubility. Also no concentration verification will be performed since the test substance is poorly soluble in water.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### EXPERIMENTAL PROCEDURE (CONT'D)

3. The blank, positive control and test substance test systems will be tested in triplicate.
4. Preparing the test medium containing the microorganisms on a large volume basis will minimize bias. In addition the test medium will be aerated for 24 hours to improve homogeneity and ensure random distribution of test organisms to all test systems. The pH of the test medium will be determined and adjusted if it falls outside the range of  $7.4 \pm 0.2$ . The initial pH of individual systems will not be determined due to the poor solubility of the test substance in water.
5. Dissolved organic carbon (DOC) analysis will not be performed due to the poor solubility of the test substance.
6. The test duration (normally 28 days) may be shortened or extended based on the biodegradation curve.

#### *Exceptions*

1. No abiotic sterile or toxicity control systems will be tested.
2. Test medium will be prepared on a large volume basis, aerated and aliquoted into each test container, instead of preparation in the individual test systems.
3. The commercial phosphate buffer, used in preparation of the test medium, has a pH value of 7.2 rather than 7.4. The phosphate buffer, purchased from VWR, has been approved by the American Public Health Association (APHA) for use in the Biological Oxygen Demand (BOD) analysis. The BOD buffer has the same composition as the buffer stated in references 1 and 2.
4. The inoculum will be mixed for 2 minutes in a blender at low speed, instead of medium speed.

#### *Preparation of the Test Systems*

The test systems will be assembled in accordance with manufacturer's directions and labeled as shown below. Test systems will be prepared as indicated in the table below on day 0. The test substance will be weighed, added to the test flask and diluted with test medium in a manner that will minimize loss due to volatilization.

APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
 MRD-03-768, STUDY NUMBER 176894A

EXPERIMENTAL PROCEDURE (CONT'D)

Test System Aerobic Respirometer	Test Substance Concentration	Test Medium
Blank - 1, 2, 3	None	Add 1 liter of test medium
Sodium Benzoate - 1, 2, 3	≈50 mg via stock solution	Add 1 liter of test medium
Test Substance -1, 2, 3	≈ 50 mg of test substance	Add 1 liter of test medium

After addition of test medium and test or positive control substance, the equipment will be assembled. Stirring will be initiated, the equipment will be checked to ensure no leaks are present, and oxygen uptake measurements will begin. No further attention is required other than printing the respirometer data and ensuring that adequate stirring is maintained during normal working hours. The water bath temperature of  $22 \pm 1^\circ\text{C}$  will be monitored by the Aquatic Toxicology Data System (ATDS) but manual temperature measurements may also be recorded. At the end of incubation, normally 28 days, the pH of the contents of the flasks will be measured.

*Calculations*

Percent biodegradation values are calculated by the respirometer software, using the ThOD, and the mass of test substance added. The ThOD will be calculated as specified in Annex IV of OECD 301 F guideline (Reference 1). The software incorporates the hourly logged oxygen uptake value along with the entered parameters into the following calculations to derive the percent biodegradation.

$$\text{Constant} = 100 \frac{1}{\text{ThOD} \times \text{mg test substance in vessel}}$$

$$\text{Percent Biodegradation (\%)} = (\text{mg } O_2 \text{ uptake by test substance} - \text{mean mg } O_2 \text{ uptake by blank}) \times \text{constant}$$

The mg of oxygen consumed by the blank represents the average oxygen consumption for the triplicate blank systems. The mean and standard deviation of the percent biodegradation for each test substance will be calculated in Microsoft® Excel 97. Due to the calculation process of Microsoft® Excel 97 some rounding differences may be noted.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### EXPERIMENTAL PROCEDURE (CONT'D)

#### *Validity of Results*

The test shall be considered valid if the difference of extremes of replicate values reported for the biodegradation of the test substance is less than 20% by day 28 and if the reference substance reaches the pass level of 60% of the theoretical oxygen demand by day 14. If either of these conditions is not met, the test should be repeated.

The oxygen uptake of the inoculum blank is normally 20-30 mg O<sub>2</sub>/L and should not be greater than 60 mg/L in 28 days. Values higher than 60 mg/L require critical examination of the data and experimental technique. If the pH value is outside the range of 6-8.5 and the oxygen consumption by the test substance is less than 60%, the test should be repeated with a lower concentration of the test substance.

#### *Classification of Ready Biodegradability*

A test substance shall be classified as readily biodegradable if the percent biodegradation reaches 60% within 28 days and the 60% degradation has been attained within 10 days of exceeding 10% biodegradation.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### REPORTS

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

1. Signature of the study director, laboratory director and date.
2. Name of the study director and laboratory director.
3. Statements of results as mean percent of biodegradation for the test and positive control substance.
4. Name and address of the testing facility and sponsor.
5. Identification of the test substance.
6. Positive control substance identification, lot number and manufacturer name.
7. The location of storage for neat test substance, raw data, and the final report.
8. Description of reagents and solutions.
9. Key study dates.
10. Inoculum information including location of source, handling, method of biomass determination, total suspended solids (TSS), and microbial concentration (CFU/mL).
11. Objectives and procedures stated in the approved protocol including any changes in the original protocol. A copy of the protocol and protocol amendments.
12. Circumstances, if any, which may have affected the quality or integrity of the data.
13. Description of methods used, including test system description.
14. Amount of each test substance used and method of addition.
15. Incubation temperature range.
16. Description of calculations, summary and analysis of the data, and a graph of the percent biodegradation of each test substance versus time.
17. Q. A. and Compliance Statements.

READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST on HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### 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 the appropriate guidelines, company standard operating procedures (SOPs), and Good Laboratory Practice regulations.

### 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 study 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.

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### REFERENCES

1. Organization for Economic Cooperation and Development (OECD), Guidelines for the Testing of Chemicals, Ready Biodegradability, 301F Manometric Respirometry Test (1992).
2. American Society for Testing and Materials (ASTM) D5864 Standard Test Method for Determining the Aerobic Aquatic Biodegradation of Lubricants and their Components (1995)
3. Organization for Economic Cooperation and Development, Principles of Good Laboratory Practice, 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.

READY BIODEGRADABILITY: OECD 301F MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
STUDY # 176894A, MRD-03-768

## APPENDIX E - PROTOCOL (CONT'D)

READY BIODEGRADABILITY: MANOMETRIC RESPIROMETRY TEST ON HEAVY PYROLYSIS FUEL OIL  
MRD-03-768, STUDY NUMBER 176894A

### PERSONNEL

Study Director.....	J. L. Givens
Sponsor Representative .....	E. Moran
Primary Study Technician.....	B. A. Kelley
Section Head, Laboratory Operations.....	R. L. Rucker
Analytical Chemistry .....	D. J. Letinski
Compound Prep .....	Staff
QA.....	Staff
Contract Administrator .....	B. J. Foster

## Robust Summary Biodegradation

<b>Test Substance:</b>	<p>Industry Stream Name : Heavy Pyrolysis Fuel Oil</p> <table border="0" style="width: 100%;"> <tr> <td style="width: 50%;"><u>CAS Number</u></td> <td style="width: 50%;"><u>CAS Inventory Name</u></td> </tr> <tr> <td>68513-69-9</td> <td>Residue, petroleum, steam-cracked light</td> </tr> <tr> <td>64741-62-4</td> <td>Clarified oils, petroleum, catalytic cracked</td> </tr> <tr> <td>69013-21-4</td> <td>Fuel oil, pyrolysis</td> </tr> <tr> <td>8002-05-9</td> <td>Petroleum</td> </tr> </table> <p>In ethylene plants cracking liquid feedstocks, the cracking furnace effluent (after heat recovery) is quenched by injection of recycled quench oil. This step results in the condensation of higher boiling hydrocarbon compounds that are typically separated from the rest of the furnace effluent as the bottoms of the oil quench tower. Lights are stripped from the excess oils generated from this quench system, resulting in the stream identified here as heavy pyrolysis fuel oil.</p>	<u>CAS Number</u>	<u>CAS Inventory Name</u>	68513-69-9	Residue, petroleum, steam-cracked light	64741-62-4	Clarified oils, petroleum, catalytic cracked	69013-21-4	Fuel oil, pyrolysis	8002-05-9	Petroleum
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<b>Method/Guideline:</b>	OECD Guideline 301F										
<b>Year (guideline):</b>	1992										
<b>Type (test type):</b>	Ready Biodegradability: Manometric Respirometry Test										
<b>GLP (Y/N):</b>	Yes										
<b>Year (study performed):</b>	2003										
<b>Inoculum:</b>	Domestic activated sludge										
<b>Exposure Period:</b>	28 Days										
<b>Test Conditions:</b> <ul style="list-style-type: none"> <li>• <b>Note: Concentration preparation, vessel type, replication, test conditions.</b></li> </ul>	<p>Triplicate test systems were used to evaluate the biodegradability of the test and positive control substances at mean concentrations of 50 mg/L and 51 mg/L, respectively. Blank test systems, which did not contain the test or positive control substance, were run concurrently in triplicate.</p> <p>The total suspended solids (TSS) of the activated sludge was determined to be 3.32 g/L. The inoculum was added at a 1% loading volume of sludge supernatant to test medium. The microbial count of the inoculum was 10<sup>5</sup> CFU/mL. One liter of test medium, which was aerated for 24 hours with carbon dioxide free air, was added to each one liter respirometer flask. The test substance was administered by direct addition on glass fiber filters into the test medium. The test system was sealed immediately after addition of the test substance. An aliquot of the positive control stock solution was added to the appropriate test flasks.</p>										

<p><b>Test Conditions (cont'd):</b></p> <p><b>Note: Concentration preparation, vessel type, replication, test conditions.</b></p>	<p>An unacclimated activated sludge inoculum was used in this study. The inoculum was obtained from the Clinton Sanitary Wastewater Treatment Plant, Annandale, NJ, USA. The treatment plant receives domestic sewage.</p> <p>All test systems were placed on a Coordinated Environmental Services (CES) automated respirometer which automatically recorded the oxygen uptake in general agreement with the OECD guideline. The 28-day study was conducted at a temperature range of <math>22 \pm 1^\circ\text{C}</math>.</p>									
<p><b>Results:</b></p> <p><b>Units/Value:</b></p> <p><b>Note: Deviations from protocol or guideline analytical method.</b></p>	<p>Biodegradation was based on oxygen consumption and the theoretical oxygen demand of the test substance as calculated using results of an elemental analysis of the test substance.</p> <p>By day 14, &gt;60% biodegradation of positive control was observed, which meets the guideline requirement. No deviations from the protocol occurred that affected the integrity of the study data.</p> <p>The test substance biodegraded to 29% and cannot be considered readily biodegradable.</p> <table border="1" data-bbox="777 961 1536 1087"> <thead> <tr> <th><u>Sample</u></th> <th><u>% Degradation*</u> <u>(day 28)</u></th> <th><u>Mean % Degradation</u> <u>(day 28)</u></th> </tr> </thead> <tbody> <tr> <td>Test Substance</td> <td>33, 31, 22</td> <td>29</td> </tr> <tr> <td>Na Benzoate</td> <td>91, 87, 89</td> <td>89</td> </tr> </tbody> </table> <p>* replicate data</p>	<u>Sample</u>	<u>% Degradation*</u> <u>(day 28)</u>	<u>Mean % Degradation</u> <u>(day 28)</u>	Test Substance	33, 31, 22	29	Na Benzoate	91, 87, 89	89
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<p><b>Conclusion:</b></p>	<p>Not readily biodegradable</p>									
<p><b>Reliability:</b></p>	<p>(1)-Reliable without restriction.</p>									
<p><b>Reference:</b></p>	<p>ExxonMobil Biomedical Sciences, Inc. 2003. Ready Biodegradability: Manometric Respirometry test. Study # 176894A</p>									
<p><b>Other (source): (FT - SO)</b></p>	<p>Olefins Panel, American Chemistry Council</p>									