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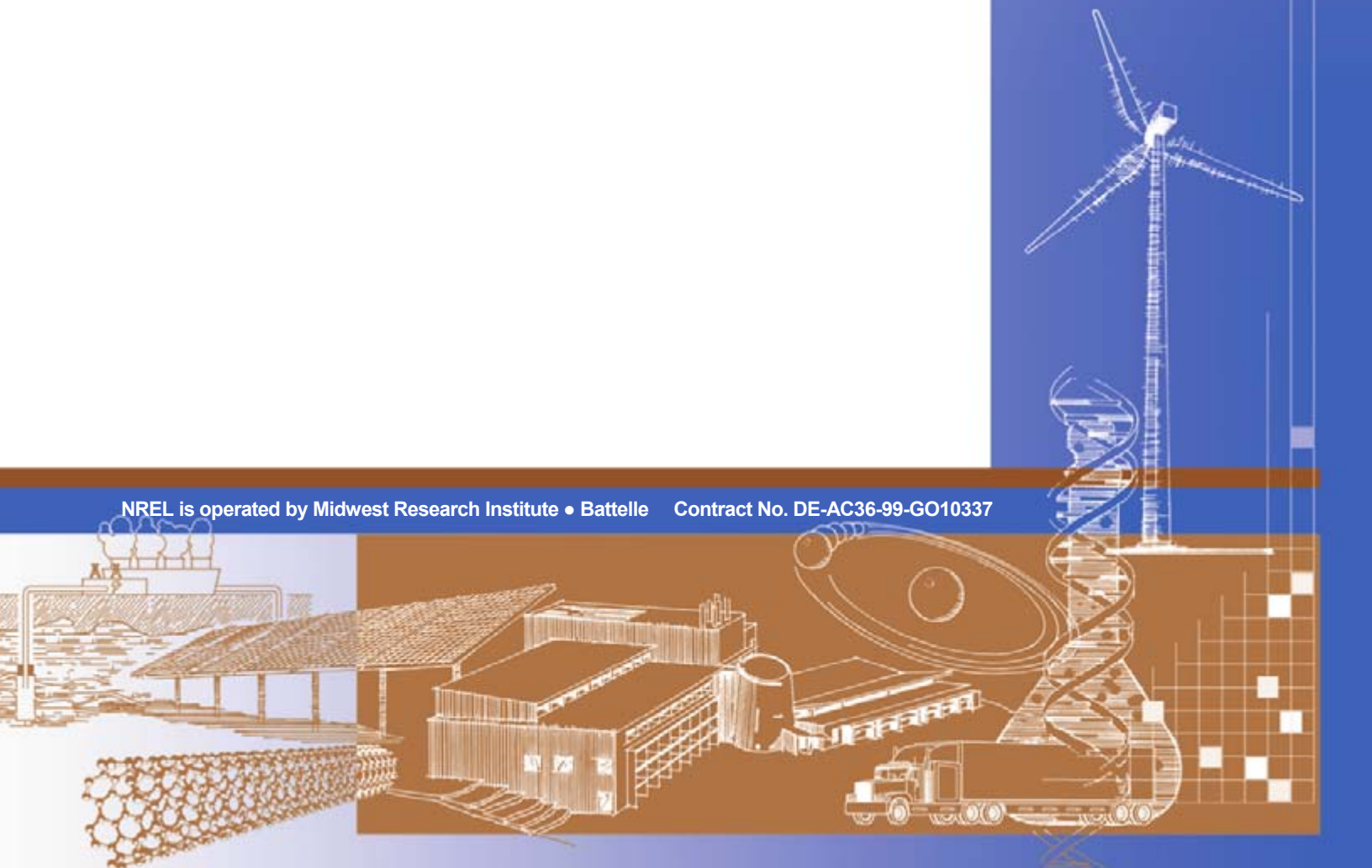
Analysis of Coconut-Derived Biodiesel and Conventional Diesel Fuel Samples from the Philippines

Task 2 Final Report

T.L. Alleman and R.L. McCormick

Milestone Report
NREL/MP-540-38643
January 2006



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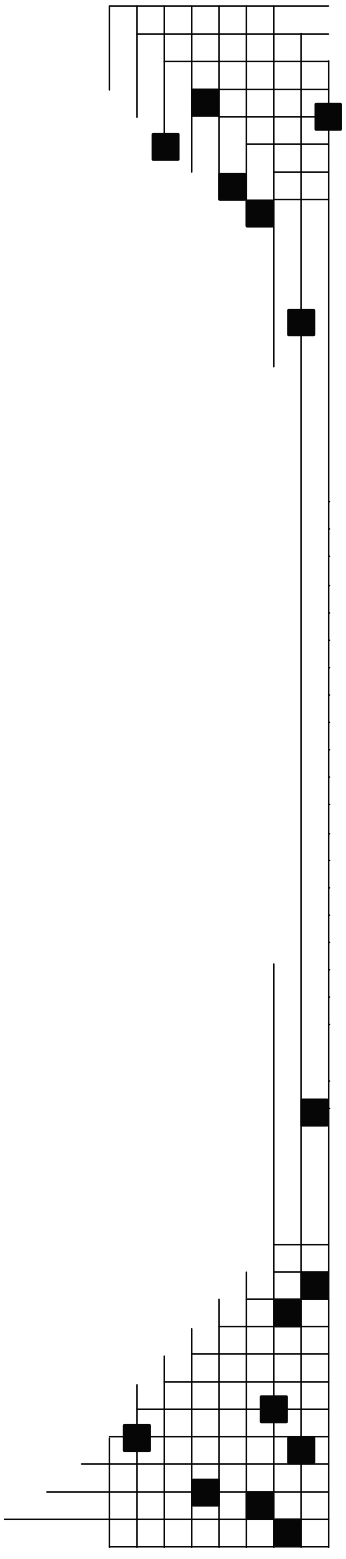

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List of Abbreviations

AET	Atmospheric Equivalent Temperature
AOCS	American Oil Chemists Society
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
ATR	Attenuated Total Reflectance
ºC	degrees Celsius
cm^{-1}	wavenumber
CME	coconut methyl ester
DA	additized diesel fuel
DUA	unadditized diesel fuel
FTIR	Fourier Transform infrared
g	gram
hr	hour
IQT	Ignition Quality Tester™
mass%	percent by mass
mg KOH/g	milligrams of Potassium Hydroxide per gram
mg/100ml	milligrams per 100 milliliters
mm^2/s	millimeters squared per second
NREL	National Renewable Energy Laboratory
PNS	Philippine National Standard
ppm	parts per million
USDOE	U.S. Department of Energy
vol%	percent by volume

Executive Summary

NREL has provided technical assistance to the Philippines in the area of biodiesel property testing and utilization. Three fuel samples were provided to NREL from the Philippines. These fuel samples were an unadditized diesel fuel, an additized diesel fuel, and a coconut derived biodiesel (coconut methyl ester or CME) fuel. Each diesel fuel sample was analyzed neat and as a blend with CME. The CME-diesel fuel blend samples were 1 volume% and 5 volume% CME in the additized and unadditized diesel fuels. Fuel property testing was also performed on the neat CME sample.

Results from the fuel property testing show that the unadditized diesel fuel and the additized diesel fuel samples met the Philippine National Standards for diesel fuel quality. Results from the fuel property testing for the CME sample shows that the current fuel quality standards were met. The 1% and 5% blends of CME in the diesel fuels also met the current Philippine National Standard for diesel fuel quality. The 5% blend of CME in diesel fuel did increase the cetane number slightly for each blend.

The water separability was gauged to determine if the diesel fuels or the CME-diesel fuel blends mixed with salt water. Test results show that the diesel fuels and the CME-diesel blends did not take up significant amounts of water, nor were stable emulsions formed for any of the fuels or fuel blends tested.

The stability of CME and CME-diesel fuel blends was determined through several test methods. In all cases, the CME sample, the diesel fuel samples, and the 5% CME-diesel blends exhibited a similar level of stability. The test results showed few insolubles were generated during the tests, which under storage conditions, may contribute to poor engine performance due to plugged fuel filters or clogged injectors.

Sixteen indicators of microbial degradation were measured over the additized and unadditized diesel fuel samples and the CME fuel sample. The results from these sixteen indicators were somewhat equivocal, but show that the CME sample and the neat diesel fuel samples have similar resistance to microbial degradation, although the mechanisms for degradation may vary.

A Fourier Transform infrared technique for determining the percentage of biodiesel in a blend was demonstrated on CME-diesel fuel blend samples. The technique was highly linear and can be used to quantitatively determine the percentage of CME in a diesel fuel sample.

Background

Biodiesel is a renewable diesel fuel produced from feedstocks like vegetable oils or animal fats. To produce biodiesel, the starting oil is reacted with alcohol in the presence of a catalyst. The products of these reactions are the fatty acid methyl esters that compose biodiesel.

Because the Philippines imports a majority of its petroleum, the Philippine government has begun to explore domestically produced biodiesel to promote energy security. The Philippine government has recently directed all government offices to use 1% biodiesel in diesel fuel where the biodiesel is produced from domestically grown coconuts. The coconut-derived biodiesel (CME or coconut methyl ester) will aid in the reduction of petroleum imports and improve fuel lubricity and may reduce exhaust emissions.

The U.S. Department of Energy's (USDOE) National Renewable Energy Laboratory (NREL) is a world leader in the effort to develop and advance renewable energy and improve energy efficiency. NREL's fuels research activities include fuels utilization impacts and the impact of fuel properties on engine and vehicle performance and emissions.

NREL has provided technical assistance to the Philippines in the area of biodiesel property testing. This report covers fuel property testing results for coconut-derived biodiesel, conventional diesel fuel samples from the Philippines, as well as 1% and 5% blends of CME in the conventional diesel fuels.

Test Matrix

Three fuel samples were provided to NREL from the Philippines. These fuel samples were an unadditized diesel fuel (DUA), an additized diesel fuel (DA), and a CME fuel. Each diesel fuel sample was analyzed neat and as a blend with CME (1 volume% and 5 volume% CME in diesel fuel). Fuel property testing was also performed on the neat CME sample. Table 1, which follows, lists the tests performed for each fuel and fuel blend. In addition, samples of CME in DA fuel were examined using an existing method for determining the percentage of biodiesel.

Table 1. Fuel Property Tests for Philippine Diesel Fuel and CME Samples.
An "X" indicates that the test was performed.

Fuel	ASTM D975	ASTM D6751	ASTM D4625	EN 14112	ASTM D6468	ASTM D2274	ASTM D1401	Modified ASTM E1259
	Standard Specification for Diesel Fuels	Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels	Standard Test Method for Distillate Fuel Storage Stability at 43ºC	Fatty Acid Methyl Esters (FAME) Determination of Oxidation Stability (Accelerated Oxidation Test)	Standard Test Method for High Temperature Stability of Distillate Fuels	Standard Test Method for Oxidation Stability of Distillate Fuel Oil (Accelerated Method)	Standard Test Method for Water Separability of Petroleum Oils and Synthetic Fluids	Standard Test Method for Evaluation of Anti-Microbials in Liquid Fuels Boiling Below 390ºC
DA	X		X	X	X	X	X	X
DUA	X		X	X	X	X	X	X
CME		X	X	X	X	X	X	X
CME + microbicide								X
1%CME in DA	X		X	X	X		X	
1% CME in DUA	X		X	X	X		X	
5% CME in DA	X		X	X	X	X	X	
5% CME in DUA	X		X	X	X	X	X	

Results and Discussion

Fuel Properties

The fuel property results from the CME sample are given in Table 2, along with the current Philippine National Standard (PNS2020:2003) and comparable results from a soy-derived biodiesel in the United States.

Table 2. Physicochemical Property Results from CME Testing.

Property	Method	CME Results	PNS2020:2003	Soy Biodiesel[1]
Flash Point, ºC	ASTM D93	107	100.0, min	157
Water & Sediment, vol%	ASTM D2709	0.0	0.050, max	<0.05
Kinematic Viscosity, mm^2/s @ 40ºC	ASTM D445	2.656	2.0-4.5	4.2
Sulfated Ash, mass%	ASTM D874	0.002	0.020	0.002
Sulfur, ppm	ASTM D5453	3	50, max	9
Copper Corrosion, 3hr @50ºC	ASTM D130	1A	No. 3, max	1A
Cetane Number	ASTM D613	70	42, min	55
Cloud Point, ºC	ASTM D2500	-5	Report	0.4
Carbon Residue, mass%	ASTM D4530	N/A**	0.050, max	Not Performed
	ASTM D524	<0.010	N/A	0.02
Acid Number, mg KOH/g	ASTM D664	0.17	0.50, max	0.35
Free Glycerin, mass%	ASTM D6584	N/A	No Standard	0.006
Monoglyceride, mass%	ASTM D6584	N/A	N/A	0.40
Diglyceride, mass%	ASTM D6584	N/A	N/A	0.21
Triglyceride, mass%	ASTM D6584	N/A	N/A	0.18
Total Glycerin, mass%	ASTM D6584	0.043	N/A	0.16
Free Glycerin, mass%	AOCS Ea6-94*	0.02	0.02, max	Not Used
Total Glycerin, mass%	AOCS Ca14-56	0.145	0.24, max	Not Used
Phosphorus, mass%	ASTM D4951	0.000	0.001, max	0.0006
Distillation, AET 90% recovered, ºC	ASTM D1160	327	360, max	352

*PNS2020:2003 references AOCS Ea6-51, which has been replaced by AOCS Ea6-94
**Not applicable

As shown in Table 2, the CME sample tested in this study met all the PNS 2020:2003 requirements. The carbon residue was tested using ASTM D524, rather than ASTM D4530. The ASTM guidelines allow for substitution of ASTM D524 in place of ASTM D4530 for carbon

residue determination. Using the correlation provided in ASTM D524, the results for carbon residue are equivalent to those obtained for ASTM D4530 for these low levels of carbon residue.

The results from the diesel fuel property testing are given in Table 3. The results from the DA and DUA fuel samples are given along with the PNS/DOE QS 004:2003, where applicable. As with the CME sample, the DUA and DA fuel samples met the Philippine fuel quality standards.

The cetane number was measured through ASTM D6890 using an ignition quality tester or IQT™. The IQT measures cetane number through combustion techniques and produces results comparable to the ASTM D613 cetane engine.[2] The cetane results from the IQT measurement were above the minimum standard for both DA and DUA samples.

At the request of Philippine stakeholders, the separability of diesel fuel and water was tested due to the practice in the Philippines of pushing diesel fuels through the pipeline with water. The test measures how known volumes of diesel fuel and water separate after mixing for 5 minutes at elevated temperatures. The results from this testing show that miscibility is low and very little water mixes with the diesel fuel sample during testing.

Table 3. Physicochemical Property Results from Additized and Unadditized Diesel Fuels.

Property	Method	DA	DUA	PNS/DOE QS 004:2003 for On-Road Vehicles	U.S. No.2 Diesel Fuel Sample
Kinematic Viscosity, mm^2/s @40ºC	ASTM D445	3.570	3.608	2.0-4.5	2.622
Flash Point, ºC	ASTM D93	85	79	55.0, min	63
Distillation, ºC	ASTM D86	N/A*	N/A	N/A	N/A
IBP		192.4	183.3	N/A	170.7
T10		218.2	214.9	N/A	208.6
T50		280.9	281.1	N/A	263
T90		355.8	355.2	370, max	318.2
FBP		389.2	386.7	N/A	347.1
Sulfated Ash, mass%	ASTM D482	<0.001	<0.001	No Standard	<0.001
Sulfur, ppm	ASTM D2622	382	355	500, max	388
Cloud Point, ºC	ASTM D2500	3	4	No Standard	-22
Water & Sediment, vol%	ASTM D2709	0.01	0.01	0.10, max	0.01
Copper Corrosion	ASTM D130	1B	1B	No Standard	1B
Cetane Number	ASTM D613	Not performed	Not performed	50, min	44.5
	ASTM D6890	55	56	No Standard	No Standard
Carbon Residue, 10% Bottoms, mass%	ASTM D524	0.08	0.09	0.15, max	0.11
Water Separability, Saline Water, 54ºC	ASTM D1401	N/A	N/A	No Standard	N/A
Oil Layer Appearance		Cloudy	Cloudy	N/A	N/A
Emulsion Appearance		N/A	N/A	N/A	N/A
Water Layer Appearance		Clear	Clear	N/A	N/A
Test Duration		5 minutes	5 minutes	N/A	N/A
Volume of Layers		40-40-0	40-40-0	N/A	N/A
Oil/Emulsion Interface		N/A	N/A	N/A	N/A
Water/Emulsion Interface		N/A	N/A	N/A	N/A

* Not applicable

The results for the CME-diesel fuel blends are presented in Table 4. CME was blended in the DA and DUA fuels at the 1 volume% and 5 volume% levels. The diesel fuel properties were not altered significantly by blending with 1% CME. A slight increase in the cetane number was noted with the 5% blends of CME. Both blends continued to meet the PNS/DOE QS 004:2003 standards.

The ASTM D1401 results show that little water is taken up by the fuel blends. A slight volume increase was recorded in the aqueous layer of the 1% CME in DA, with a lacy (bubbles present) appearance, although no emulsion layer was recorded. In contrast, no volume change in the layers was recorded for the 1% CME in DUA sample, although the aqueous layer was hazy (translucent). For both the 5% CME blends, no changes were noted in either the appearance of the layers or in the volumes of each layer.

Table 4. Physicochemical Property Results from Blends of CME and Unadditized and Additized Diesel Fuels.

Property	Method	1%CME in Additized Diesel Fuel	1%CME in Unadditized Diesel Fuel	5%CME in Additized Diesel Fuel	5%CME in Unadditized Diesel Fuel
Kinematic Viscosity, mm^2/s @40ºC	ASTM D445	3.548	3.535	3.494	3.503
Flash Point, ºC	ASTM D93	86	78	86	78
Distillation, ºC	ASTM D86				
IBP		199.8	185.4	190.2	182.3
T10		221.6	213.2	211.9	219.4
T50		282.7	278.5	276.9	280.9
T90		356.7	353.1	349.9	354.9
FBP		391.5	387.9	388.5	387.9
Sulfated Ash, mass%	ASTM D482	<0.001	<0.001	<0.001	<0.001
Sulfur, ppm	ASTM D2622	Not Requested	Not Requested	366	350
Cloud Point, ºC	ASTM D2500	4	5	4	4
Water & Sediment, vol%	ASTM D2709	0.01	0.01	0.01	0.01
High Temperature Stability, 180 minutes, Avg. % Reflectance	ASTM D6468	90	98	96	98
Copper Corrosion	ASTM D130	1B	1B	1B	1B
Cetane Number	ASTM D6890	55	55	58	57
Carbon Residue, 10% Bottoms, mass%	ASTM D524	0.09	0.10	0.08	0.08
Water Separability, Saline Water, 54ºC	ASTM D1401				
Oil Layer Appearance		Cloudy	Cloudy	Cloudy	Cloudy
Emulsion Appearance		N/A*	N/A	N/A	N/A
Water Layer Appearance		Lacy	Hazy	Clear	Clear
Test Duration		5 minutes	5 minutes	5 minutes	5 minutes
Volume of Layers		41-39-0	40-40-0	40-40-0	40-40-0
Oil/Emulsion Interface		N/A	N/A	N/A	N/A
Water/Emulsion Interface		N/A	N/A	N/A	N/A

*Not Applicable

Stability

Fuel stability, while not part of current fuel property standards in the United States, is nevertheless important so tests were performed to understand how CME blending affects fuel property changes during storage. Fuel stability testing was performed using accelerated methods to approximate field aging. The methods use varying degrees of acceleration, from mild to severe. Table 5 shows the stability results from all the fuels and fuel blends.

All the neat fuel and fuel blend samples exceeded 8 hours on the EN14112 oxidation stability test. The European standard (EN14214) for biodiesel (B100) on this test is 6 hours minimum. There is currently no oxidation stability standard for biodiesel in the United States.

The neat fuels and the 5% blends of CME in the DA and DUA fuel samples were tested using the ASTM D2274 and D6468 methods for accelerated and high temperature stability, respectively. The 1% blends of CME in the diesel fuels were not tested because the 5% CME blend represented the most extreme case in terms of stability. The neat fuels and the 5% CME blends had very high percent reflectance on the D6468 test, thus indicating few solids were present in the fuels at the conclusion of the test. The insolubles for D2274 were lowest for the neat diesel fuels. The highest insoluble result was recorded with the neat CME fuel sample. Although the CME sample results were an order of magnitude greater than the neat diesel fuels (1.55 mg/100mL compared to 0.12 mg/100mL), the absolute level was low. For example, a typical pipeline specification for diesel fuel in the U.S. is 2.5mg/100 mL maximum for the ASTM D2274 test. All fuels tested in this study produced deposits well below this level. The influence of the CME sample on the blend results is evident, with the results increasing to 0.63 mg/100mL and 0.54 mg/100mL for the DA and DUA blends, respectively.

Storage stability testing was conducted using ASTM D4625 for the DUA and DA fuel samples, the CME sample, and a blend of 5% CME in the DUA fuel. In this method, the fuel storage stability is determined using mildly accelerated conditions (43ºC). For petroleum fuels, there is an excellent correlation between D4625 and performance of fuels in storage. One week on the D4625 test approximates one month of storage in an underground tank. At specific intervals during testing, the insolubles in the sample are measured and classified as either filterable or adherent. Figure 1 illustrates how the insoluble levels varied over the test period (the data are in Table 6).

The DA and DUA fuel samples had very low levels of insolubles over the test period. The variability observed in these levels is likely due to the very low levels measured. In contrast, the CME sample recorded relatively higher levels of insolubles over the test period. These levels are still quite low and suggest that storage of neat CME or CME blend samples for periods up to 12 months is feasible. However if B100 CME is to be stored for 12 months, the inclusion of an antioxidant additive may be prudent. Again, the influence of the CME sample on the blend is evident in the results. The insolubles measured over the test period are increased relative to the base diesel fuels, although are still very low.

Table 5. Fuel Stability Test Results.

Property	Method	CME	DUA	DA	1% CME in DA	1%CME in DUA	5% CME in DA	5% CME in DUA
Oxidative Stability	EN14112							
Time		Exceeded 8 hours	Exceeded 8 hours	Exceeded 8 hours	Exceeded 8 hours	Exceeded 8 hours	Exceeded 8 hours	Exceeded 8 hours
Temperature		110ºC	110ºC	110ºC	110ºC	110ºC	110ºC	110ºC
High Temperature Stability,180 minutes, Avg % Reflectance	ASTM D6468	100	97	94	Not tested	Not tested	96	98
Accelerated Stability, mg/100mL	ASTM D2274	1.55	0.12	0.12	Not tested	Not tested	0.63	0.54

Table 6. ASTM D4625 Fuel Stability Results.

	5% CME in DUA (Sample 1)	*5% CME in DUA (Sample 2)*	*DA (Sample 1)*	*DA (Sample 2)*	*DUA (Sample 1)*	*DUA (Sample 2)*	*CME (Sample 1)*	*CME (Sample 2)*
Week 0								
Filterable	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.91
Adherent	0.03	0.06	0.03	0.03	0.11	0.03	0.03	0.03
Total	0.03	0.23	0.03	0.03	0.11	0.03	0.03	0.94
Week 4								
Filterable	0.11	0.00	0.00	0.03	0.00	0.14	0.09	0.09
Adherent	0.00	0.09	0.00	0.06	0.09	0.00	0.03	0.26
Total	0.11	0.09	0.00	0.09	0.09	0.14	0.12	0.35
Week 8								
Filterable	0.26	0.26	0.03	0.09	0.03	0.17	1.89	2.14
Adherent	0.06	0.14	0.17	0.14	0.00	0.11	0.00	0.00
Total	0.32	0.40	0.20	0.23	0.03	0.28	1.89	2.14
Week 12								
Filterable	0.26	0.23	0.00	0.00	0.00	0.00	2.86	2.26
Adherent	0.03	0.17	0.17	0.11	0.14	0.11	0.20	0.06
Total	0.29	0.40	0.17	0.11	0.14	0.11	3.06	2.32

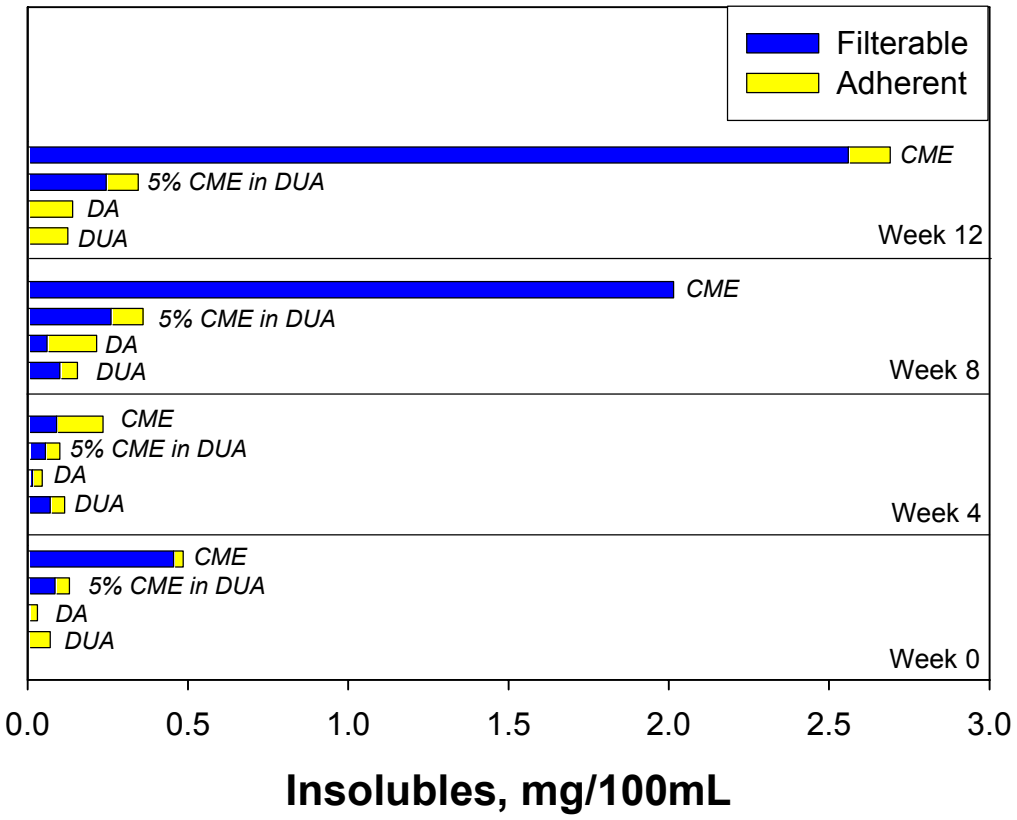

Figure 1. ASTM D4625 Results for Philippine Diesel Fuels, CME, and CME-Diesel Fuel Blends.

Microbial Degradation

The susceptibility to microbial degradation was tested through ASTM E1259. Sixteen indicators for biodegradability were examined for the neat diesel fuel and the neat CME samples. The null hypothesis for this testing was that CME and conventional diesel fuel samples do not differ significantly for biodeterioration risk. Table 7 lists each of the indicators and whether the null hypothesis is supported or refuted. The results show that the microbial degradation of neat CME sample is likely equivalent to that of conventional diesel fuel sample, but may occur by different mechanisms. The complete biodegradation report is attached as Appendix 1.

Experience in the United States and other countries had shown that microbial degradation results for neat fuels should not be extrapolated to blended fuels. Because of this, the results presented here for the neat CME, DA, and DUA fuel samples may not be representative of the results for CME blend samples. Additional testing is required to fully understand the influence of low levels of CME in diesel fuel samples on biodeterioration susceptibility. However, it is not anticipated that blending of CME into diesel fuel at 5 volume% or lower will significantly impact biodeterioration susceptibility.

Table 7. Results from Indicators for ASTM E1259 Biodegradability for CME Compared to Conventional Diesel Fuel.

Indicator	Support or Refute Null Hypothesis	Fuel More Susceptible to Microbial Degradation
Gross Appearance	Supports	Equivalent
Fuel Chemistry		
Entrained Water	Refutes	CME
Total Acid Number	Refutes	CME
Corrosivity	Refutes	CME
Bottom Water Chemistry		
pH	Refutes	Diesel Fuel
Alkalinity/Acidity	Refutes	Diesel Fuel
Hardness	Refutes	Diesel Fuel
Total Dissolved Solids	Supports	Equivalent
Total Organic Carbon	Supports	Equivalent
Bottom Water Microbiology		
Adenosine Triphosphate (ATP)	Refutes	Diesel Fuel
Oxygen Demand	Refutes	Diesel Fuel
Culturable Bacteria	Refutes	Diesel Fuel
Culturable Fungi	Refutes	Diesel Fuel
Fuel Microbiology		
ATP	Equivocal	
Culturable Bacteria	Refutes	Diesel Fuel
Culturable Fungi	Refutes	Diesel Fuel

Blend Level Determination

To determine the percentage of biodiesel in a sample of diesel fuel, a Fourier Transform infrared (FTIR) method has been applied. The method utilizes an Attenuated Thermal Reflectance (ATR) liquid cell and approximately 2 mL of sample. The spectra were collected from 48 scans together at a slow mirror speed. Spectra were taken from 2,000 cm^{-1} to 600 cm^{-1} and software baseline corrected (Nicolet supplied software) to bring the baseline to zero at 2,000 cm^{-1}. The peak at 1,745 cm^{-1} was used to determine the percent biodiesel in the sample. A small absorbance was noted from diesel fuel at this intensity and was subtracted from the peak intensity for the biodiesel blends. This technique is currently being discussed as an ASTM method to be included in future ASTM specifications for biodiesel blends. A copy of the method is in Appendix 2.

Multiple blends were produced using the CME in a typical US diesel fuel meeting the 500 ppm sulfur standard. A calibration curve was produced using blends with varying percentages of CME in diesel fuel. A Beer's law fit of the curve had a very high correlation coefficient (>0.99) and is illustrated in Figure 2. The robustness of the technique will allow for quantitative determination of unknown concentrations of biodiesel in diesel fuel.

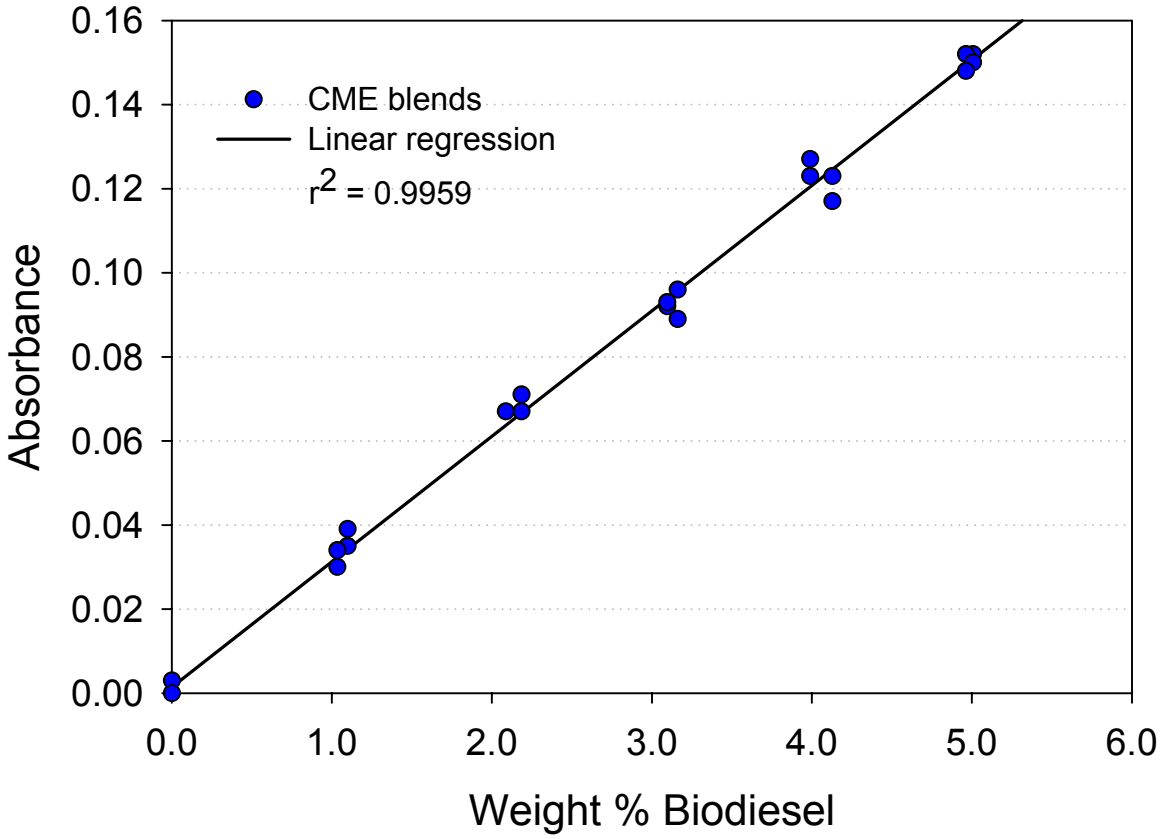

Figure 2. Calibration Curve of CME-Diesel Fuel Blends as Measured by FTIR Spectroscopy.

Summary and Recommendations

Fuel property, stability, and microbial deterioration tests were conducted on neat CME, neat DA and DUA fuel samples, and 1% and 5% blends of CME in each diesel fuel. Results from the fuel property testing for these samples show that the current fuel quality standards were met.

Fuel stability testing revealed that all the samples tested had adequate storage stability. The microbial deterioration testing proved more complex to interpret. The results show that CME and the DA and DUA samples have similar susceptibility to microbial degradation, although the mechanisms vary. In order to fully assess the microbial susceptibility of the biodiesel blends likely to be used in the Philippines, additional testing on blended fuels with several replicates is recommended.

The Philippines uses salt water to push fuels through their pipeline system. The tendency of fuels to take up water was examined for the neat diesel fuels and the 1% and 5% blends of CME in diesel fuel. The tests showed little miscibility of the fuel samples with water. FTIR testing of CME-diesel fuel blends showed the technique is applicable to coconut-derived biodiesel and could be used for quantitative determination of biodiesel percentage in blends.

References

1. "Survey of the Quality and Stability of Biodiesel and Biodiesel Blends in the United States in 2004", NREL-TP-540-38836, http://www.nrel.gov/docs/fy06osti/38836.pdf, October 2005.
2. Diesel Fuel Ignition Quality Tester, online, http://www.aet.ca/IQT_Info_Brochure.pdf, Accessed August 2005.

Appendix 1.

Subcontractor Report on Microbial Degradation

NREL SUBCONTRACT NO. ADK-5-55501-01
EVALUATION OF BIODIESEL FUEL BIODETERIORATION SUSCEPTIBILITY

FINAL REPORT

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Executive Summary

This study was undertaken in order to evaluate the biodegradability of 100% coconut methyl ester (CME-100) relative to conventional, non additized low sulfur diesel (NALSD) and additized low sulfur diesel (ALSD) fuels. The null hypothesis was:

> CME-100 biodegradation risk is not significantly different from conventional LSD biodeterioration risk when products are stored under typical above ground storage tank conditions for up to three months.

Five microcosm test systems were set up; each containing 2 L fuel over 500 mL synthetic bottom-water. The microcosms were designated as follows: A) NALSD; B) ALSD; C) CME-100; D) microbicide-treated CME-100; and E) filter sterilized CME-100 over sterile bottom-water (sterile control). The microcosm exposure period was 90-days. At T_0, microcosms A, B, C and D were inoculated with an uncharacterized mixed culture that had been preconditioned to grow in fuel over water. Microcosms were incubated in the dark at 23 ± 0.5 °C.

Gross observations were made on all microcosms at T_0 and monthly thereafter. Fuel and bottom-water samples were collected from microcosms A & E for chemical testing at T_0. Microcosm A and E bottom-water adenosine triphosphate (ATP) concentration was tested monthly. All fuel and bottom-water tests were run on all microcosms at $T_{3\text{-months}}$.

The results of this study were somewhat equivocal, but generally did not support the null hypothesis. Sixteen parameters were used to compare CME-100 biodegradability against both additized and non-additized LSD. Data for six of the 16 parameters supported the null hypothesis. Although CME-100 was more hazy than LSD at $T_{1\text{-month}}$ and $T_{2\text{ months}}$, the CME-100 could not be differentiated from the ALSD or NALSD on the basis of gross appearance, corrosivity; bottom-water alkalinity, TDS, TOC, or O.D.; or fuel-phase ATP concentration at $T_{3\text{-months}}$. Karl Fischer water, TAN, bottom-water pH, and bottom-water hardness data indicated that CME-100 was significantly more susceptible than LSD to biodeterioration. Fuel and bottom-water ATP and culturable bacteria and fungi data indicated that CME-100 was less likely than LSD to support culturable microbes. However, the ~ 5 Log CFU bacteria/mL recoveries from CME-100 bottom-water made microcosms C, D, and E high biodeterioration risk systems.

The overall results suggest that CME-100 and LSD have comparable but somewhat different biodeterioration susceptibilities. CME-100 may be less susceptible than LSD to biodeterioration.

Background

NREL is working in cooperation with the Philippines biodiesel commercial development effort. As part of that effort, stakeholders are interested in understanding how CME-100 susceptibility to biodeterioration compares with that of conventional diesel fuel.

The current draft proposed revision to ASTM E1259, *Practice for Evaluation of Antimicrobials in Liquid Fuels Boiling Below 390°C*, provides a protocol for investigating both product biodegradability and microbicide performance in laboratory scale fuel systems (microcosms).

BCA originally proposed a testing program that included replicate microcosms, incubated for a period of six-months. The proposed test parameter list included carbon-number distribution and distillation profile; two parameters that reflect substantial fuel chemistry changes. Through a series of teleconference discussions, the final testing program as reported in this document was defined. The three-month test duration reflected stakeholder estimates of the maximum storage period for CME-100. Cost considerations necessitated the elimination of carbon-number distribution and distillation profiles from the test battery. Additionally, cost considerations resulted in the designation of microcosms A & E as surrogates (most and least biodegraded) for the other three microcosms for microbiological tests run a T_0, $T_{1\text{-month}}$, and $T_{2\text{-months}}$. These test design compromises were made with the mutual understanding of all stakeholders that the resulting data matrix, though somewhat limited, would probably be sufficient to test the study's primary hypothesis:

> **Hypothesis:** CME-100 biodegradation risk is not significantly different from conventional LSD biodeterioration risk when products are stored under typical above ground storage tank conditions for up to three months.

The final test plan was not designed to provide data that would support detailed explanations for differences in the relative biodeterioration of CME-100 and LSD in the test microcosms.

Materials and methods

Facilities

All microcosms were assembled and maintained at EMSL Analytical, Inc., 107 Haddon Avenue, Westmont, NJ 08108. Fuel chemistry tests were performed by Clark Laboratories, LLC, 4000 Tech Center Drive, Monroeville, PA 15146.

Challenge Population

Stage 1 Microcosms

Triplicate *Stage 1* microcosms were prepared by dispensing 100 mL synthetic bottom-water (SBW) into 1.0 L *Mason* jars and dispensing 500 mL NALSD[1] on top of the bottom-water. *Bacto Minimal Broth Davis without Dextrose* (Becton-Dickinson, Baltimore, MD) – 0.1 % w/v in deionized water (10.6 g/L) – was used as synthetic bottom-water. The SBW was sterilized by filtering it through a 0.2 µm cellulose acetate filter.

Each *Stage 1* microcosm was inoculated with 1 mL of uncharacterized contaminated bottom-water that had been collected from fuel storage tanks. Contaminated bottom-water samples were provided by Fuel Quality Services, Inc., Flowery Branch, GA. After inoculation, microcosms were incubated in the dark at 25 ± 2 °C for three weeks. Microcosms were then tested for culturable bacteria and fungal counts Each *Stage 1* microcosm was reinoculated with an additional 1 mL of contaminated bottom-water and with 10 µL of a suspension of fungal biomass prepared from fungal colonies that had grown on the media used to enumerate microcosm fungi (see below).

Stage 1 microcosms were incubated for an additional two-weeks then examined visually and tested for ATP concentration. A second round of *Stage 1* microcosms was prepared. Each of the triplicate *Stage 1, Round 2* microcosms was inoculated with 10 mL of pooled *Stage 1, Round 1* bottom-water. *Stage 1, Round 2* microcosms were incubated for two weeks then tested for robust growth. For the purposes of this project, *robust* was defined as:

- $\geq 1 \times 10^6$ CFU bacteria/mL bottom-water by viable count method (spread plate)
- $\geq 1 \times 10^5$ CFU fungi/bottom-water mL by viable count method (spread plate)
- $\geq 1 \times 10^3$ RLU ($\log_{10}$ RLU ≥ 3) bottom-water ATP by BCA SOP 006 (Appendix A)

After two-weeks and confirmation of robust growth in the *Stage 1, Round 2* microcosms, 67 mL bottom-waters from each of these microcosms was pooled to be used as the inoculum for the *Stage 2* microcosm.

Stage 2 Microcosm

The *Stage 2* microcosm was used as the microbial challenge source for the Test microcosms. A 3.78 L wide-mouthed glass jar was used for *Stage 2*. An 800 mL portion of SBW was dispensed into the glass jar. Next, the microcosm was inoculated with 200 mL of pooled *Stage 1, Round 2* microcosm bottom-water (figure 1). Finally, 2.0 L NALSD was decanted into the *Stage 2* microcosm (figure 2). The microcosm was

[1] All fuels were provided by NREL, Golden, CO. NREL provide approximately 15 L of LSD, additized LSD and CME-100 in 5-gal (18.9) polypropylene pails. Fuel was stored in the same room, under the same conditions as the microcosms throughout the study.

incubated in the dark at 25 ± 2 °C for one month. The robust microbial population in the *Stage 2* microcosm was used to inoculate *Test* microcosms A, B, C & D.

Figure 1. *Stage 2* microcosm bottom-water. 200 mL pooled bottom-water from *Stage 1, Round 2* microcosms have been added to 800 mL SBW.

Figure 2. *Stage 2* microcosm just after set-up: NALSD over SBW depicted in figure 1.

Test Microcosms

Five 3.78 L unused, wide-mouth glass jars were set up as *Test* microcosms. Each jar received 200 mL sterile SBW. Fuel was added to Test Microcosms A through D as follows:

Microcosm	Fuel
A	NALSD
B	ALSD
C	CME-100
D	CME-100 + microbicide

Each microcosm received 2.0 L fuel. Microcosm D was treated with 220 µL Kathon® FP1.5 to give 100 µL/L (220 ppm $^{v}/_{v}$) dose, as supplied (1.5 ppm active ingredient).

A 2.0 L volume of CME-100 was filter sterilized using a 0.2 µm pore size cellulose acetate filter. The filter sterilized CME-100 was then dispensed into microcosm E.

Except during monthly observations and sampling evolutions, all microcosms were stored in the dark. Incubation, observations and sampling were all completed in a climate controlled room with the temperature at 25 ± 2 °C.

Sampling

All samples were collected using 10 mL sterile serological pipets. Fluid transfers were made using a pipetter bulb. Samples for microbiological testing were transferred to 50 mL, screw-cap, sterile, disposable, polyethylene centrifuge tubes. Samples for chemical testing were transferred to 250 mL wide-mouth, brown-glass bottles. Fuel samples were then packaged in accordance with DOT regulations and shipped to Clark Laboratories for analysis. Water samples for chemical analysis and all microbiological tests except ATP and O.D. were transferred to the appropriate lab at EMSL.

Testing

Gross Observations

Gross observations were made on all *Test* microcosms at T_0, $T_{1\text{-month}}$, $T_{2\text{-months}}$, and $T_{3\text{-months}}$. The fuel phase in each microcosm was rated for haze (ASTM D 4176-02, *Standard Test Method for Free Water and Particulate Contamination in Distillate Fuels (Visual Inspection Procedures)*) and color (ASTM D1500-04a *Standard Test Method for ASTM Color of Petroleum Products (ASTM Color Scale)*).

® Kathon is a registered trademark of Rohm & Haas Company, Philadelphia, PA.

The fuel-water interface was rated for the presence of an invert emulsion layer, pellicle (membrane) or both. The following interface characteristics were reported:

- Presence (Y or N)
- Thickness (mm)
- Stalactites (intrusions into the water phase; Y or N)
- Stalagmites (intrusions into the fuel phase; Y or N)
- Consistency (flocculent, membranous, dispersible, non-dispersible)
- Adherence to glass jar surface

The water-phase was rated for turbidity, color and the presence of sediment. Water turbidity was rate using the ASTM D4176 haze rating. Color was rated according to ASTM D1500 and sediment was rated as percentage of bottom coverage.

A photographic record was made of each microcosm immediately after gross observations were recorded. Photographs were taken using a Nikon Coolpix 995 digital camera.

Microbiology

ATP

Adenosine Triphosphate (ATP) was determined using a New Horizons Diagnostics (NHD, Columbia, MD) Model 4560 Bioluminometer. The Protocol is detailed in Appendix A. In summary, 50 µL bottom-water samples were concentrated onto a filter and rinsed twice with a mild surfactant solution. A strong surfactant was then used to lyse cells and extract ATP. The released ATP was then transferred onto a *Luciferin-Luciferase* impregnated pad which was then placed into the Bioluminometer. Light emitted from the reaction of ATP with the Luciferin-Luciferase enzyme-substrate pair was recorded as Log_{10} relative light units[2] (RLU).

For fuel samples, cells were concentrated by filtering 25 mL of fuel through a 0.45 µm pore-size filter, rinsing twice with a mild surfactant and extracting the ATP with a strong surfactant, as described above for bottom-water samples.

Culturable Bacteria and Fungi

Bottom-water culturable bacteria and fungal viable counts were performed by EMSL Analytical. Culturable bacteria were enumerated by the standard plate count method (Method 9125C *Spread Plate Method*, *Standard Methods for the Examination of Water and Wastewater*; APHA, Washington, DC). Aliquants (0.1 mL) of serial ten-fold dilutions were plated onto tryptic soy agar (TSA; Becton Dickenson, Baltimore, MD). Cultural fungi were enumerated similarly, except that 0.1 mL aliquants of serial dilutions

[2] RLU: 1.0 RLU = 1 pg ATP. Typical ATP concentration/cell = 1 to 5 fg (10^{-15} g).

were plated onto malt dextrose agar (MDA; Becton Dickenson, Baltimore, MD). For both bacteria and fungi, inoculated plates were incubated inverted at 25 ± 0.5 °C. Bacterial colonies were counted after 24 to 76 hours incubation. Fungal colonies were counted after five days.

Fuel-phase culturable bacteria and fungi were enumerated in accordance with ASTM D6974 *Practice for Enumerating Viable Bacteria and Fungi in Liquid Fuels – Filtration and Culture Procedures*.

Oxygen Demand

Oxygen demand (O.D.) was determined in accordance with BCA SOP 004 *Modified Oxygen Demand for Monitoring biocontamination of Fuel System Bottoms-Water*. This SOP is included in Appendix A. In summary, a 25 mL sample of bottom-water is transferred from a microcosm to a 50 mL disposable centrifuge tube and shaken vigorously for 30 sec. A T_0 dissolve oxygen (DO) reading is taken, the centrifuge tube is sealed and allowed to stand for two-hours. After two-hours, a second DO reading is taken. The O.D. is the percent difference between the DO at T_0 and the DO at T_{2h}.

Chemistry

Bottom-water chemistry tests were performed by EMSL Analytical.
Table 1 lists the tests and the methods used.

Table 1. Water chemistry test methods used for the CME-100 biodeterioration study

Parameter	EMSL Method	ASTM Method
Alkalinity	310.1	D1067
Hardness	130.2	D1126
pH	150.1	D1293
Total dissolved solids (TDS)	160.1	N/A
Total organic carbon (TOC)	415.1	D4839

Fuel Chemistry tests were performed by Clark Laboratories. The following Table 2 lists the tests and methods used.

Table 2. Fuel chemistry test methods used for the CME-100 biodeterioration study

Parameter	ASTM Method
Water by Karl Fischer	D6304
Total acid number (TAN)	D664
Total base number (TBN)	D4739

Fuel Corrosivity

Fuel corrosivity testing was performed by EMSL Analytical in accordance with NACE Standard TM0172 modified as follows.
The test specimens were 5.01 mm dia x 59 mm polished carbon steel cylinders. For corrosivity testing, 300 mL of fuel was dispensed into a 400 mL glass beaker with a magnetic stirring bar. The beaker was placed into a water bath which was then placed onto a hotplate magnetic stirrer. The fuel temperature was maintained at 38 °C and stirring was maintained at a speed sufficient to keep the fuel sample homogeneous, but not cause visible turbulence on the fuel surface.

Once the fuel was at 38 °C, the test specimen was suspended into the beaker so that is was completely submerged and its lower end was ~ 15 mm above the beaker's bottom. After 30 minutes, 30 mL SBW was injected into the beaker. The exposure period continued for an additional 3.5 h, during which the beaker was covered to prevent fluid loss.

After 4.0h total exposure, the specimen was removed form the fuel-water mixture, cleaned with acetone and inspected for evidence of corrosion.

Results

Challenge Population

Stage 1 round 1 microcosms we set up on 30 November 2004. The original inoculum source was *Water sample D from Ed's microcosm started 9-17-04*[3] (received by BCA on 23 November 2004). A robust community should yield ≥ 1,000 RLU ($\log_{10}$ RLU ≥ 3). The FQS sample had 104 ± 11 RLU ($\log_{10}$ RLU = 2.02). To compensate for the relatively low ATP concentration, 10 mL of sample was used to inoculate each of three *Stage 1 round 1* microcosms. The *BACTO Minimal Broth Davis without Dextrose* that had been ordered, had not yet arrived at EMSL. Consequently, an alternative bottom-water preparation was formulated by dispersing 1 g of local garden soil into 1.0 L deionized water.

As of 20 December, there was no gross evidence of growth in any of the three microcosms. EMSL resuspended colonies from a Sani-Check™ AB test strip into tryptic soy broth (TSB) and colonies from a Sani-Check YM test strip onto (MDA). Within 48h, the TSB had become turbid and fungal colonies had grown on the MDA. Each of the

[3] Microbially contaminated bottom-water was provided by Fuel Quality Services, Inc., Flowery Branch, GA.

™ Sani-Check is a trademark of Biosan Laboratories, Warren, MI. AB and YM test strips are growth media impregnated filter pads. When they are dipped into an aqueous solution, the pads rehydrate. The AB test strips support bacterial growth and the YM test strips support fungal growth.

Stage 1 microcosms was inoculated with 10 mL of TSB culture and 10 mL of resuspended growth from an MDA plate.

When observed on 10 January 2005, all three *Stage 1 round 1* microcosms showed gross evidence of microbial activity (Table 3).

Table 3. Stage 1, round 1 microcosm gross observations; 10 January 2005

Parameter	Microcosm		
	A	**B**	**C**
Fuel			
Haze Rating	1	1	1
ASTM Color	3	3	3
Interface			
Visible?	Y	Y	Y
Thickness (mm)	< 1 mm	< 1mm	< 1mm
Stalactites?	N	N	N
Stalagmites?	N	N	N
Consistency	Membranous pellicle	Membranous pellicle	Membranous pellicle
Adheres to glass?	Y	Y	Y
Bottom-water			
Turbidity	Haze 3	Haze 3	Haze 3
Color	1	1	1
Sediment	N	N	N

Figure 3. *Stage 1, Round 1* microcosms 60 days after first inoculation and 18 days after reinoculation.

Bottom-water was drawn from each microcosm and tested for ATP concentration. Average Log_{10} RLU = 2.8 ± 0.07 (630 RLU). Based on the ATP results, a second round of Stage 1 microcosms was set up and inoculated. A 10 mL aliquant was drawn from each *Stage 1 round 1* microcosm and pooled into a 50 mL disposable centrifuge tube. Each aliquant included membranous flocs of biomass. The pooled bottom-water samples were shaken vigorously for 30 sec then dispensed as 10 mL aliquots into the three *Stage 1 round 2* microcosms.

On 31 January, the *Stage 1 round 2* microcosms were observed and tested for ATP concentration. All three microcosms show gross symptoms of microbial activity (Table 3). The average ATP concentration (log_{10} RLU) for the three microcosms was 3.2 ± 0.1. The ATP concentration met the *robust growth* criterion. Consequently 66.8 mL of bottom-water from each *Stage 1 round 2* microcosm was pooled to create a 200 mL inoculum for the *Stage 2* microcosm. The pooled inoculum was tested for ATP concentration, culturable bacterial count and cultural fungal count. The test results were:

Parameter	Result
ATP (log_{10} RLU/50 µL)	3.5
Log_{10} bacteria CFU/mL	4.60
Log_{10} fungi CFU/mL	1.83

The *Stage 2* microcosm was retested on 01 March, just before bottom-water from this microcosm was used to challenge *Test* microcosms A through D. Duplicate ATP analysis yielded log_{10} 3.3 ± 0.14. Figure 4 shows the substantial invert emulsion layer that had formed at the fuel-water interface of the *Stage 2* microcosm.

Figure 4. *Stage 2* microcosm fuel-water interface 5-weeks post-challenge.

Test Microcosms

Gross Observations

Effect of Filtration on CME-100

As noted under **Materials and Methods** CME-100 was filter sterilized for use in the sterile control (microcosm E). The filtration process affected CME-100's appearance, imparting a slight green opalescent color to the product (figure 5).

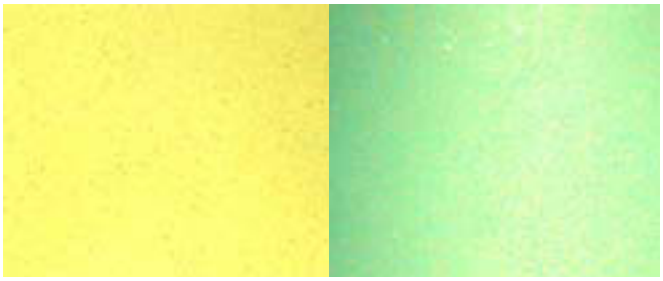

Figure 5. Color comparison; unfiltered (left) and filtered (right) CME-100.

The filter sterilized CME-100 retained this green tint for the duration of the test.

Effect of Exposure to Microbial Contamination

Table 4 summarizes the gross observation data for Test microcosm A. Interestingly, between months 2 and 3, the fuel lost some of its color and the haze diminished. Also, the morphology of the fuel-water interface changed from a well defined, continuous membranous layer into a flocculent discontinuous zone. This change may have reflected the impact of microbial activity. Figure 6 illustrates the change in microcosm A's appearance between months zero and three.

Table 5 presents the gross observation data for Test microcosm B. Similarly to microcosm A, the ALSD microcosm's gross appearance changed between months two and three (figure 7). The membranous layer at the fuel-water interface become flocculent and the fuel color decreased from ASTM 7 to ASTM 5. Moreover, the bottom-water color lightened from ASTM 4 to ASTM 1.

Microcosm C (CME-100) gross observations are listed in Table 6 and illustrated in figure 8. The primary change in this microcosm was the loss of haze between months two and three.

Gross observation data for the Kathon FP1.5 treated microcosm (D) appear in Table 7. Figure 9 presents photographs of Microcosm D. At $T_{3\text{-months}}$, the microbicide treated microcosm had a non-dimensional (< 1mm thick), adherent membrane layer that covered

approximately 98 percent of the fuel-water interface. There was no other gross indication of microbial activity in this microcosm.

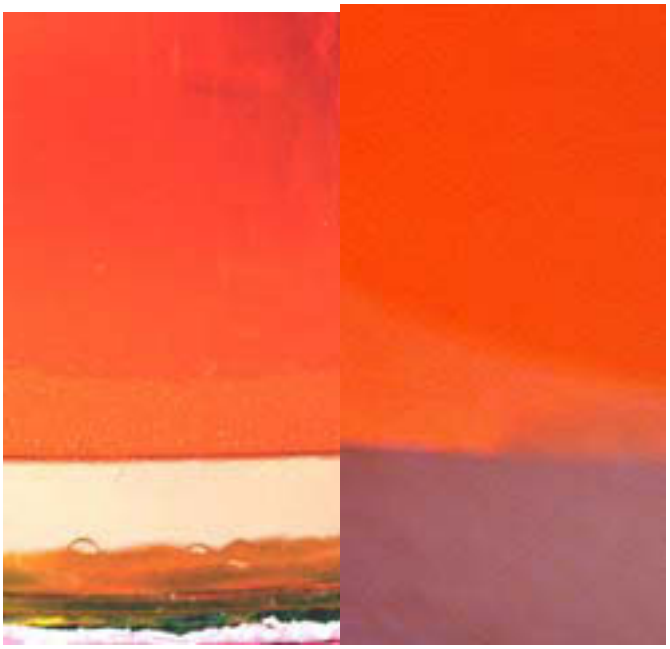

Figure 6. Microcosm A (NALSD) at T_0 (left) and $T_{3\text{-months}}$ (right)

Table 4. Microcosm A, NALSD, gross observations.

Parameter	Time			
	0	1-month	2-months	3-months
Fuel				
Haze Rating	1	1	4	2
ASTM Color	4	6	7	5
Interface				
Visible?	N	Y	Y	Y
Thickness (mm)	N/A	< 1 mm	< 1 mm	< 1mm
Stalactites?	N	N	N	N
Stalagmites?	N	Y (globules)	N	N
Consistency	N/A	Membranous pellicle	Membranous pellicle; adherent	Flocculent, partially dispersible, not continuous; not adherent
Adheres to glass?	N/A	N	N	Y
Bottom-water				
Turbidity	Haze 2	Haze 6	Haze 6	Haze 6
Color	Water White	4	4	1
Sediment	N	N	20%; flocculent	30%; flocculent

Table 5. Microcosm B, ALSD, gross observations.

Parameter	Time			
	0	1-month	2-months	3-months
Fuel				
Haze Rating	1	1	2	2
ASTM Color	4	6	7	5
Interface				
Visible?	N	Y	Y	Y
Thickness (mm)	N/A	< 1 mm	< 1 mm	< 1mm
Stalactites?	N	N	N	N
Stalagmites?	N	Y (globules)	N	N
Consistency	N/A	Membranous pellicle	Membranous pellicle; adherent	Flocculent, partially dispersible, not continuous; adherent
Adheres to glass?	N/A	Y	Y	Y
Bottom-water				
Turbidity	Haze 2	Haze 6	Haze 6	Haze 6
Color	Water White	4	4	1
Sediment	N	N	10%; flocculent	20%; flocculent

Figure 7. Microcosm B (ALSD) left to right: T_0, $T_{1\text{-month}}$, $T_{2\text{-months}}$ & $T_{3\text{ months}}$

Table 6. Microcosm C, CME-100, gross observations.

Parameter	Time			
	0	1-month	2-months	3-months
Fuel				
Haze Rating	2	6	6	1
ASTM Color	2	2	2 (green tint)	2
Interface				
Visible?	N	Y	Y	Y
Thickness (mm)	N/A	< 1 mm	< 1 mm	< 1mm
Stalactites?	N	N	N	N
Stalagmites?	N	N	N	N
Consistency	N/A	Flat, uniform pellicle	Membranous pellicle	Membrancous pellicle
Adheres to glass?	N/A	Y	Y	Y
Bottom-water				
Turbidity	Haze 2	Clear	Clear	Haze 2
Color	Water White	Water-white	Water-white	1
Sediment	N	N	N	N

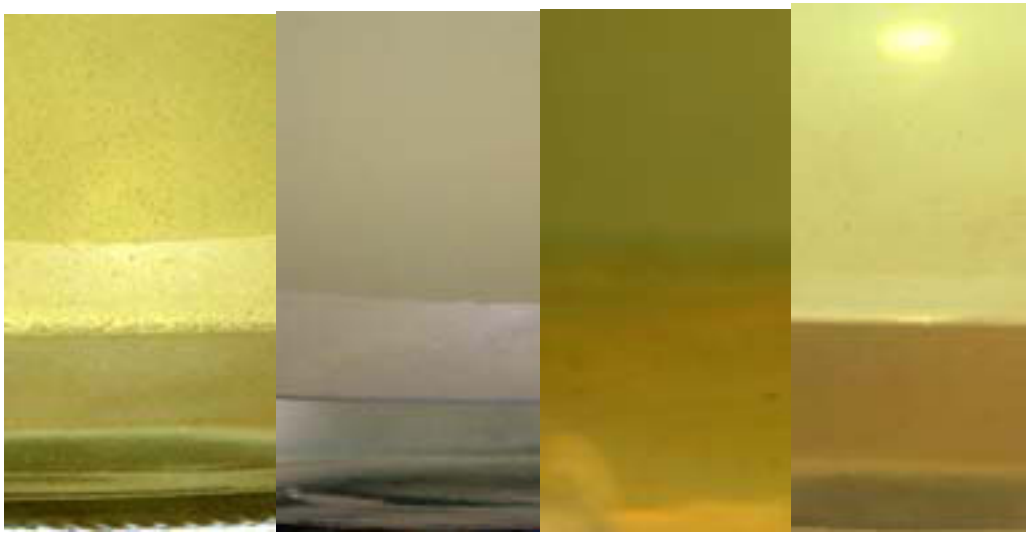

Figure 8. Microcosm C (CME-100) left to right: T_0, $T_{1\text{-month}}$, $T_{2\text{-months}}$ & $T_{3\text{ months}}$

Table 7. Microcosm D, Kathon FP1.5 treated CME-100, gross observations.

Parameter	Time			
	0	1-month	2-months	3-months
Fuel				
Haze Rating	6	6	6	2
ASTM Color	2	2	2	2
Interface				
Visible?	N	Y/N	Y/N	Y
Thickness (mm)	N/A	0 mm	0 mm	< 1 mm
Stalactites?	N	N	N	N
Stalagmites?	N	N	N	N
Consistency	N/A	Flat, non-dimensional; may be optical illusion	Some particles at fuel-water interface	Flat, non-dimensional, adherent
Adheres to glass?	N/A	N	N	Y
Bottom-water				
Turbidity	Haze 6	Clear	Clear	Haze 2
Color	Water-white	Water-white	Water-white	Water-white
Sediment	N	N	N	N

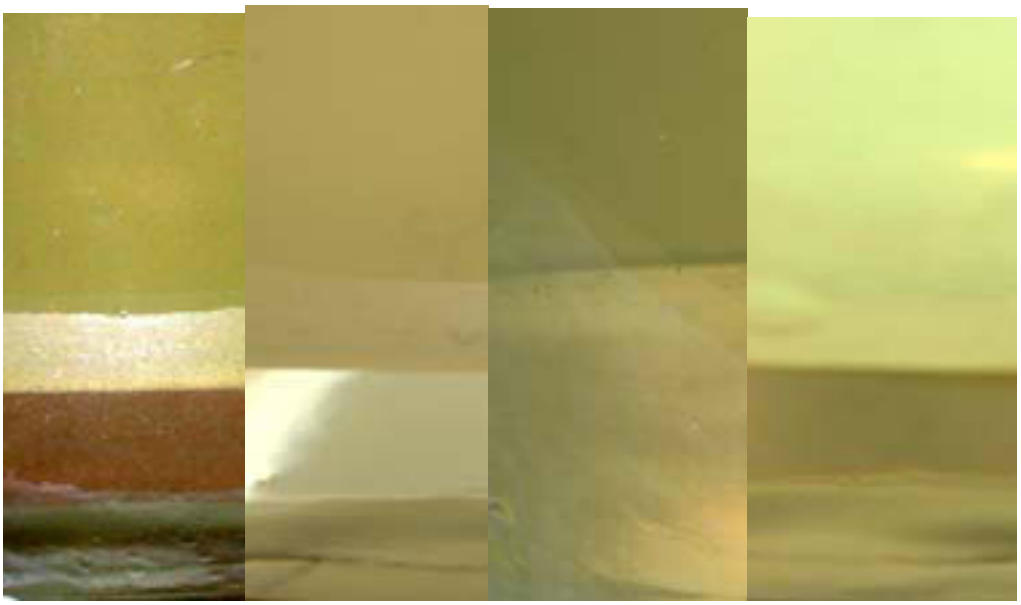

Figure 9. Microcosm D (Microbicide treated CME-100) left to right: T_0, $T_{1\text{-month}}$, $T_{2\text{-months}}$ & $T_{3\text{-months}}$

At $T_{3\text{-months}}$ microcosm E's appearance was very similar to microcosm D's. The haze that had characterized the CME-100 in microcosm E through the first two months had cleared by the end of the testing period (Table 8 and figure 10) although the filter-sterilized

CME-100 still had a darker green tint than unfiltered CME-100. Microcosm E had developed a non-dimensional, non-adherent fuel-water interface membrane between months two and three. Also, the bottom-water had become slightly translucent by the end of the study.

Table 8. Microcosm E, filter-sterilized CME-100, gross observations.

Parameter	Time			
	0	**1-month**	**2-months**	**3-months**
Fuel				
Haze Rating	6	6	6	1
ASTM Color	2 (green)	2 (green)	2 (green)	1
Interface				
Visible?	N	N	N	Y
Thickness (mm)	N/A	0 mm	0 mm	< 1 mm
Stalactites?	N	N/A	N/A	N
Stalagmites?	N	N/A	N/A	N
Consistency	N/A	N/A	Trace of oily film, not a complete layer	Flat, non-dimensional, adherent
Adheres to glass?	N/A	N/A	N/A	Y
Bottom-water				
Turbidity	Haze 6	Clear	Clear	Haze 2
Color	Water-white	Water-white	Water-white	Water-white
Sediment	N	N	N	N

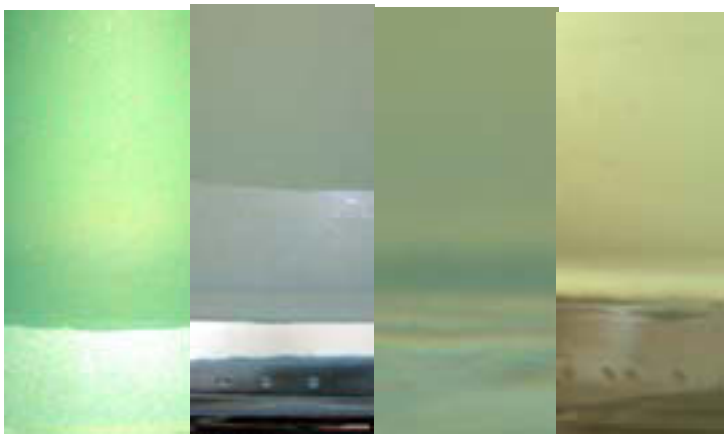

Figure 10. Microcosm E (filter-sterilized CME-100) left to right: T_0, $T_{1\text{-month}}$, $T_{2\text{-months}}$ & $T_{3\text{-months}}$

Fuel Chemistry

Fuel chemistry analyses were performed by Clark Laboratories. Data reports from Clark are provided in Appendix B.

Initially, three chemical parameters were to be monitored during the test period: Karl-Fischer water, total acid number (TAN) and total base number (TBN). A $T_{0\text{-month}}$ CME-100 sample was tested for water, TAN and TBN, but the CME-100 caused instrument problems when TBN was analyzed by ASTM Method D4739. Consequently, the TBN of microcosm C, D and E fuels was not determined at $T_{3\text{-months}}$.

Entrained Water

Table 9 presents the Karl-Fischer water data. The percent change of water in CME-100 was computed as:

$$(1) \qquad [(W_{\text{Microcosm X@ T3-mo}} - W_{\text{Microcosm E@ T0-mo}}) \div W_{\text{Microcosm E@ T0-mo}}] \times 100$$

Where W is Karl Fischer water content and X is microcosm C, D or E, respectively. The water content of microcosm C, D and E increased by > 330% during the course of the study.

Table 9. Water content of microcosm fuels (mg water/kg fuel)

Microcosm	Test time (months)		%
	0	3	change
A	n.d.	< 50	n.d
B	n.d.	86	n.d.
C	n.d.	2,258	331%
D	n.d.	2,256	331%
E	524	2,311	341%

Microbial activity did not seem to affect water partitioning. The microcosm B additive package may have been responsible for some water dispersion into microcosm B. The below detection limit (BDL) result for microcosm A and the very similar amounts of water in all three CME-100 microcosms suggest strongly that CME-100 has a substantially greater tendency than LSD to retain water. Had water dispersion been biologically mediated, the water content of CME-100 from microcosms D and E would have been significantly lower than that of CME-100 from microcosm C.

Total Acid Number

The CME-100 TAN is approximately 10x that of the LSD. Moreover, the $T_{3\text{-month}}$ TAN for untreated, microbially challenged CME-100 (microcosm C) was slightly higher than it was for either microbicide treated or sterile control CME-100 (Table 10).
Without replicate data, it is unclear whether the TAN differences amongst microcosms C, D and E was significantly different, or within the normal range of test variability. The TAN of microcosm C was at $T_{3\text{-months}}$ was 12 percent greater than the average TAN for CME-100 from microcosms D and E at $T_{3\text{-months}}$, and 31% greater than the microcosm E $T_{0\text{-months}}$ TAN.

Table 10. Total acid number of microcosm fuels (mg KOH/g fuel)

Microcosm	Test time (months)	
	0	3
A	n.d.	0.05
B	n.d.	0.06
C	n.d.	0.51
D	n.d.	0.45
E	0.39	0.46

Total Base Number

As noted above, CME-100 TBN was not testable. The $T_{0\text{-months}}$ microcosm E value (1.0) was substantially greater than the $T_{3\text{-months}}$ values for either LSD from microcosms A and B (both < 0.1 mg KOH/g fuel). Table 11 presents the TBN data.

Table 11. Total base number of microcosm fuels (mg KOH/g fuel)

Microcosm	Test time (months)	
	0	3
A	n.d.	<1.0
B	n.d.	<1.0
C	n.d.	n.d.
D	n.d.	n.d.
E	1.0	n.d.

Fuel Corrosivity

Microbial activity can increase the corrosivity of both fuel and fuel associated water. The primary mechanisms for this effect are identified below under *pH*. Biosurfactants are detergent molecules produced by microbes. Biosurfactants will increase water dispersion into fuel. The corrosive properties of the dispersed water will contribute to the fuel's

corrosivity. Moreover, bioconversion of non-polar fuel molecules into charged species will affect the fuel's corrosivity.

The corrosivity data are presented in Table 12. Table 10a lists the NACE TM0172 corrosivity ratings for the microcosm fuel samples. Table 10b captured the extent of coupon corrosion at the end of the exposure period. There was no evidence of corrosion on either T_0 or $T_{3\text{-months}}$ coupons. **Consequently, fuel corrosivity testing did not differentiate between conventional LSD and CME-100.**

Table 12. Fuel corrosivity

a. NACE TM0172 corrosivity rating

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	A
B	n.d.	n.d.	n.d.	A
C	n.d.	n.d.	n.d.	A
D	n.d.	n.d.	n.d.	A
E	B++	n.d.	n.d.	A

b. Percent of test coupon surface corroded

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	< 0.1
B	n.d.	n.d.	n.d.	< 0.1
C	n.d.	n.d.	n.d.	< 0.1
D	n.d.	n.d.	n.d.	< 0.1
E	< 0.1	n.d.	n.d.	< 0.1

Bottom-water Chemistry

Bottom-water chemistry was tested to evaluate the impact of fuel type and microbial activity on the primary characteristics of the bottom-waters in the different microcosms. Bottom-water chemistry testing was completed by EMSL. Their data reports are provided in Appendix B.

pH

Any pH drop reflects the net production of acidic molecules. Many microbial metabolites are weak organic acids. These acids may, in turn react with chloride, sulfate and nitrate salts in the water-phase, producing weak organic bases and strong inorganic acids (hydrochloric, sulfuric and nitric acids, respectively). These biogenic strong inorganic acids play a major role in microbially influenced corrosion (MIC). The pH data from microcosm bottom-water samples appears in Table 13.

The microcosm E bottom-water pH dropped substantially during the test period. The drop was both absolute and relative to all other microcosms. Were it not for the low alkalinity test results (Table 12), it might be assumed that the microcosm E pH result reflected analytical error. There is no apparent cause for the pH in the filter-sterilized control to have fallen by > 3 units during the test period.

At $T_{3\text{-months}}$, bottom-water from the two challenged CME-100 microcosms were significantly different ($F\text{-ratio}_{[1,2]} = 63.8$; $F\text{-critical}_{0.01\ [1,2]} = 18.5$). The substantial pH drop in the filter-sterilized control, combined with the significant differences between the LSD and CME-100 microcosms mean that **the pH data did not support the null hypothesis.**

Table 13. Microcosm bottom-water pH

Microcosm	Test time (months)	
	0	3
A	n.d.	6.79
B	n.d.	6.86
C	n.d.	6.21
D	n.d.	6.33
E	7.13	4.70

Alkalinity

Alkalinity was tested to determine whether microbial production of organic acids reduced the alkalinity of microcosm bottom-waters. Most often pH changes will occur only after the alkalinity has decreased substantially. Table 14 shows the alkalinity data for the test microcosms. As discussed in the preceding paragraph, Microcosm E bottom-water was substantially different from the other four microcosms. Instead of having reserve alkalinity, microcosm E bottom-water was strongly acidic (acidity = 3,700 mg $CaCO_3$/ L). The alkalinity of microcosm B (ALSD) bottom-water was also substantially different from all of the other microcosms. The most likely explanation for this was additive partioning into the water-phase. This type of partitioning is common in fuel storage

systems. Generally, additive depletion from the fuel-phase is immeasurably small (less than the variability due to experimental error of the test method), but may reach saturation in the water-phase. Typically, this additive partitioning phenomenon is reflected in alkalinity, TDS and TOC results. It may occur abiotically or be microbially mediated. In the latter case, biosurfactants and other metabolites may alter additive solubility characteristics, partition-coefficients or both. Since microcosm C was the only additized fuel used in this study, it's not possible to differentiate between biotic and abiotic processes that may have contributed to this partitioning. Since microcosm C and D bottom-water alkalinities were in the same range as microcosm A, **the alkalinity data supported the null hypothesis.** It is possible that the energy imparted to the CME-100 during the filter-sterilization process contributed to the unexpected pH and alkalinity decreases in this microcosm.

Table 14. Microcosm bottom-water alkalinity (mg $CaCO_3$/L water)

Microcosm	Test time (months)	
	0	3
A	n.d.	1,800
B	n.d.	3,500
C	n.d.	1,500
D	n.d.	1,000
E	1,800	<20

Hardness

As water hardness increases, microbial populations tend to become more robust. The dissolved salts are essential nutrients for microbes. In static microcosms such as the ones set up for this test, the only source of $CaCO_3$ and the other salts captured in the hardness titration would be those previously suspended or dissolved in the fuel. Consequently, there should be negligible changes in hardness during the course of the test.

The hardness data are presented in Table 15. Bottom-water hardness under LSD was significantly less than the hardness under CME-100. One way analysis of variance (ANOVA) gave an observed F-ratio $_{[1,3]} = 30.7$ (F-critical $_{0.01\,[1,3]} = 7.7$). The average hardness of CME-100 bottom-water (108 mg $CaCO_3$/L) was approximately three-times (2.8x) as high as the average hardness of bottom-water under LSD (40 mg $CaCO_3$/L). Since the T_0 hardness determination was unlikely to have been affected by partitioning between the fuel and water, the most likely explanation for the difference is that an interaction between the LSD and bottom-water in microcosms A and B caused the hardness to decrease in those systems. Alternatively, since there were no replicate

microcosms, the differences may have reflected microcosm to microcosm variability. **The difference between LSD and CME-100 microcosm bottom-water hardness does not support the null hypothesis.**

Table 15. Microcosm bottom-water hardness (mg $CaCO_3$/L water)

Microcosm	Test time (months)	
	0	3
A	n.d.	32.3
B	n.d.	48.6
C	n.d.	130.0
D	n.d.	93.6
E	2.0	100.0

Total Dissolved Solids

The TDS data reflect dissolve organic and inorganic solids in the water-phase. Microbial activity will cause TDS to increase by a combination of one or more mechanisms. New biomass, resulting from cell growth and proliferation increases both TOC and TDS. Waste metabolites excreted by active microorganisms also contribute to both TOC and TDS. Biosurfactants and organic acids produced by the contaminant population will cause fuel constituents to partition into the water-phase. This process also contributes to both TDS and TOC increases. Consequently, microcosms A, B and C would be expected to have greater TDS and TOC concentrations than microcosms D and E. Differences between A and B would reflect the impact of the additive used in the ALSD. Differences between A and C would reflect the relative tendency for TDS to develop in bottom-waters associated with NALSD and CME-100, respectively. Data from microcosm D would reflect the impact of microbicide treatment on the net TDS change.

Table 16 presents the TDS data. The dissolved solids contribution of the Davis Minimal Broth medium used to formulate synthetic bottom-water was 10g/L. During the three-month test period, bottom-water TDS increased by an average of 31% relative to TDS at T_0. It is likely that this increase was not biogenic, since the highest $T_{3\text{-months}}$ TDS value was in the filter sterilized CME-100 microcosm. As noted before, without replication, it is impossible to differentiate experimental variation from variation due to fuel type. There is no apparent difference in bottom-water TDS amongst the microcosms. Consequently, **the TDS data support the null hypothesis.**

Table 16. Microcosm bottom-water total dissolved solids (g dissolved solids/L water)

Microcosm	Test time (months)	
	0	3
A	n.d.	12
B	n.d.	13
C	n.d.	13
D	n.d.	12
E	9.8	14

Total Organic Carbon

As discussed in the preceding subsection, TOC changes are affected by many of the same processes that drive TDS changes. Additionally, TOC analysis captures both dissolved and particulate organic carbon. As noted before, changes in bottom-water TOC concentrations are generally proportional to microbial activity.

However, non-microbially mediated partitioning of fuel constituents into the water and oxidation byproduct particles settling out of the fuel phase will also contribute to TOC. Data from the untreated CME-100, microbicide treated and filter sterilized microcosms will differentiate abiotic TOC generation from biologically mediated TOC generation. The results (Table 17) from these three microcosms were sufficiently close to indicate that the TOC increases were not microbially mediated.

The data show that TOC increased an average of 13-fold during the three-month storage period. Test results are from microcosms A and B are counter-intuitive. Additive partitioning would have caused microcosm B bottom-water TOC to be substantially greater than the value for microcosm A. The pH, alkalinity and hardness data suggest that additive did partition from the ALSD. However, the microcosm B TOC concentration was only 25% that of microcosm A. These results suggest that significant additive portioning did not occur in microcosm B. The TOC concentrations in microcosm C, D and E were indistinguishable form the concentration in microcosm A; **supporting the null hypothesis.**

Table 17. Microcosm bottom-water total organic carbon (mg TOC/L water)

Microcosm	Test time (months)	
	0	3
A	n.d.	1,400
B	n.d.	350
C	n.d.	1,300
D	n.d.	1,400
E	97.8	1,300

Bottom-water Microbiology

No single parameter captures an adequate profile of microbial contamination in fuel systems. The basis for this statement will be addressed in the **Discussion** section, below. To capture the critical aspects of both biomass accumulation and microbial activity, three types of data were collected. Culture data were used to estimate changes in culturable bacteria and fungi in both fuel and water-phase samples. Since many microbes are not culturable, ATP data provide an alternative measure of total biomass. Oxygen demand (O.D.) is proportional to metabolic activity. High numbers of dormant or moribund microbes consume less oxygen than do metabolically active communities. Consequently, O.D. is an excellent measure of a population's biodeteriogenic activity.

Adenosine Triphosphate

Bottom-water ATP data appear in Table 18. Microcosm A and E bottom-water ATP was determined monthly (figure 11). For the other three microcosms, bottom-water ATP was tested only at $T_{3\text{-months}}$.

Table 18. Microcosm bottom-water ATP (log RLU/50 µL water)

Microcosm	Test time (months)			
	0	1	2	3
A	1.4	4.1	3.6	4.7
B	n.d.	n.d.	n.d.	4.1
C	n.d.	n.d.	n.d.	1.8
D	n.d.	n.d.	n.d.	2.0
E	0.2	-	0.4	0.9

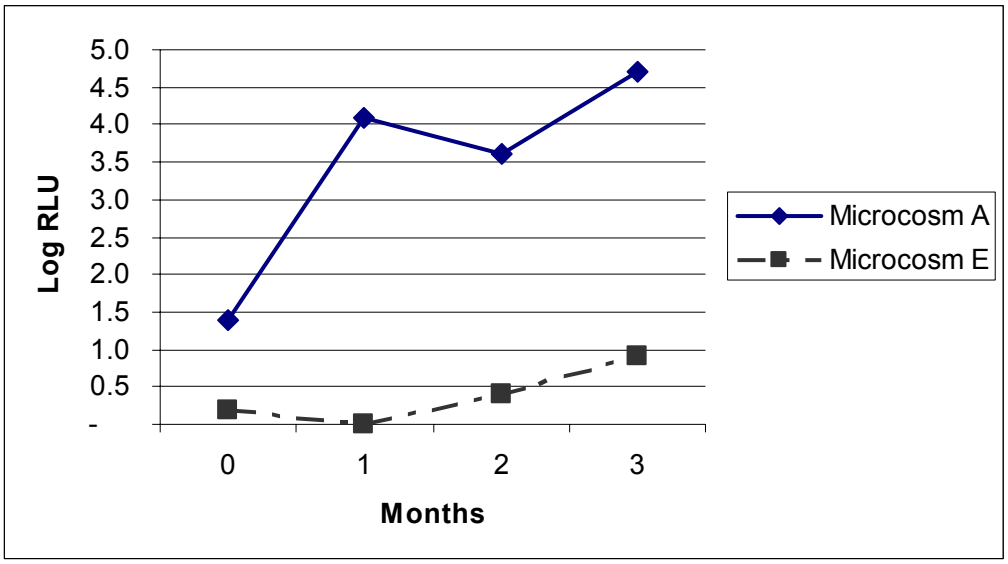

Figure 11. ATP concentration as a function of exposure time in NALSD (microcosm A) and filter sterilized CME-100 (microcosm E)

The ATP data show that during the first month after inoculation, the ATP concentration in the challenged NALSD control bottom-water increased by 2.7 orders of magnitude, and remained high through out the remaining two months of the study. In contrast, the ATP concentration in the filter-sterilized CME-100 control microcosm, as Log RLU remained at <1.0 throughout the test. The bottom-water ATP concentrations in the challenged, untreated and microbicide treated CME-100 microcosms were indistinguishable at $T_{3\ -months}$. Both the NALSD and ALSD bottom-waters had high ATP concentrations at $T_{3\text{-months}}$. These data suggested that **CME-100 did not support bottom-water microbial proliferation as well as LSD.** The F-ratio $_{[1,2]}$ = 46.7 (F-critical $_{0.01\ [1,2]}$ = 18.5) where the treatments (fuel type) were LSD or CME-100. **The bottom-water ATP data do not support the null hypothesis.**

Fuel-phase ATP data are shown in Table 19. Microcosms A, B and C all have considerable ATP concentrations. The difference between fuel ATP in microcosms A (NALSD) and C (CME-100) is significant. However, the difference between fuel ATP in microcosms B (ALSD) and C (CME-100) is not significant. Nor is the difference between microcosms D (microbicide treated CME-100) and E (filter-sterilized CME-100). **The fuel-phase ATP test results do not support nor refute the null hypothesis** although differences between CME-100 and LSD are affected by LSD additive use.

The results suggest that ALSD and CME-100 may be less likely than NALSD to support microbial transport. Interestingly, fuel-phase ATP concentration does not appear to covary with the fuel's water content.

Table 19. Microcosm fuel ATP (log RLU/25 mL fuel)

Microcosm	ATP Log RLU
A	3.1
B	1.7
C	2.0
D	0.9
E	-

Oxygen Demand

Table 20 presents the D.O. (mg O_2/L) and O.D. test results. The T_{0h} and T_{2h} data for microcosm bottom-water O.D. at one and three-months are shown in 20a and 20b respectively. Summary O.D. data are shown in 20c. The D.O. of the inoculated NALSD microcosm bottom-water was 77% at the end of the first month and 91% at the end of the test. These results are consistent with the ATP test results, showing a substantial and metabolically active biomass. Similarly, the filter-sterilized CME-100 bottom-water had a negligible D.O. throughout the study; consistent with the low ATP concentration data.

The O.D. data for microcosms B, C and D also paralleled the bottom-water ATP data from those systems. The O.D. in microcosm B was 16%. This was substantially lower than that in the NALSD microcosm, but still significant. In contrast, O.D. in challenged, untreated CME-100, microbicide treated CME-100 and filter-sterilized CME-100 were all negligible. **The O.D. data do not support the null hypothesis. CME-100 appears to be substantially more bioresistant than LSD as reflected in bottom-water O.D.**

Culturable Bacteria

Bacterial enumeration data are shown in Table 21[4]. At $T_{0\text{-months}}$ log colony forming units (CFU) of bacteria/mL was below the method's lower detection limits (Log CFU bacteria/mL < 3.0).

By the end of the storage period, the bottom water culturable bacteria population had increased to > $10^{8.5}$ CFU bacteria/mL. Although the bottom-water cultural bacteria populations were also quite dense in the three CME-100 microcosm bottom-waters, there was a 3.5 to 3.7 log suppression of growth relative to the LSD microcosms. Neither filter sterilization nor microbicide treatment affected the $T_{3\text{-months}}$ CME-100 bottom-water

[4] Culturable bacteria and fungi testing were performed at EMSL. Data reports are provided in Appendix B.

Table 20. Microcosm bottom-water oxygen demand (% Δ $mgO_2/L/2hours$)

a. $T_{1\text{-month}}$

Microcosm	D.O. (mg O_2/L) @ T_0			
	T_0	T_{2h}	ΔD.O.	% Change
A	5.01	1.17	3.84	77%
B	n.d	n.d	n.d	n.d
C	n.d	n.d	n.d	n.d
D	n.d	n.d	n.d	n.d
E	5.54	5.09	0.45	8%

b. $T_{3\text{-months}}$

Microcosm	D.O. (mg O_2/L) @ T_3			
	T_0	T_{2h}	ΔD.O.	% Change
A	5.72	0.54	5.18	91%
B	5.76	4.85	0.91	16%
C	5.98	5.76	0.22	4%
D	6.00	5.94	0.06	1%
E	6.67	6.69	(0.02)	0%

c. Summary

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	77%	n.d.	91%
B	n.d.	n.d.	n.d.	16%
C	n.d.	n.d.	n.d.	4%
D	n.d.	n.d.	n.d.	1%
E	n.d.	8%	n.d.	0%

culturable bacteria population density. The high population density in microcosm E may have reflected the proliferation of bacteria that had been introduced during sampling. However the most likely explanation is that it is a reflection of the inadequacy of vacuum filtration for filter sterilization. The **bottom-water cultural bacteria data did not support the null hypothesis.**

Table 21. Microcosm bottom-water culturable bacteria counts (Log CFU/mL)

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	> 8.5
B	n.d.	n.d.	n.d.	>8.5
C	n.d.	n.d.	n.d.	4.8
D	n.d.	n.d.	n.d.	4.9
E	< 3.0	n.d.	n.d.	5.0

Fuel-phase bacterial culturability test results are shown in Table 22. At $T_{0\text{-months}}$, $\log_{10}$ CFU bacteria/L < 1.0. By the end of the storage period, fuel phase culturable counts in microcosms A and B were 3.4 and 3.1 $\log_{10}$ CFU bacteria/L respectively. Without T_o data from NALSD and ALSD, it is impossible to determine whether the bacterial population densities had remained stable or had increased in the LSD fuels. The positive microcosm E test results support the hypothesis posed in the previous paragraph. Vacuum filtration was probably not an adequate process for sterilizing the CME-100. However the negligible ATP concentrations detected in microcosm E bottom-water (Table 18) and fuel (Table 19), coupled with the zero O.D. (Table 20) indicate that culturable bacteria recovered from microcosm E were not metabolically active in the microcosm.

The **fuel-phase cultural bacteria data did not support the null hypothesis.**

Table 22. Microcosm fuel-phase culturable bacteria counts ($\log_{10}$ CFU/L)

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	3.4
B	n.d.	n.d.	n.d.	3.1
C	n.d.	n.d.	n.d.	-
D	n.d.	n.d.	n.d.	-
E	< 1.0	n.d.	n.d.	2.0

Tables 23 and 24 present bottom-water and fuel-phase fungal data. At $T_{0\text{-months}}$ the filter-sterilized CME-100 microcosms did not have detectible fungi in either the fuel or

bottom-water. Bottom-water culturable fungi data paralleled the bacterial results. The NALSD and ALSD microcosm bottom-waters had 3.0 and 5.6 $\log_{10}$ CFU fungi/mL respectively. Colony counts from microcosms C and D were below detection limits, but E had 2.0 Log CFU fungi/mL.

Significant numbers of fungi were recovered from both LSD microcosms, but not from any of the CME-100 microcosms. Consequently, **the culturable fungi data from both fuel and bottom-water samples did not support the null hypothesis.**

Table 23. Microcosm bottom-water culturable fungi counts ($\log_{10}$ CFU/mL)

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	3.4
B	n.d.	n.d.	n.d.	3.1
C	n.d.	n.d.	n.d.	<0
D	n.d.	n.d.	n.d.	<0
E	< 3.0	n.d.	n.d.	2.0

Table 24. Microcosm fuel-phase culturable fungi counts ($\log_{10}$ CFU/L)

Microcosm	Test time (months)			
	0	1	2	3
A	n.d.	n.d.	n.d.	3.0
B	n.d.	n.d.	n.d.	1.7
C	n.d.	n.d.	n.d.	<0
D	n.d.	n.d.	n.d.	<0
E	< 1.0	n.d.	n.d.	<0

Discussion

Biodiesel biodeterioration has been reported routinely in trade meetings and non-peer reviewed literature, but has not been described in the peer reviewed literature. Typical anecdotal reports suggest that biodiesel fuel systems experience filter plugging more frequently than similar systems containing conventional diesel fuel. Increased component corrosion has been noted as well, although this phenomenon has been reported less frequently.

Typically, biodiesel fuels are marketed as blends comprised of 10 to 20 percent biodiesel stock in conventional petroleum distillate. Biodiesel stocks are methyl esters of vegetable oils or animal fats. Currently, rapeseed (*Brassica napus*) and soy (*Glycine max*) are the two predominant biodiesel source crops. However, biodiesel is being produced from an increasing number of corps.

Uncut biodiesel stocks (B-100) must meet specifications established by the International Standards Organization (ISO) or ASTM International (ISO 14214 and ASTM D6751, respectively). Neither standard addresses B-100 or biodiesel blend biodegradability.

Biodiesel biodeterioration susceptibility is a function of numerous factors. ASTM Manual 47 and ASTM D-6469 discuss the various factors contributing to fuel and fuel system biodeterioration. For the purposes of this report, it's sufficient to note that microbial contamination, product chemistry, water availability and temperature are the four dominant factors affecting fuel biodeterioration.

The present study focused on CME-100 biodegradability during storage over water at moderate temperature (25°C). The data generated during this study cannot be extrapolated to assess the likelihood of CME blend (CME-20, etc.) biodegradability or CME-100 biodegradability at warmer temperatures (e.g. > 30°C). Although replicate tests were not performed, normal variability for each of the methods used is known, and assumed to apply to the data reported herein. Consequently, only differences greater than five-times the expected range of experimental error were considered significant.

The experimental matrix was designed to test:

> **Null Hypothesis:** CME-100 biodegradation risk is not significantly different from conventional LSD biodeterioration risk when products are stored under typical above ground storage tank conditions for up to three months.

The hypothesis was tested on the basis of microcosm gross appearance, fuel corrosivity, fuel chemistry, fuel microbiology, bottom-water chemistry and bottom-water microbiology. Data from microcosms A (microbially challenged NALSD) and C (microbially challenged CME-100) were used to test the null hypothesis. Comparison of data from microcosms A and B reflected differences between NALSD and ALSD biodeterioration risk. Comparison of data from microcosm C and D permitted assessment of microbicide treatment effectiveness against biodeterioration.

BCA has a standard guideline for scoring biodeterioration risk based on classes of parameters (gross observations, fuel chemistry, etc.). The decision tree for gross observations is provided in Appendix A. Although there were specific differences amongst the microcosms (see Tables 5 through 8 and figures 6 through 10), there was not significant difference in $T_{3\text{-months}}$ risk scores for the five test microcosms:

Microcosm	Risk Score
A	19
B	20
C	20
D	17
E	16

The presence of a defined third layer between the fuel and water phases accounted for 8 points of the 17 and 16 point scores for microcosms D and E, respectively. The microbiological data suggest that the apparent invert emulsion layer in these two microcosms was not produced microbially. Instead, they may have resulted from an abiotic interaction between CME-100 and water. Gross observation scores for A, B and C were indistinguishable. Consequently, **the gross observation data support the null hypothesis.**

Without chemical analysis, it's impossible to determine the extent to which the microcosm A though C invert-emulsion layers were biogenic. However, microcosms D and E appeared distinctly different form the others. This suggested that microbicide treatment did inhibit gross appearance changes to some degree.

The only fuel chemistry parameter that this study shares with BCA's biodeterioration risk survey parameter matrix is Karl-Fisher water. The BCA risk score for this parameter are:

% water ($^{v}/_{v}$)	Risk Score
< 0.01	0
0.01 – 0.1	1
0.1 – 0.2	3
> 0.2	5

All of the fuels, including the microcosm E CME-100, had > 0.2% water at $T_{3\text{-months}}$. Consequently all would be scored as fuels at high risk for biodeterioration. However, all CME-100 fuels (microcosms C, D and E) had water values three orders of magnitude greater than those detected in either the NALSD or ALSD. Thus, **fuel-phase water content did not support the null hypothesis.**

All TAN results were below the ASTM D6751 specification upper control limit (0.8 mg KOH/g), however, TAN values for CME-100 samples from microcosms C through E were all an order of magnitude greater than either the NALSD or ALSD TAN values. Consequently, **TAN data did not support the null hypothesis**.

None of the microcosm fuels were corrosive. Consequently, **the corrosivity data supported the null hypothesis.**

Table 25 summarizes BCA's risk scores for the bottom-water microbiological parameters tested in this study.

Table 25. Biodeterioration risk decision tree; bottom-water microbiology

Parameter	Criterion	Risk Score
Oxygen Demand (O..D.)	< 10 %	1
	10 to 50%	2
	> 50 %	5
ATP (Log RLU)	< 2.5	1
	2.5 to 3.5	2
	3.5 to 4.0	3
	> 4.0	5
Bacteria (log CFU)	<2	1
	2 to 4	2
	> 4	5
Fungi (log CFU)	< 2	1
	2 to 3	2
	> 3	5

At $T_{3\text{-moths}}$, the O.D. risk scores for both conventional diesel fuel microcosms were high (5). In contrast O.D. risk for all CME-100 microcosms was low (1). **The O.D. results did not supporte the null hypothesis**. Similarly, risk scores for CME-100 microcosm bottom-water ATP concentrations were all 1 and for the conventional diesel microcosms were both 5. **Thus ATP data did not support the null hypothesis**. T**he culturable bacteria and fungi data also did not support the null hypothesis**. Bacterial and fungal recoveries from the three CME-100 microcosms were consistently lower than recoveries from the LSD microcosms.

Table 26 summarizes the null hypothesis assessment for each parameter measured. Interestingly, most of the chemical data suggest that CME-100 is more susceptible than LSD to biodeterioration. However, all of the microbiologic data suggested that LSD was more susceptible than the CME-100.

The use of replicate microcosms facilitates differentiation between normal system variability and variation attributable to the treatment (initial fuel chemistry). The data spread between LSD microcosm and CME-100 microcosm results were substantial (≥

10x) for the parameters that for which data did not support the null hypothesis. This suggests that the observed differences between LSD and CME-100 were real. However, the results from a single microcosm may not be representative of the typical dynamics within a commercial operation. Triplicate microcosms address the need to minimize testing costs balanced against the risk of making incorrect interpretations on the basis of a single microcosm.

Table 26. Summary of null hypothesis testing; relative LSD and CME-100 relative biodeterioration risk.

Parameter	Results Support Null Hypothesis (Y or N)	More Susceptible Product (CME-100 or LSD)
Gross observations	Y	-
Fuel Chemistry		
Entrained water	N	CME-100
Total Acid Number	N	CME-100
Corrosivity	N	CME-100
Bottom-water Chemistry		
pH	N	LSD
Alkalinity/Acidity	N	LSD
Hardness	N	LSD
TDS	Y	-
TOC	Y	-
Bottom-water Microbiology		
ATP	N	LSD
O.D.	N	LSD
CFU bacteria/mL	N	LSD
CFU fungi/mL	N	LSD
Fuel Microbiology		
ATP	Equivocal Result	-
CFU bacteria/L	N	LSD
CFU fungi/L	N	LSD

In this study, filtration imparted a green tint to the CME-100. Moreover, the filter sterilized CME-100 microcosm bottom-water pH plummeted during the test period. Initial total alkalinity of 1,800 mg $CaCO_3$/L fell to 3,700 mg $CaCO_3$/L acidity between T_0 and $T_{3\text{-months}}$. No change approaching this magnitude occurred in any of the other microcosms. Without replicate microcosms, it was impossible to determine whether this phenomenon was somehow related to the filtration process, or was just an aberration in this single microcosm.

The original test design included simulated distillation and carbon number distribution. These two parameters reflect chemical changes in the fuel more precisely than the TAN, TBN and water data that were collected. They may also have provided some clues

regarding the major changes in microcosm E. Complete sets of tests at $T_{1\text{-month}}$ and $T_{2\text{-months}}$ would also have provided rate information that would have facilitated more thorough interpretation of the test results.

Rate data are important for designing cost-effective condition monitoring programs. Testing frequency should be based on the anticipated rate at which system change is expected to occur. The test results demonstrated that significant changes occur in CME-100 storage systems during a period less than the three-month test period used for this project.

Typically, O.D. and ATP concentration data are more sensitive than culture tests as indicators of microbial activity. Bacteria and fungi unable to elaborate into colonies on the growth media used may be active within the environment from which the sample was collected. Less frequently, microbes that are moribund or dormant in the sampled environment recover and flourish on the nutrient media. This appears to be the case in this study. Despite low ATP concentrations and O.D., microcosm C, D and E bottom-waters all yielded substantial numbers of culturable bacteria and fungi. Without replicate microcosms, it's impossible to determine if this phenomenon is representative of CME-100 stored over incidental water. Notwithstanding the weak correlation between non-conventional microbiological data and culture tests results, the data show that CME-100 is no more likely than LSD to support microbial growth, and seems substantially less likely to support metabolic activity – the engine of biodeterioration.

The testing reported herein considered only CME-100. Research that I've performed in the past has demonstrated that chemistries that are inhibitory at high concentrations may not be at lower concentrations. Testing performed with a variety of one to four –carbon alcohols, for example, demonstrated that methanol, ethanol, propanol (normal and iso) and butanol (normal, secondary and tertiary) all inhibited microbial growth at concentrations down to ~ 17%. The effects between 10% and 17% varied with the alcohol tested, but at 10% all stimulated growth. This dynamic of inhibiting growth at high concentrations but stimulating growth at lower concentrations is well documented. *Hormonisis* is the term used for this dose-dependant effect. In South America, where gasoline is blended with 20% ethanol, microbial contamination problems are non-existent. In the U.S. and U.K., where ethanol is blended into gasoline at 10%, biodeterioration problems occur routinely. Consequently, it is reasonable to speculate that despite the apparent bioresistance of CME-100, CME-5, CME-10 and CME-20 may be substantially less bioresistant.

Conclusions

The results of this study were somewhat equivocal, but generally did not support the null hypothesis. Sixteen parameters were used to compare CME-100 biodegradability against both additized and non-additized LSD. Data for six of the 16 parameters supported the

null hypothesis. Although CME-100 was more hazy than LSD at $T_{1\text{-month}}$ and $T_{2\text{ months}}$, the CME-100 could not be differentiated from the ALSD or NALSD on the basis of gross appearance, corrosivity; bottom-water alkalinity, TDS, TOC, or O.D.; or fuel-phase ATP concentration at $T_{3\text{-months}}$. Karl Fischer water, TAN, bottom-water pH, and bottom-water hardness data indicated that CME-100 was significantly more susceptible than LSD to biodeterioration. Fuel and bottom-water ATP and culturable bacteria and fungi data indicated that CME-100 was less likely than LSD to support culturable microbes. However, the ~ 5 $\log_{10}$ CFU bacteria/mL recoveries from CME-100 bottom-water made microcosms C, D, and E high biodeterioration risk systems.

The overall results suggest that CME-100 and LSD have comparable but somewhat different biodeterioration susceptibilities. Although the CME-100 microcosms had lower microbial population densities, biomass and metabolic activity, CME-100's chemical properties are characteristic of high biodeterioration risk fuels. It's possible that CME-100's high lauric (n-dodecanoic) acid content gives the product antimicrobial properties that compensate for the increased biodeterioration risk due to high TAN and water content. **The microbiological data all support the hypothesis that CME-100 is less suitable than LSD as a medium for microbial activity.**

Since CME-100 is most probably going to be used as a blend stock, it's important to understand the relationship between blend concentration and biodeterioration resistance. Testing CME-10, CME-20 and CME-40 would provide important market application information. Comparing these blends with similar blends from outer common methyl ester stocks – particularly rapeseed and soy – would provide important competitive performance information.

Recommendations

The data from this study were inconclusive but very encouraging. I recommend that based on the generally positive results reported in this document, a second phase of testing be undertaken.

I recommend the following test plan:

Fuels:

- Conventional Low Sulfur Diesel
- Conventional Ultra Low Sulfur Diesel
- Biodiesel Blend Stock (B-100) from:
  - Coconut
  - Rapeseed
  - Soy
- Fuel blends from each stock:
  - B-40
  - B-20

- B-10

Sampling times (all microcosms):

- T_0
- $T_{1\text{-month}}$
- $T_{2\text{-months}}$
- $T_{3\text{-months}}$

Test parameters: the same parameters used in this study modified as follows:

- Fuel corrosivity: immerse coupons for the duration of the study and compare coupons at $T_{1,\ 2\ \text{and}\ 3\text{-months}}$.
- Fuel chemistry: add simulated distillation and carbon number distribution at times T_0 and $T_{3\text{-months}}$. Also use a methyl-ester compatible modification[5] of the TBN test.

I further recommend that all fuels be tested in triplicate microcosms. As discussed earlier, replicates will provide the essential basis for differentiating between the effects of fuel chemistry and normal experimental variation.

Favorable results from the proposed expanded study are expected to have a dramatic effect on CME marketability. Differentiating CME blends from other biodiesel on the basis of biodeterioration resistance would create unique pricing opportunities and give CME lends a distinct advantage in markets where climate in particularly conducive to biodeterioration.

[5] S. Westbrook of SWRI has advised me that his lab has developed a modified TBN test for B-100 stocks that circumvents the equipment incompatibility problems that Clark Laboratories encountered during this project.

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NREL SUBCONTRACT NO. ADK-5-55501-01
EVALUATION OF BIODIESEL FUEL BIODETERIORATION SUSCEPTIBILITY
FINAL REPORT

APPENDIX A – BCA TEST METHODS

- **Modified Oxygen Demand for Monitoring Biocontamination of Fuel System Bottoms-water**
- **ATP in Fuel System Bottoms-water**

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Modified Oxygen Demand for Monitoring Biocontamination of Fuel System Bottoms-water

Objective:

To use dissolved oxygen measurements in order to estimate bioactivity in water-miscible metalworking fluid (MWF) samples.

Theory:

At typical MWF temperatures, oxygen concentrations ($[O_2]$) range from 6 to 11 mg O_2/liter of fluid.

Active aerobic bacteria and fungi consume oxygen. Consequently, they will deplete $[O_2]$ rapidly if they are active in a bottoms-water sample allowed to stand without aeration. By measuring $[O_2]$ immediately after agitating a sample, and again after two-hours of standing, we can compute the rate of oxygen consumption. The traditional Biochemical Oxygen Demand test (BOD_5) is a five-day procedure used to estimate the concentration of biodegradable organic matter in wastewater. This 2-hour modification of the BOD_5 enables system operators to estimate microbial loads, or bioburdens. The greater the difference (Δ DO) between $[O_2]$ at time zero (T_0) and two-hours (T_{2h}), the greater the bioburden.

Active microbial communities will deplete $[O_2]$ more quickly than dormant communities will. This means that ΔDO test results may not always correlate with viable count (dip-slide) results. A small, active community (fewer CFU/mL) will have a greater ΔDO than a larger (more CFU/mL), less active community. However, the former will be more likely to cause biodeterioration problems than the latter.

Materials:

Dissolved oxygen meter – for example, Corning Checkmate™ Dissolve Oxygen System
Sensor and accessories (electrolyte solution and replacement membranes)
50 mL screw-cap disposable centrifuge tube
Zero oxygen solution
Distilled water in wash bottle
Lab wipes
250 mL beaker
500 mL Boston round sample bottle
10 mL volumetric or serological pipette
Pipette pump

Procedure

Meter calibration

Follow dissolved oxygen (D.O.) meter manufacturer's instructions for maintenance and calibration.

Testing

1. Collect MWF sample using an appropriate bottom sampling device, or directly into 50 mL centrifuge tube.
2. Use 10 mL pipette to draw 35 to 40 mL water from sample, and transfer to clean centrifuge tube. If sample was collected in centrifuge tube ensure that volume is 35 to 40 mL.
3. Within 15 minutes of sampling, or as soon as sample temperature equilibrates to room temperature, tighten screw cap and agitate bottle vigorously for 30 seconds.
4. Immediately remove cap and immerse D. O. probe into water and measure $[O_2]$ in mg O_2/liter and percent D.O. Also record sample temperature.
5. Rinse D.O. probe with distilled water, rinsing residual bottoms-water into the 250 mL beaker and drying the probe gently with a lab wipe. Place the probe in distilled water until next use.
6. Cover the centrifuge tube, securing the screw-cap lid tightly. Don't over-torque the lid, or lid may split.
7. Let sample stand for 2-hours.
8. At the end of 2-hours, carefully remove sample centrifuge tube cap, and repeat steps 3 and 4.

Reporting

Enter data into a table similar to the example below:

Sample ID	**Time T_0**			**Time T_{2h}**			**ΔDO**	
	mg O_2/L	**% O_2**	**T °C**	**mg O_2/L**	**% O_2**	**T °C**	**mg O_2/L**	**% Change**

Compute % change as follows:

1) $\Delta DO = [O_2]_{T2h} - [O_2]_{T0}$

2) % Change = $(\Delta DO \div [O_2]_{T0}) \times 100$

Interpretation

% DO Change	Risk Descriptor
≤ 10 %	Low
10% < DO Change ≤ 50%	Moderate
50% < DO Change	High

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ATP in Fuel System Bottoms-water

Objective:

To use a Luciferin-Luciferase enzyme-fluorochrome complex that will react specifically with the molecule, *adenosine triphosphate* (ATP), to create a bioluminescence reaction that can be measured quantitatively using a sensitive, accurate, bioluminometer.

Theory:

Adenosine triphosphate (ATP) is the primary energy transfer molecule in all living cells. The principle energy generating metabolic pathways – the Krebs Cycle and Embden-Myerhoff Pathway react adenosine diphosphate (ADP) with polyphosphate to produce ATP. This ATP is then used to drive those metabolic pathways that consume energy.

Luciferin is a bioluminescent molecule found in glow-worms and fireflies. In the presence of the enzyme, luciferase, and ATP, luciferin emits light at a characteristic wavelength. This light is seen with the naked eye as the glow of fireflies and glow-worms. The intensity of the light emission is proportional to the ATP concentration available to react with the luciferin-luciferase complex.

Consequently, Profile 1 bioluminescence reading, in lumens, is directly proportional to the ATP concentration within the sample tested.

The ATP concentration in a sample depends on the total bioburden. Generally speaking, ATP concentration increases as the number of cells per mL increases. However, the amount of ATP per cell depends on both genetics and physiology. Different species have different typical specific ATP concentrations (average ATP per cell). For a given species, metabolically active cells will have greater specific ATP concentrations than will dormant cells. Consequently, ATP can be used as an indicator of total biomass and the metabolic potential of a contaminant population.

Hydrocarbons and other chemical substances likely to be found in bottom-water may interfere with the ATP assay. This protocol includes preliminary sample preparation steps that eliminate these interferences.

Materials

Profile 1 Model 4560 Bioluminometer (New Horizons, Inc., Columbia, MD)
Profile 1 *Filtervette*, 1 per test
Profile 1 *Filtervette* 5-place holder, 2
Profile 1 *Filtervette* blotters, 4 per 5 tests
Profile 1 *Filtervette* filtration plunger

Profile 1 freeze-dried luciferin-luciferase reaction pads (tickets), 1 per test
New Horizons SRA reagent, one vial per 50 samples
New Horizons BRA reagent, one vial per 50 samples
New Horizons ATP Standard, one vial
Pipette, microliter, 50 µL
Pipette, microliter, 15 µL
Microliter pipette tips, disposable, sterile for 50 µL and 15 µL pipettes, 1 each per sample.

Photo 1. Model 4560 Bioluminometer and supplies.

Procedure

Bioluminometer calibration

1. With sample drawer closed, bioluminometer should read 0 (see Photo 1).
2. Place 15 µL of BRA onto an unused ticket, place ticket into its position in sample drawer, close drawer and read bioluminescence. Value should be < 50 lumens.
3. Place 50 µL ATP standard onto unused ticket, place ticket into its position in sample drawer, close drawer and read bioluminescence. Value should be 10,000 ± 4,000 lumens.
4. If unit fails any of these three calibration/quality assurance tests, re-run test. If unit fails on second attempt, refer to manufacturer's manual for further guidance.

Testing

1. Set up two Filtervette 5-place holders; place one or two Filtervette blotters onto base of each holder.
2. Place a Filtervette cuvette into one of the holders in the Filtervette 5-place holder.
3. Attach an unused pipette tip into the 50 µL pipette.
4. Draw a 50 µL sub-sample from the sample container. NOTE: if the sample bottle contains fuel over water, use a sterile, glass (1 to 10 mL) pipette to transfer bottom-water to a sterile container (for example a 50 mL centrifuge tube), then draw the required 50 µL water sample.

5. Dispense the 50 µL sample portion into the Filtervette cuvette.

6. Add 3 to 5 drops of SRA reagent.

7. Draw the Filtervette filtration plunger's plunger out approximately ¾ from the plunger's barrel, place the barrel's rubber seal onto the top of the Filtervette cuvette and apply pressure gently to the plunger in order to create sufficient pressure within the cuvette to drive the fluid through the cuvette's filter base.

8. Repeat Steps 6 & 7 once more.
9. Remove a ticket from the 5-ticket pouch, open the ticket and place the open ticket under one of the openings of the second Filtravette 5-place holders. NOTE: the ticket should be between the blotter and the cuvette holder opening.

10. Remove the cuvette from the first holder and place it into the opening under which the ticket has been positioned. Ensure that the cuvette's base surrounds the pad of freeze-dried luciferin-luciferase reagent located near the center of the bottom half of the ticket.

11. Place a pipette tip onto the 15 µL pipet.
12. Draw a 15 µL portion of BRA reagent and pipet it into the cuvette, just above the cuvette's bottom-filter pad.

13. Repeat Step 7.
14. As quickly as possible, remover the ticket from beneath the cuvette, refold the ticket, place the ticket into its position in the bioluminometer's sample drawer and close the drawer.

15. Observe the digital display on the bioluminometer.
16. When the numbers on the display remain constant (approximately 5 sec) record the value displayed.

Precautions:

1. The Filtravette's base is made of a filtration medium that is used to concentrate the sample and separate interferences from ATP. When using the micropipettes to dispense sample or BRA reagent, position the pipette-tip near the cuvette's base so that fluid is delivered to the base and not the Filtravette's walls.
2. Steps 13 and 14 deliver a droplet of BRA reagent to the ticket surface. Avoid allowing the droplet to come into contact with the Filtervette 5-place holder. Such contact may reduce the apparent ATP concentration (the ATP in the BRA fluid left behind on the holder won't be measured) and may contaminate the next sample. If BRA from the ticket adheres to the Filtravette holder, dry the wetted surface and retest the sample.
3. To prevent cross contamination, do not reuse pipette tips. If a sample is being measured in replicate, a single tip may be used for all of the replicate sub-samples.
4. Test variability depends on several factors including:
    a. Analytical technique
        i. Consistency of sample portion size (pipeting technique)
        ii. Precautionary items 1 through 3 listed above
        iii. Consistency and brevity of time lapse between completion of step 13 and completion of step 14

b. Biomass distribution within sample. Biomass flocs may contribute to considerable variability amongst sub-samples. Optimally each sample should be tested in triplicate. Alternatively, triplicate analyses should be performed on 10% of the samples tested. Compute the average standard deviation amongst the triplicate analysis data sets and report all data as observed value ± this standard deviation.

Interpretation

Bioluminometer Reading		**Risk Descriptor**
Linear Scale	**Log_{10} Scale**	
≤ 100	≤ 2	Low
100 < ATP ≤ 500	2 < ATP ≥ 2.7	Medium
>500	> 2.7	High

NREL SUBCONTRACT NO. ADK-5-55501-01
EVALUATION OF BIODIESEL FUEL BIODETERIORATION SUSCEPTIBILITY
FINAL REPORT

APPENDIX B– LABORATORY REPORTS

- Clark Laboratories Report 23878 of 3/1/05 – CME-100 Chemistry
- Clark Laboratories Report 24834 of 6/9/05 – Microcosm A Fuel chemistry
- Clark Laboratories Report 24835 of 6/9/05 – Microcosm B Fuel Chemistry
- Clark Laboratories Report 24836 of 6/9/05 – Microcosm C Fuel Chemistry
- Clark Laboratories Report 24837 of 6/9/05 – Microcosm D Fuel Chemistry
- Clark Laboratories Report 24838 of 6/9/05 – Microcosm E Fuel Chemistry
- EMSL Analytical Case No: 360500219 of 03/09/05 Modified NACE TM0172
- EMSL Analytical Case No: 360500555 of 06/08/05 Modified NACE TM0172
- EMSL Analytical Case No: 370501236 of 03/28/05 Water Chemistry
- EMSL Analytical Case No: 370501236 of 06/24 /05 Water Chemistry
- EMSL Analytical Case No: 370501576 of 03/7/05 Water and Fuel Microbiology
- EMSL Analytical Case No: 370501576 of 06/20/05 Water and Fuel Microbiology

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Clark Laboratories Report 23878 of 3/1/05 – CME-100 Chemistry

BCA Inc.
Fred Passman
3 Carlyle Court

Printon, NJ 08543-3659

Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC

Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2255

Lube Supplier			
Unknown	BCA Inc. Special Testing Coconut Coconut Methylester-1003 Test Group(s): D16 D14 D13	BCA Special Coconut Meth-1003	Level 3 Make: unknown Model: Level 4 Make: Model: Serial No:
Lube Type			
Unknown			

Machine Condition: **Unknown**

Lubricant Condition: **Unknown**

Lab #	238378
Work Order	
Date	3/1/05
Time on Unit	0.0
Time on Oil	0.0
Mach Cond	6
Lube Cond	6

KF [D6304-04]				03/01/05	RANGE
Water by Karl Fischer				324.20 ppm	

TAN (D664-04) mg KOH/g				03/01/05	RANGE
TAN				0.39	

TBN (D4739-02) mg KOH/g				03/01/05	RANGE
TBN				1.0	

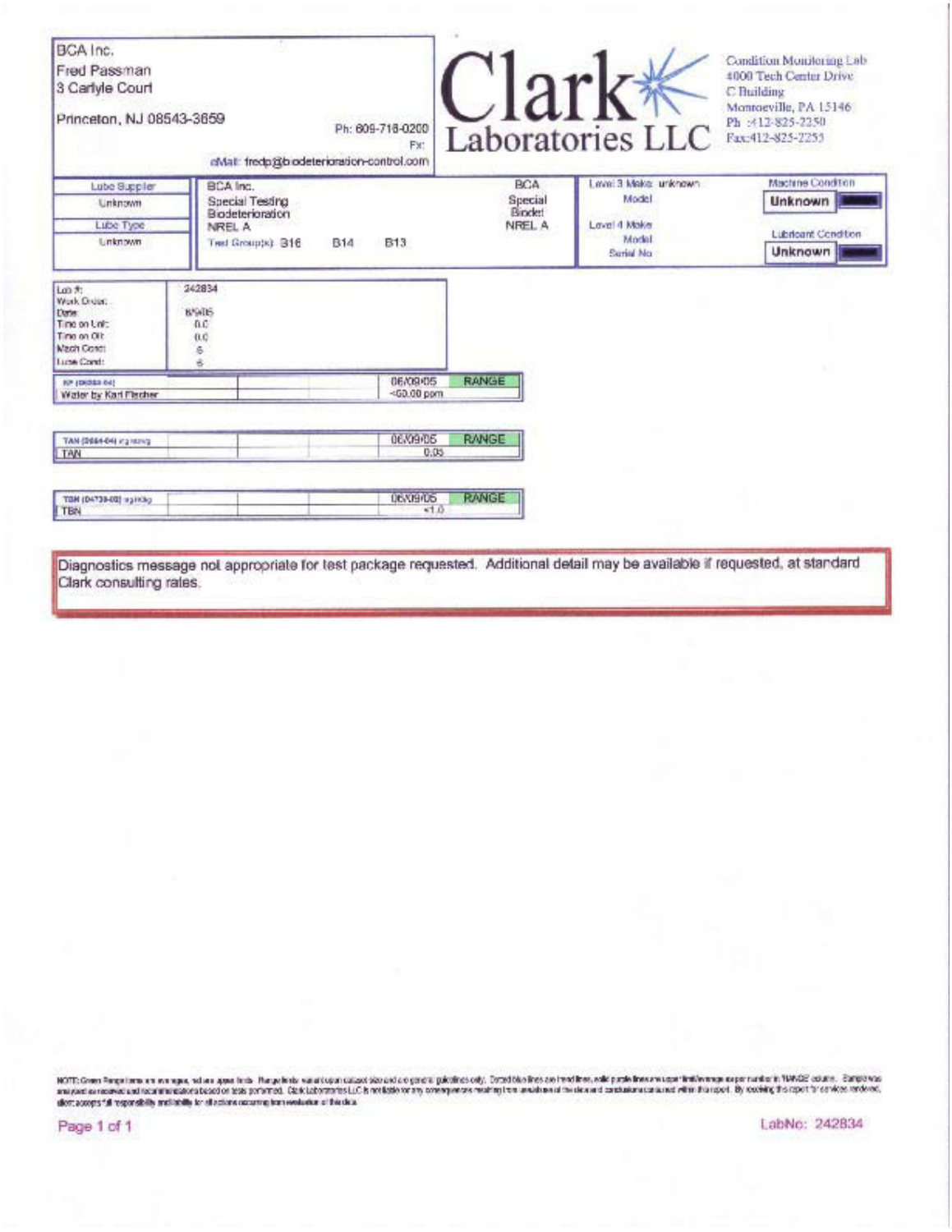
Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

NOTE: Base Number by ASTM D4739-02 may not be appropriate due to ester matrix interferences, which caused erratic curve plotting. Acid number by D664 seems to be unaffected.

NOTE: Green Range items are averages, red are upper limits. Range limits variant upon dataset size and are general guidelines only. Dotted blue lines are trend lines, solid purple lines are upper limit/average as per number in 'RANGE' column. Sample was analyzed as received and recommendations based on tests performed. Clark Laboratories LLC is not liable for any consequences resulting from use/abuse of the data and conclusions contained within this report. By receiving this report for services rendered, client accepts full responsibility and liability for all actions [illegible]

Page 1 of 1 LabNo: 238378

Clark Laboratories Report 24834 of 6/9/05 – Microcosm A Fuel chemistry



BCA Inc.
Fred Passman
3 Carlyle Court
Princeton, NJ 08543-3659
Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC
Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2253

Lube Supplier	BCA Inc.	BCA	Level 3 Make: unknown	Machine Condition
Unknown	Special Testing	Special	Model	Unknown
Lube Type	Biodeterioration	Biodet	Level 4 Make	Lubricant Condition
Unknown	NREL A	NREL A	Model	Unknown
	Test Group(s): B16 B14 B13		Serial No	

Lab #:	242834
Work Order:	
Date:	6/9/05
Time on Unit:	0.0
Time on Oil:	0.0
Mach Cond:	S
Lube Cond:	S

			06/09/05	RANGE
Water by Karl Fischer			<0.00 pcm	

			06/09/05	RANGE
TAN			0.05	

			06/09/05	RANGE
TBN			<1.0	

Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

NOTE: Green Range limits are averages, values appear limits. Range limits based upon dataset size and are general guidelines only. Dotted blue lines are trend lines, solid purple lines are upper limit/average or upper number in RANGE column. Sample was analyzed as received and recommendations based on tests performed. Clark Laboratories LLC is not liable for any consequences resulting from omissions in the data and conclusions contained within this report. By receiving this report for services rendered, client accepts all responsibility and liability for all actions occurring from evaluation of this data.

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LabNo: 242834

Clark Laboratories Report 24835 of 6/9/05 – Microcosm B Fuel chemistry

BCA Inc.
Fred Passman
3 Carlyle Court

Princeton, NJ 08543-3659

Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC

Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2255

Lube Supplier	BCA Inc.	BCA	Level 3 Make: unknown	Machine Condition
Unknown	Special Testing	Special	Model	Unknown
Lube Type	Biodeterioration	Biodet	Level 4 Make:	
Unknown	NREL B	NREL B	Model	Lubricant Condition
	Test Group(s): B16 B14 B13		Serial No:	Unknown

Lab #:	242835
Work Order:	
Date:	6/9/05
Time on Unit:	0.0
Time on Oil:	0.0
Mach Cond:	6
Lube Cond:	6

KF (D6304-04)				06/09/05	RANGE
Water by Karl Fischer				86.00 ppm	

TAN (D664-04) mg KOH/g				06/09/05	RANGE
TAN				0.08	

TBN (D4739-02) mg HCl/g				06/09/05	RANGE
TBN				<0.0	

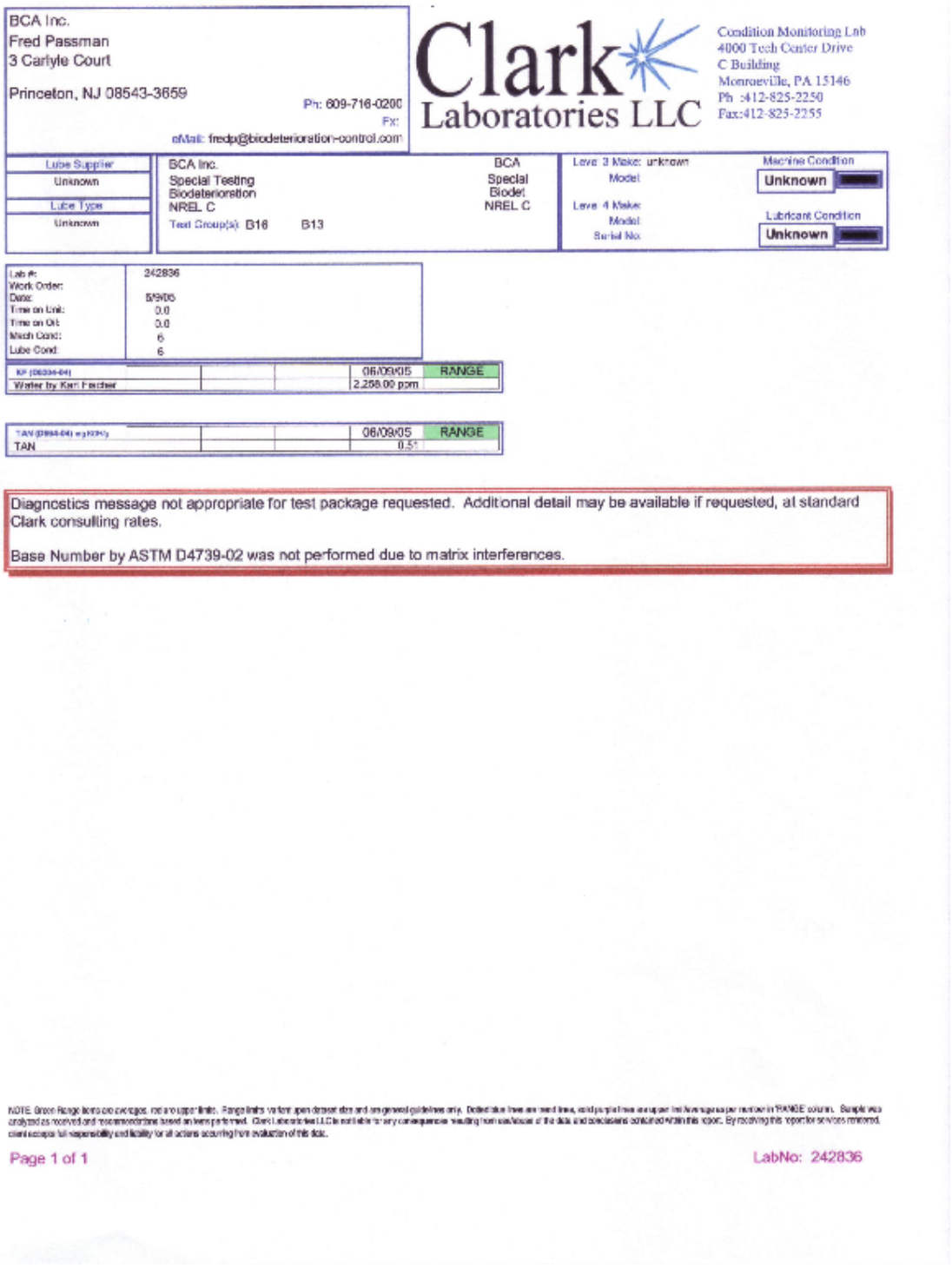
Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

NOTE: Green Range bars are averages, red are upper limits. Range limits vary upon dataset size and are general guidelines only. Dotted blue lines are trend lines, solid purple lines are upper limit average as per number in "RANGE" column. Samples was analyzed as received and recommendations based on tests performed. Clark Laboratories LLC is not liable for any consequences resulting from use/abuse of the data and conclusions contained within this report. By receiving this report for services rendered, client accepts full responsibility and liability for all actions occurring from evaluation of this data.

Page 1 of 1

LabNo: 242835

Clark Laboratories Report 24836 of 6/9/05 – Microcosm C Fuel chemistry



BCA Inc.
Fred Passman
3 Carlyle Court

Princeton, NJ 08543-3659

Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC

Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2255

Lube Supplier	BCA Inc.	BCA	Level 3 Make: unknown	Machine Condition
Unknown	Special Testing	Special	Model	Unknown
Lube Type	Biodeterionstion	Biodet	Level 4 Make	
Unknown	NREL C	NREL C	Model	Lubricant Condition
	Test Group(s): B16 B13		Serial No.	Unknown

Lab #:	242836
Work Order:	
Date:	5/9/05
Time on Unit:	0.0
Time on Oil:	0.0
Mech Cond:	6
Lube Cond:	6

			06/09/05	RANGE
Water by Karl Fischer			2,258.00 ppm	

			06/09/05	RANGE
TAN			0.51	

Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

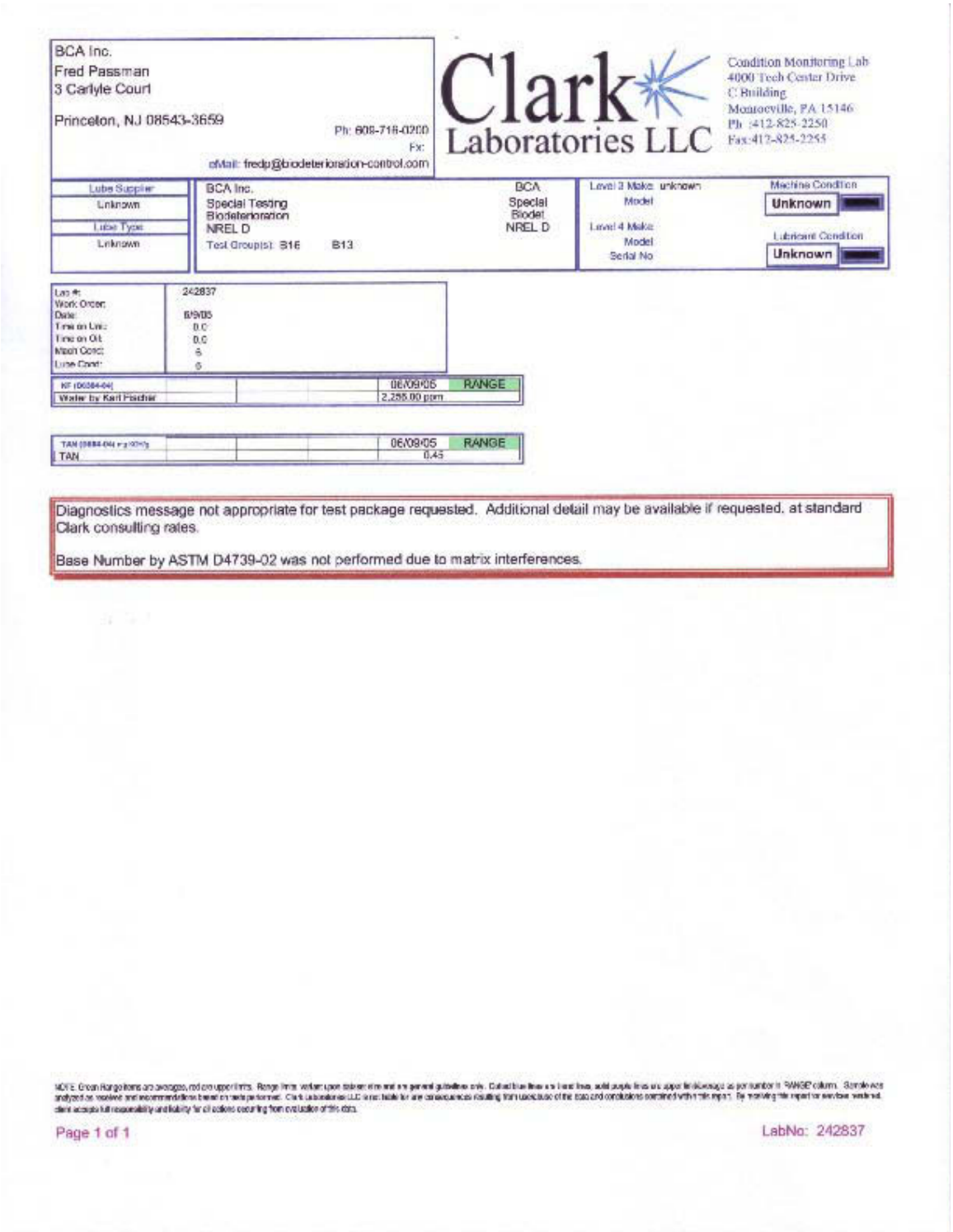
Base Number by ASTM D4739-02 was not performed due to matrix interferences.

NOTE: Green Range bars are averages, not are upper limits. Range limits vary for each data set and are general guidelines only. Dotted blue lines are next lines, and purple lines are upper limit lines appropriate number in RANGE column. Sample was analyzed as received and recommendations based on tests performed. Clark Laboratories LLC is not liable for any consequences resulting from use/reuse of the data and conclusions contained within this report. By receiving this report for services rendered, client accepts full responsibility and liability for all actions occurring from evaluation of this data.

Page 1 of 1

LabNo: 242836

Clark Laboratories Report 24837 of 6/9/05 – Microcosm D Fuel chemistry



BCA Inc.
Fred Passman
3 Carlyle Court

Princeton, NJ 08543-3659

Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC

Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2255

Lube Supplier	BCA Inc.	BCA	Level 3 Make: unknown	Machine Condition
Unknown	Special Testing	Special	Model	Unknown
Lube Type	Biodeterioration	Biodet	Level 4 Make:	Lubricant Condition
Unknown	NREL D	NREL D	Model	Unknown
	Test Group(s): B16 B13		Serial No	

Lab #:	242837
Work Order:	
Date:	6/9/05
Time on Unit:	0.0
Time on Oil:	0.0
Mach Cond:	5
Lube Cond:	5

KF (D6304-04)			06/09/05	RANGE
Water by Karl Fischer			2,288.00 ppm	

TAN (D664-04) e1 (KOH/g)			06/09/05	RANGE
TAN			0.43	

Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

Base Number by ASTM D4739-02 was not performed due to matrix interferences.

NOTE: Green Range Icons are averages, red are upper limits. Range limits written upon laboratory review are general guidelines only. Colored blue lines are trend lines, solid purple lines are upper limits/averages as per number in 'RANGE' column. Samples are analyzed as received and recommendations based on tests performed. Clark Laboratories LLC is not liable for any consequences resulting from use/misuse of the data and conclusions contained within this report. By receiving this report or sample received, client accepts full responsibility and liability for all actions occurring from evaluation of this data.

Page 1 of 1

LabNo: 242837

Clark Laboratories Report 24838 of 6/9/05 – Microcosm E Fuel chemistry

BCA Inc.
Fred Passman
3 Carlyle Court

Princeton, NJ 08543-3659

Ph: 609-716-0200
Fx:
eMail: fredp@biodeterioration-control.com

Clark Laboratories LLC

Condition Monitoring Lab
4000 Tech Center Drive
C Building
Monroeville, PA 15146
Ph :412-825-2250
Fax:412-825-2255

Lube Supplier	BCA Inc.	BCA	Level 3 Make: unknown	Machine Condition
Unknown	Special Testing	Special	Model:	Unknown
Lube Type	Biodeterioration	Biodet	Level 4 Make:	Lubricant Condition
Unknown	NREL E	NREL E	Model:	Unknown
	Test Group(s): B16 B13		Serial No:	

Lab #:	242838
Work Order:	
Date:	6/9/05
Time on Unit:	0.0
Time on Oil:	0.0
Mach Cond:	5
Lube Cond:	5

		06/09/05	RANGE
Water by Karl Fischer		2,311.00 ppm	

		06/09/05	RANGE
TAN		0.48	

Diagnostics message not appropriate for test package requested. Additional detail may be available if requested, at standard Clark consulting rates.

Base Number by ASTM D4739-02 was not performed due to matrix interferences.

NOTE: Green Range bars are averages, not one-sided limits. Ranges for individual samples are general guidelines only. Dotted baselines are trend lines, while purple lines are upper limit/average as per number in RANGE column. Samples were analyzed as received and recommendations based on past performance. Clark Laboratories LLC is not liable for any consequences resulting from evaluation of the data and conclusions contained within this report. By receiving this report for analysis, client accepts full responsibility and liability for all actions occurring from evaluation of the data.

LabNo: 242838

EMSL Analytical Case No: 360500219 of 03/09/05 Modified NACE TM0172

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Materials Science Division

Attn.: *Dr. Jason Dobranic*
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500219
Sample(s) Received: 03/04/05
Date of Analysis: 03/07/05
Date Printed: 03/09/05
Reported By: L. Katipelli

- Laboratory Report -

Modified NACE Standard TM0172

For

Project: NREL Biodiesel Biodegradability

Analyzed by: K. Lalitha Reddy — *Lalitha R. Katipelli, Materials Engineer* — March 08, 2005 — *Date*

QA/QC : Jian Hu — *Jian Hu, Ph.D., Senior Materials Scientist* — March 08, 2005 — *Date*

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Attn.: *Dr. Jason Dobranic*
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500219
Sample(s) Received: 03/04/05
Date of Analysis: 03/07/05
Date Printed: 03/09/05
Reported By: L. Katipelli

Procurement of Samples and Analytical Overview:

The sample for analysis arrived at the Materials Science Division of EMSL Analytical's corporate laboratory in Westmont, NJ on March 04, 2005. The package arrived in satisfactory condition with no evidence of damage to the contents. The sample was submitted for the purpose of determining corrosive properties according to NACE TM0172 standard test method (modified).

Testing Procedure:

A. A cylindrical test specimen of polished carbon steel 5.01 mm (diameter) x 59 mm (length) was used.

B. 300 mL of the sample ("NREL CME Filtered") was poured into a 400 mL glass beaker, which was placed in a water bath. This assembly was placed on a heater; the temperature of the fuel inside the beaker was maintained at 38°C. A magnetic stirrer was placed inside the beaker. Stirring was maintained at a setting that created sufficient agitation but did not cause visible turbulence on the surface of the fuel sample; the setting was marked.

C. After the temperature of the fuel sample reached 38°C, the test specimen was suspended so that the specimen was completely submerged in the sample and its lower end was ~15 mm from the bottom of the beaker.

D. After 30 minutes, 30 mL of a provided solution (Davis Salts) was injected into the beaker. Stirring was continued for 3.5 hours at 38°C while the beaker was covered to avoid any loss of liquid.

E. The test specimen was removed from the mixture, cleaned with acetone, and inspected.

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Attn.: Dr. Jason Dobranic
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500219
Sample(s) Received: 03/04/05
Date of Analysis: 03/07/05
Date Printed: 03/09/05
Reported By: L. Katipelli

Results and Discussion:

Rating of the test specimen is based on the scale provided in NACE TM0172 standard test method.

Rating	Percent of Test Surface Corroded
B++	Less than 0.1 (2 or 3 spots of no more than 1 mm diameter)

EMSL Analytical Case No: 360500555 of 06/08/05 Modified NACE TM0172

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Materials Science Division

Attn.: Dr. Jason Dobranic
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500555
Sample(s) Received: 06/08/05
Date of Analysis: 06/17/05
Date Printed: 06/17/05
Reported By: L. Katipelli

- Laboratory Report -

Modified NACE Standard TM0172

Analyzed by: K. Lalitha Reddy — *Lalitha R. Katipelli, Materials Scientist* — June 17, 2005 — *Date*

QA/QC: Jian Hu — *Jian Hu, Ph.D., Senior Materials Scientist* — June 17, 2005 — *Date*

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Attn.: Dr. Jason Dobranic
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500555
Sample(s) Received: 06/08/05
Date of Analysis: 06/17/05
Date Printed: 06/17/05
Reported By: L. Katipelli

Procurement of Samples and Analytical Overview:

The samples for analysis arrived at the Materials Science Division of EMSL Analytical's corporate laboratory in Westmont, NJ on June 08, 2005. The package arrived in satisfactory condition with no evidence of damage to the contents. The samples were submitted for the purpose of determining corrosive properties according to NACE TM0172 standard test method (modified).

Testing Procedure:

Each of the 5 tests were performed as follows:

1. A cylindrical test specimen of polished carbon steel 5.0 mm ± 0.1mm(diameter) x 60mm ±1.0mm (length) was used.
2. 300 mL of the fuel sample was poured into a 400 mL glass beaker, which was placed in a water bath. This assembly was placed on a heater; the temperature of the fuel inside the beaker was maintained at 38°C. A magnetic stirrer was placed inside the beaker. Stirring was maintained at a setting that created sufficient agitation but did not cause visible turbulence on the surface of the fuel sample; the setting was marked.
3. After the temperature of the fuel sample reached 38°C, the test specimen was suspended so that the specimen was completely submerged in the sample and its lower end was ~15 mm from the bottom of the beaker.
4. After 30 minutes, 30 mL of a provided solution (Davis Mim.) was injected into the beaker. Stirring was continued for 3.5 hours at 38°C while the beaker was covered to avoid any loss of liquid.
5. The test specimen was removed from the mixture, cleaned with acetone, and inspected.

EMSL Analytical, Inc.

107 Haddon Avenue, Westmont, NJ 08108
Phone: (856) 858-4800

Attn.: Dr. Jason Dobranic
EMSL- Westmont
Microbiology Lab
107 Haddon Avenue
Westmont, NJ 08108
Phone: 856-858-4800 Fax: 856-858-4960

EMSL Case No.: 360500555
Sample(s) Received: 06/08/05
Date of Analysis: 06/17/05
Date Printed: 06/17/05
Reported By: L. Katipelli

Results and Discussion:

Rating of the test specimens is based on the scale provided in NACE TM0172 standard test method.

Sample ID	Description	Rating	Percent of Test Surface Corroded
A	LSD CIRC	A	0
B		A	0
C	CME-100	A	0
D	CME-100 + Kathon	A	0
E	F.S. CME-100 + Sterile D.S.	A	0

EMSL Analytical Case No: 370501236 of 03/28/05 Water Chemistry

Asbestos • Lead • Environmental • Materials & Indoor Air Analysis

EMSL Analytical

http://www.emsl.com

3 Cooper St.
Westmont, NJ 08108
Phone: (856) 858-4800
Fax: (856) 858-4571

Attn: **Cherrie**
INTER DEPT ANALYSIS
For Samples Logged into one dept
but analyzed by another
Westmont, NJ 08108

3/28/2005

Phone
Fax:

The following report covers the analysis performed on samples submitted to EMSL Analytical on 3/4/2005. The results are tabulated on the attached data pages for the following client designated project:

The reference number for these samples is EMSL Order #010500753. Please use this reference when calling about these samples.

If you have any questions, please do not hesitate to contact me at (856) 858-4800.

Reviewed and Approved By:

Laboratory Director or other
approved signatory
NJ-NELAP Accredited:04653

ACCREDITED IN ACCORDANCE WITH nelac

The test results contained within this report meet the requirements of NELAC and/or the specific certification program that is applicable, unless otherwise noted.

Page 1 of 2

EMSL Analytical
3 Cooper St., Westmont, NJ 08108
Phone: (856) 858-4800 Fax: (856) 858-4571 Email: swesson@emsl.com

Attn: **Cherrie**
INTER DEPT ANALYSIS
For Samples Logged into one dept
but analyzed by another
Westmont, NJ 08108
Fax: Phone:

Customer ID: +04INTDEP
Customer PO: 370501236
Received: 03/04/05 4:45 PM
EMSL Order: 010500753
EMSL Proj:
Report Date: 3/28/2005

Client Sample Description 370501236 *Collected:* 3/4/2005 *Lab ID:* 0001

Test	*Method*	*Parameter*	*Concentration*	*Units*	*RL*	*Analysis Date/Time*	*Analyst*
Hardness as CaCO3	130.2	Hardness as CaCO3	2.02	mg/L	2	3/21/2005 12:30 PM	hpandhi
pH	150.1	pH	7.13	ph Units	n/a	3/8/2005 01:30 PM	mschafer
Total Dissolved Solids	160.1	Total Dissolved Solids	9800	mg/L	5	3/8/2005 04:25 PM	shume
Alkalinity as CaCO3	310.1	Alkalinity	1600	mg/L	2	3/9/2005 02:00 PM	hpandhi
Total Organic Carbon	415.1	Total Organic Carbon	97.8	mg/L	1	3/23/2005 05:09 PM	hpandhi

Page 2 of 2

EMSL Analytical Case No: 370501236 of 06/24/05 Water Chemistry

Asbestos • Lead • Environmental • Materials & Indoor Air Analysis

EMSL Analytical

http://www.emsl.com

3 Cooper St.
Westmont, NJ 08108
Phone: (856) 858-4800
Fax: (856) 858-4571

Attn: **Jason Dobranic**
INTER DEPT ANALYSIS
For Samples Logged into one dept
but analyzed by another
Westmont, NJ 08108

6/24/05

Phone
Fax:

The following report covers the analysis performed on samples submitted to EMSL Analytical on 6/7/05. The results are tabulated on the attached data pages for the following client designated project:

Project ID: BCA

The reference number for these samples is EMSL Order #010502114. Please use this reference when calling about these samples.

If you have any questions, please do not hesitate to contact me at (856) 858-4800.

Reviewed and Approved By:

Laboratory Director or other
approved signatory
NJ-NELAP Accredited:04653

ACCREDITED IN ACCORDANCE WITH nelac

The test results contained within this report meet the requirements of NELAC and/or the specific certification program that is applicable, unless otherwise noted.

Page 1 of 3

EMSL Analytical
3 Cooper St., Westmont, NJ 08108
Phone: (856) 858-4800 Fax: (856) 858-4571 Email: swesson@emsl.com

Attn: **Jason Dobranic**
INTER DEPT ANALYSIS
For Samples Logged into one dept
but analyzed by another
Westmont, NJ 08108
Fax: Phone:

Customer ID: +04INTDEP
Customer PO: 370501236
Received: 06/07/05 3:40 PM
EMSL Order: 010502114
EMSL Proj: BCA
Report Date: 6/24/05

Client Sample Description A — *Collected:* 6/6/05 4:30:00 PM — *Lab ID:* 0001

Test	Method	Parameter	Concentration	Units	RL	Analysis Date/Time	Analyst
Hardness as CaCO3	130.2	Hardness as CaCO3	32.3	mg/L	2	6/17/05 08:00 AM	hpandhi
pH	150.1	pH	6.79	ph Units	n/a	6/14/05 01:30 PM	tlech
Total Dissolved Solids	160.1	Total Dissolved Solids	12000	mg/L	5	6/14/05 02:00 PM	tlech
Alkalinity as CaCO3	310.1	Alkalinity	1800	mg/L	2	6/17/05 09:30 AM	hpandhi
Total Organic Carbon	415.1	Total Organic Carbon	1400	mg/L	1	6/21/05 02:00 PM	hpandhi

Client Sample Description B — *Collected:* 6/6/05 4:30:00 PM — *Lab ID:* 0002

Test	Method	Parameter	Concentration	Units	RL	Analysis Date/Time	Analyst
Hardness as CaCO3	130.2	Hardness as CaCO3	48.6	mg/L	2	6/17/05 08:00 AM	hpandhi
pH	150.1	pH	6.86	ph Units	n/a	6/14/05 01:30 PM	tlech
Total Dissolved Solids	160.1	Total Dissolved Solids	13000	mg/L	5	6/14/05 02:00 PM	tlech
Alkalinity as CaCO3	310.1	Alkalinity	3500	mg/L	2	6/17/05 09:30 AM	hpandhi
Total Organic Carbon	415.1	Total Organic Carbon	350	mg/L	1	6/21/05 02:00 PM	hpandhi

Client Sample Description C — *Collected:* 6/6/05 4:30:00 PM — *Lab ID:* 0003

Test	Method	Parameter	Concentration	Units	RL	Analysis Date/Time	Analyst
Hardness as CaCO3	130.2	Hardness as CaCO3	130	mg/L	2	6/17/05 08:00 AM	hpandhi
pH	150.1	pH	6.21	ph Units	n/a	6/14/05 01:30 PM	tlech
Total Dissolved Solids	160.1	Total Dissolved Solids	13000	mg/L	5	6/14/05 02:00 PM	tlech
Alkalinity as CaCO3	310.1	Alkalinity	1500	mg/L	2	6/17/05 09:30 AM	hpandhi
Total Organic Carbon	415.1	Total Organic Carbon	1300	mg/L	1	6/21/05 02:00 PM	hpandhi

Page 2 of 3

EMSL Analytical
3 Cooper St., Westmont, NJ 08108
Phone: (856) 858-4800 Fax: (856) 858-4571 Email: swesson@emsl.com

Attn: **Jason Dobranic**
INTER DEPT ANALYSIS
For Samples Logged into one dept
but analyzed by another
Westmont, NJ 08108

Fax: Phone:

Customer ID: +04INTDEP
Customer PO: 370501236
Received: 06/07/05 3:40 PM
EMSL Order: 010502114
EMSL Proj: BCA
Report Date: 6/24/05

Client Sample Description D — *Collected:* 6/6/05 4:30:00 PM — *Lab ID:* 0004

Test	Method	Parameter	Concentration	Units	RL	Analysis Date/Time	Analyst
Hardness as CaCO3	130.2	Hardness as CaCO3	93.6	mg/L	2	6/17/05 08:00 AM	hpandhi
pH	150.1	pH	6.33	ph Units	n/a	6/14/05 01:30 PM	tlech
Total Dissolved Solids	160.1	Total Dissolved Solids	12000	mg/L	5	6/14/05 02:00 PM	tlech
Alkalinity as CaCO3	310.1	Alkalinity	1000	mg/L	2	6/17/05 09:30 AM	hpandhi
Total Organic Carbon	415.1	Total Organic Carbon	2400	mg/L	1	6/21/05 02:00 PM	hpandhi

Client Sample Description E — *Collected:* 6/6/05 4:30:00 PM — *Lab ID:* 0005

Test	Method	Parameter	Concentration	Units	RL	Analysis Date/Time	Analyst
Hardness as CaCO3	130.2	Hardness as CaCO3	100	mg/L	2	6/17/05 08:00 AM	hpandhi
pH	150.1	pH	4.70	ph Units	n/a	6/14/05 01:30 PM	tlech
Total Dissolved Solids	160.1	Total Dissolved Solids	14000	mg/L	5	6/14/05 02:00 PM	tlech
Acidity as CaCO3	2310B	Acidity	3700	mg/L	2	6/24/05 03:30 PM	jwalker
Alkalinity as CaCO3	310.1	Alkalinity	<20.0	mg/L	2	6/17/05 09:30 AM	hpandhi
Note: 502114-5 for Alkalinity reported with a higher detection limit due to - Limited Sample volume and matrix.							
Total Organic Carbon	415.1	Total Organic Carbon	1300	mg/L	1	6/21/05 02:00 PM	hpandhi

Page 3 of 3

EMSL Analytical Case No: 370501576 of 03/07/05 Water and Fuel Microbiology

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3650
Attn: Dr. Fred Pasanen
Project: NREL

EMSL Reference: 370501576
Date Received: 3/1/2005
Date Analyzed: 3/2/2005
Date Reported: 3/7/2005

Culturable Bacteria - Plate Count Method (EMSL M009)

Sample	Location	Media	Bacterial Count	Concentration	Comments
1	Time 0 Sterile Control, Bottom Water	TSA	No growth	< 1000 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100184

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL

EMSL Reference: **370501576**
Date Received: 3/1/2005
Date Analyzed: 3/4/2005
Date Reported: 3/7/2005

Culturable Fungi - Plate Count Method (EMSL M005)

Sample	Location	Media	Fungal Count	Concentration	Comments
2	Time 0 Sterile Control, Bottom Water	MEA	No growth	< 1000 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL

EMSL Reference: **370501576**
Date Received: 3/1/2005
Date Analyzed: 3/4/2005
Date Reported: 3/7/2005

Enumeration of Viable Bacteria in Liquid Fuels (ASTM D6974)

Sample	Location	Media	Bacterial Count	Concentration	Comments
3	Time 0 Sterile Control, Fuel	TSA	No growth	< 10 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL

EMSL Reference: **370501576**
Date Received: 3/1/2005
Date Analyzed: 3/4/2005
Date Reported: 3/7/2005

Enumeration of Viable Fungi in Liquid Fuels (ASTM D6974)

Sample	Location	Media	Fungal Count	Concentration	Comments
4	Time 0 Sterile Control, Fuel	MEA	No growth	< 10 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical Case No: 370501576 of 06/02/05 Water and Fuel Microbiology

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL Time= Final

EMSL Reference: 370501576
Date Received: 6/6/2005
Date Analyzed: 6/16/2005
Date Reported: 6/20/2005

Culturable Bacteria - Plate Count Method (EMSL M009)

Sample	Location	Media	Bacterial Count	Concentration	Comments
A	LSD	TSA	>300	>300,000,000 CFU/L	
B		TSA	>300	>300,000,000 CFU/L	
C	CME-100	TSA	60	60,000 CFU/L	
D	CME-100 + Kathon	TSA	75	75,000 CFU/L	
E	F.S. CME-100 + Sterile D.W	TSA	110	110,000 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL Time= Final

EMSL Reference: **370501576**
Date Received: 6/6/2005
Date Analyzed: 6/16/2005
Date Reported: 6/20/2005

Culturable Fungi - Plate Count Method (EMSL M005)

Sample	Location	Media	Fungal Count	Concentration	Comments
A	LSD	MEA	>300	>300,000,000 CFU/L	
B		MEA	37	370000 CFU/L	
C	CME-100	MEA	No Growth	<10 CFU/L	
D	CME-100 + Kathon	MEA	No Growth	<10 CFU/L	
E	F.S. CME-100 + Sterile D.W	MEA	1	1000 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL Time= Final

EMSL Reference: 370501576
Date Received: 6/6/2005
Date Analyzed: 6/16/2005
Date Reported: 6/20/2005

Enumeration of Viable Bacteria in Liquid Fuels (ASTM D6974)

Sample	Location	Media	Bacterial Count	Concentration	Comments
A	LSD	TSA	49	2450 CFU/L	
B		TSA	24	1200 CFU/L	
C	CME-100	TSA	No Growth	>10 CFU/L	
D	CME-100 + Kathon	TSA	No Growth	>10 CFU/L	
E	F.S. CME-100 + Sterile D.W	TSA	2	100 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

EMSL Analytical, Inc.
107 Haddon Avenue, Westmont, NJ. 08108 (856) 858-4800

Client: BCA, Inc.
3 Carlyle Court
Princeton, NJ 08543-3659
Attn. Dr. Fred Passman
Project: NREL Time= Final

EMSL Reference: **370501576**
Date Received: 6/6/2005
Date Analyzed: 6/16/2005
Date Reported: 6/20/2005

Enumeration of Viable Fungi in Liquid Fuels (ASTM D6974)

Sample	Location	Media	Fungal Count	Concentration	Comments
A	LSD	MEA	22	1100 CFU/L	
B		MEA	1	50 CFU/L	
C	CME-100	MEA	No Growth	<10 CFU/L	
D	CME-100 + Kathon	MEA	No Growth	<10 CFU/L	
E	F.S. CME-100 + Sterile D.W	MEA	No Growth	<10 CFU/L	

CFU = Colony Forming Unit

Concentration is reported in CFUs/L unless otherwise noted

AIHA EMLAP Lab ID # 100194

Approved EMSL Signatory
Jason Dobranic, PhD
National Director of Microbiology

1 of 1

Appendix 2.

FTIR/ATR Method for Biodiesel Blend Determination

Standard Test Method for Determination of Biodiesel in Diesel Fuel Oil Using Mid Infrared Spectroscopy

This method is derivative to ASTM D 6277 Standard Test Method for Determination of Benzene in Spark-Ignition Engine Fuels Using Mid Infrared Spectroscopy

Summary of Test Method

A sample of diesel fuel is introduced into a liquid sample cell. A beam of infrared light is imaged through the sample onto a detector, and the detector response is determined. Wavelengths of the spectrum that correlates highly with biodiesel or interferences are selected for analysis by mathematically selecting areas of the whole spectrum. A multivariate mathematical analysis converts the detector response for the selected areas of the spectrum of an unknown to a concentration of biodiesel

Instrument:
Nicolet Magna 550 Fourier Transform Mid-Infrared Spectrometer, 4000 to 650 cm^{-1}, resolution 4 cm^{-1}

Cell:
Axiom Analytical, Inc, FT-IR Attenuated Total Reflectance (ATR) Tunnel Cell, TNL-120A, ZnSe Element 60 degree, housing with 0.125 inch Swagelok connections.

ATR element material ZnSe
beam condensing optics conical, non-focusing optics
integral to cell body
element configuration circular cross section with
coaxial conical ends
cone half angle 60°
element length 1.55 in.
element diameter 0.125 in.
angle of incidence at
sample interface 53.8°
maximum range of
incidence angles 6 1.5°
standard absorbance
(1428 cm-1 band of acetone) 0.38 6 0.02 AU
material of construction 316 stainless steel
seals Chemraz or Kalraz o-rings

Calibration

Analysis type:	Partial Least Squares
Pathlength type:	Constant
Component name:	Biodiesel
Standards:	biodiesel in diesel fuel (0-25% biodiesel) Number of standards and concentrations to be determined
Spectrum Range:	1810.8-1669.5
Baseline type:	Two points, point1 1838.0, point2 1547.0
Measurement location:	1810.8-1669.5
Corrections:	No corrections
Other:	use mean centering technique
Factors used:	1

Preliminary Results: Based on two soy-based sources of B100, this method will measure the biodiesel +/-7% relative (@20% +/-1.4%). Better accuracy is expected with known source of biodiesel.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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T.L. Alleman and R.L. McCormick

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NREL tested Philippines coconut biodiesel samples of neat and blended fuels. Results show that the current fuel quality standards were met with very few exceptions. Additional testing is recommended.

15. SUBJECT TERMS
coconut; biodiesel; Philippines; fuel quality standards; fuel property; fuel stability; microbial deterioration; CME; coconut methy ester; DA; additized diesel fuel; DUA; unadditized diesel fuel

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