Biodegradability, BOD_5, COD and Toxicity of Biodiesel Fuels

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The purpose of this chapter is to summarize the University of Idaho research related to biodegradability, biochemical oxygen demand, chemical oxygen demand and toxicity of biodiesel. These studies were conducted in the mid-nineties using neat oils and biodiesel from a variety of feedstocks including soy, canola, rapeseed, and others. Both methyl and ethyl esters were included in most studies. Phillips 2-D low sulfur reference diesel fuel (2-D) was used for a comparison petroleum diesel in all of the studies. In some studies, blends of the 2-D reference fuel with the test vegetable oil based fuels were included as noted in each section.

Fuel nomenclature is as follows:

- Phillips 2-D low-sulfur diesel control fuel (2~D)
- 100 % rapeseed methyl ester (RME)
- 100 % rapeseed ethyl ester (REE)
- 50 % RME - 50 % 2-D (50RME)
- 50 % REE - 50 % 2-D (50REE)
- 20 % RME - 80 % 2-D (20RME)
- 20 % REE - 80 % 2-D (20REE)

Biodegradability

The biodegradability of various biodiesel fuels in the aquatic and soil environments was examined by the CO2 evolution method, chromatography (GC) analysis, and seed germination. The fuels examined included neat rapeseed oil, neat soybean oil, the methyl- and ethyl- esters of rapeseed oil and soybean oil, and Phillips 2-D reference petroleum diesel. Blends of biodiesel petroleum diesel at different volumetric ratios, including 80/20, 50/50, 20/80, were also examined in the aquatic phase.

There are many test methods for accessing the biodegradability of an organic compound. Among them, the CO, evolution test (shaker flask system) and gas chromatography (GC) analysis are most common and were employed as the major method for the aquatic and soil experiments, respectively. One important difference between them is that C02 evolution measures ultimate degradation (mineralization) in which a substance is broken down to the final products, C02 and water while GC analysis measures primary degradation only in which the substance is not necessarily transformed to the end products. Finally, re-vegetation of soils contaminated by fuel spills is often a desirable goal, seed germination was employed to evaluate toxicity of biodiesel on plants in the soil system.

The C02 evolution method employed in this work followed the EPA standard method 560/6-82-003 for determining biodegradability of chemical substances (Zhang et al., 1995). A brief review of this method follows.

A specially equipped 2 liter Erlenmeyer flask (Fig. 1) contained 100 ml of inoculum (prepared from soil, activated sewage, raw domestic sewage water 14 days before the experiment), 1 ml of each stock solution, 900 ml deionized distilled water (DIW), and the 10 mg carbon of a test substance (10 mg/L).

A reservoir holding 10 ml of barium hydroxide solution was suspended in the flask to trap the C02 evolved from the media. After inoculation, the test flasks were sparged with C02 free air to ensure aerobic conditions and that C02 was trapped only from microorganism's metabolizing the test substance. The flasks were then sealed and incubated with shaking in a dark room for a 28 day period.

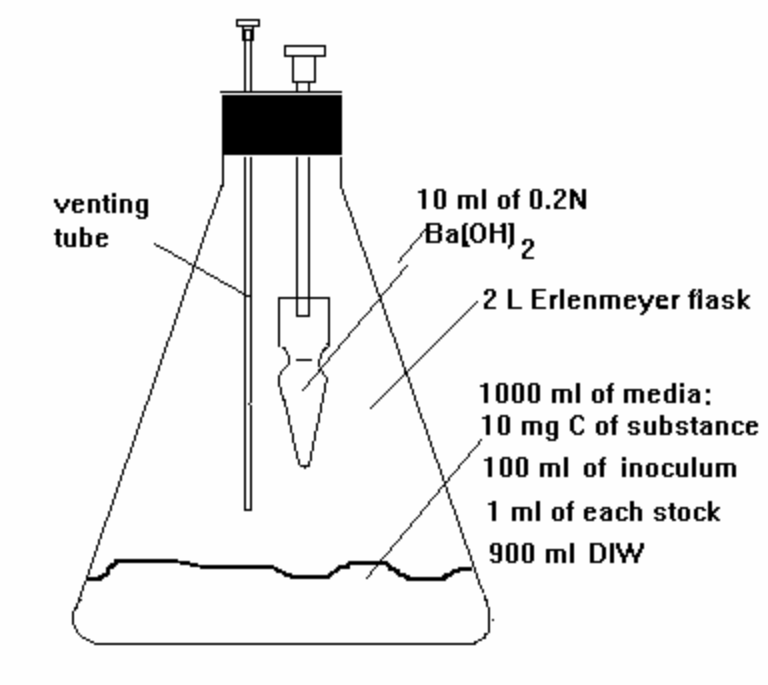

Figure 1. Shake Flask System for measuring biodegradability in the aquatic environment.

Periodically, the 10 ml of Ba(OH) 2 plus 10 ml DIW was removed for $C0_2$ measurement by titration with 0.1 N HC1 to the phenolphthalein end point. The reservoir was refilled with fresh Ba(OH) 2. All the samples were analyzed at least five times in a 28 day period to allow for a smooth biodegradation plot.

The GC method involved extraction of the samples with a solvent and injection of a

portion of the extract into a gas chromatograph. Quantitation was accomplished by using internal and external standards. In the extraction, the sample was first acidified to a pH of 2.0 or lower by adding 5 ml hydrochloric acid (1:1). One ml of internal standard (methyl 17:0 for biodiesel and 2-fluorobiphenyl for diesel) was then added to the sample and shaken to mix well. Finally, 30 ml of solvent (hexane for biodiesel and methylene chloride for 2-D) was added and the mixture was vigorously shaken for 1 minute. The layers were allowed to separate and the solvent layer was passed through a funnel containing sodium sulfate. The extract was transferred to a vial, sealed, and kept in a refrigerator (4 °C) prior to GC analysis.

Soil flask test - a 500 ml Erlenmeyer flask system was used in the soil flask tests. Thirty grams of dry weight soil were placed in the flask and thoroughly amended with the required amount of a test substance (10,000 mg/L) weighed by an accurate balance and stock solution. DIW was also added if necessary to bring the soil to 30% moisture level. Each flask was sealed and incubated at room temperature.

At each time interval, 2 grams of the soil (dry weight) were removed for extraction and GC analysis. The 2 g soil sample was placed into a 24 ml vial with a Teflon cap. The soil was mixed with the same volume of anhydrous sodium sulfate to absorb moisture in the sample. 1 ml of internal standard (the same as those in the aquatic system) was added into the vial to determine the extraction efficiency and serve as a quantitative standard. Immediately after adding the internal standard, 9 ml of extraction solvent (the same as those in the aquatic system) were added. Two milliliters of the extraction then was conducted in a bath sonicator for 30 minutes with less than 10 vials at one time. The extract was transferred into a vial, sealed, and kept in a refrigerator (4 °C) prior to GC analysis.

Seed germination involved four 32 cm x 6 cm (diameter x height) plates holding 2.0 kg soil (dry weight) which was contaminated with one of four different test substances including biodiesel REE, RME, and NR, and 2-D at an approximately average concentration of 50,000 mg/L. A plate with no substrate was used as the control. 100 seeds of Legacy Alfalfa were seeded in each of the fuel-contaminated plate and control at day 1, week 1, 3, and 6. The plates were covered by a thin plastic film (with small holes), kept in a green house to maintain a favorable temperature for microorganisms and plants, and watered periodically to maintain required moisture.

Results - The average cumulative percent theoretical C02 evolution from six biodiesel fuels NR, NS, REE, RME, SEE, and SME and 2-D in 28 days is summarized is shown in Figure 2 (All six samples in duplicate experiments were averaged and an arithmetic mean, standard deviation, and RSD% were calculated). The maximum percent C02 evolution from REE, RME, SEE, SME were between 84-89%, the same as that of dextrose. The statistical analysis indicates there is no difference in their biodegradability. The maximum percent C02 evolution from neat rapeseed oil and neat soybean oil were 78 and 76%, respectively, which are slightly lower than their modified products. Their

higher viscosity is one of the possible reasons accounting for this. The C02 evolution from 2-D was only 18.2% (averaged from several experiments).

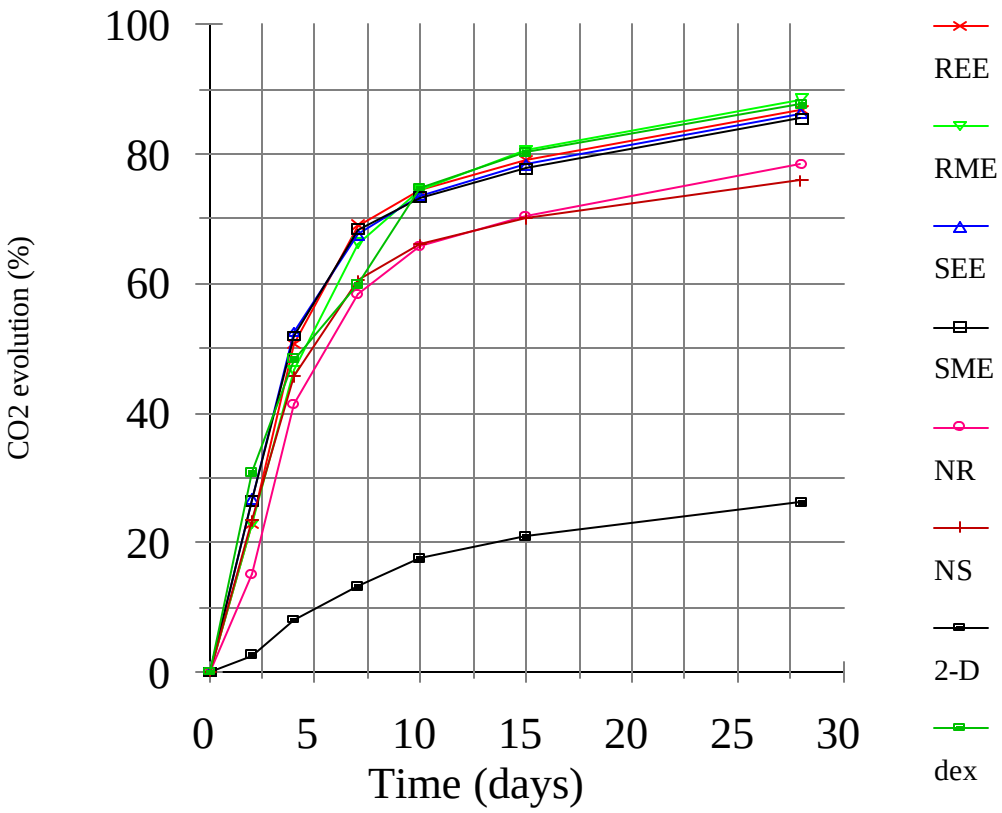

Figure 2. Biodegradability of Biodiesel compared to dextrose and 2-D reference fuel by the shake flask test.

The results demonstrate that all the biodiesel fuels are "readily biodegradable". Moreover, co-metabolism was observed in the biodegradation of the biodiesel diesel blend in the aquatic phase: in the presence of REE, the degradation rate and extent of petroleum diesel increased to twice that of petroleum diesel alone.

The GC analysis showed much faster degradation for REE and 2-D, Figure 3. The disappearance of REE and 2-D at day 1 reached 64% and 27%, respectively. At day 2, all the fatty acids in REE were not detectable while only 48% of 2-D had disappeared. However, the ratios of percent primary degradation to ultimate degradation for REE and 2-D were quite different, 1.2 versus 2.7. The lower ratio for REE indicates that most of biodiesel was transformed to the end products and the higher ratio for diesel implies that most of the diesel, about two-thirds out of 48% primary degradation, was changed to an intermediate product. To answer what are these intermediates needs further study.

Blends - The percent C02 evolution from REE/2-D blends, as shown in Figure 4 and Fig. 5, increased linearly with the increase of REE concentration in the blend. The higher the volume of REE in the blend, the higher the percentage of CO2 evolution.

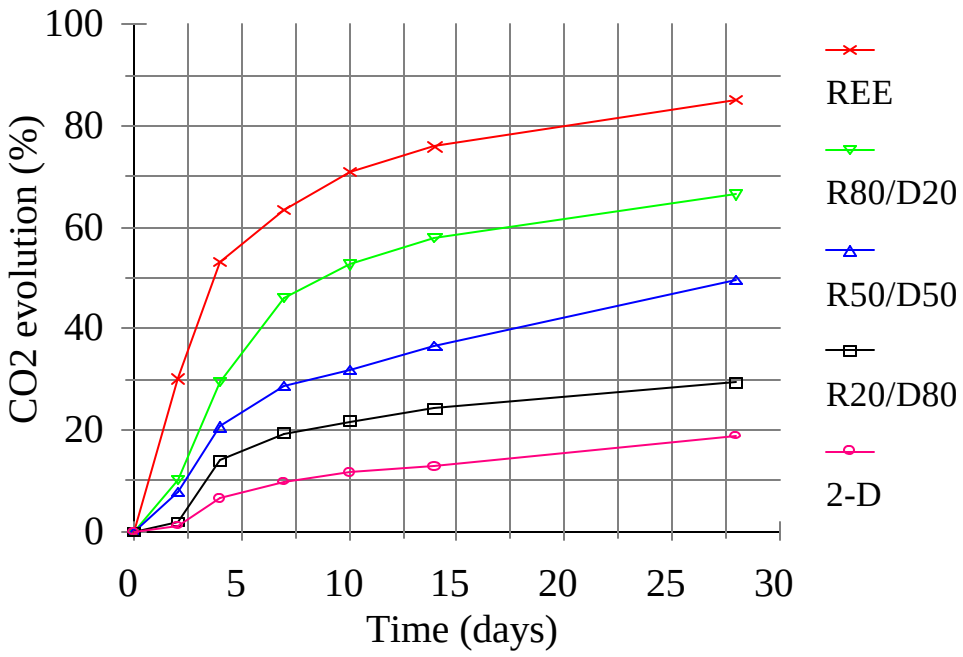

Figure 3. Biodegradability of blends of biodiesel and 2-D reference fuel as measured with the shake flask test.

The relation can be described by a linear equation Y = 0.629 X + 20.16 with R2 =0.992(95% confidence limit), where X = the percent concentration of REE in the blend and Y = the cumulative percent CO2 evolved in 28 days.

Again, GC analysis showed much faster and higher primary degradation in the REE/2-D 50/50 blend, 64% and 96% in day 1 and 2 respectively. In addition, co-metabolism was observed. The 2-D in the blend was degraded twice as fast as the 2-D alone as the substance; 63% versus 27% at day 1. This suggests that in the presence of REE, microorganisms use the fatty acids as an energy source to promote the degradation of 2-D.

Soil Flask Results - the average substrate disappearance versus time for five different biodiesel fuels and diesel at the initial concentration of 10,000 mg/L is summarized in Figure 6 (three samples for each substance were averaged). In 28 days, the percent substance disappearance for five biodiesel fuels reached 83-95%, with an average of 88% and for diesel was 52%.

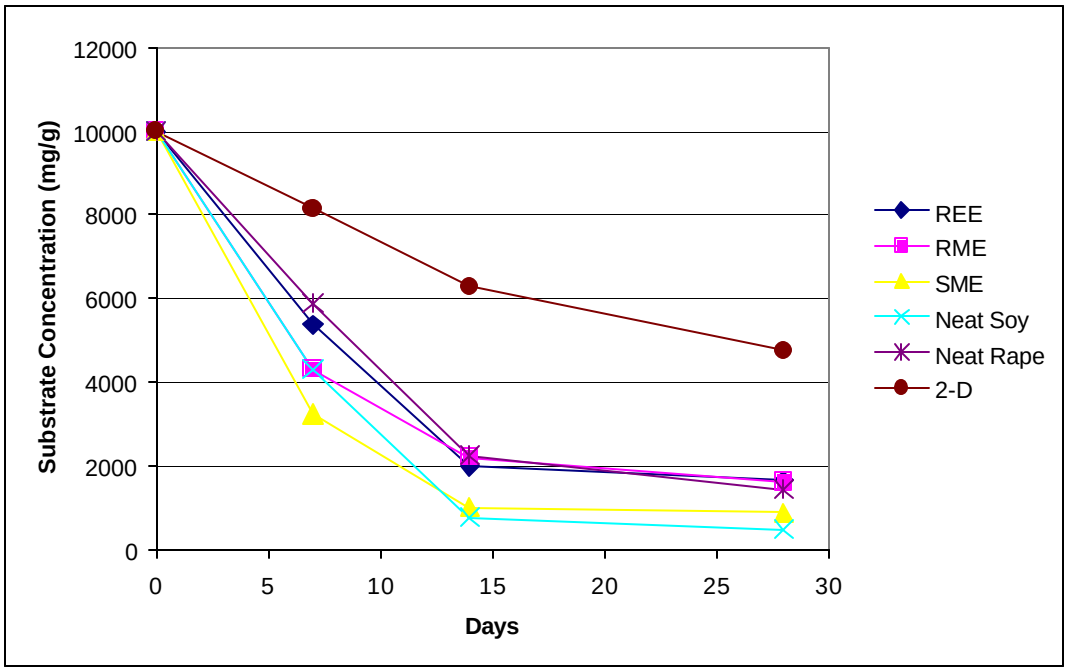

Figure 4. Substrate disappearance vs. time for five biodiesel fuels and diesel at an initial concentration of 10,000 mg/L in soil.

The results of the seed germination tests are shown in Table 1. Biodiesel fuels REE. RME, and NR-contaminated soils had lower seed germination rates at day 1 and week 1 seeding than week 3 and 4 since fungus grew rapidly across the plate in two weeks after contamination. About 20 of the seeds germinated but died underground. However, after week 3 when most of the biodiesel was degraded and fungus began to disappear visually, the seed germination rates in biodiesel-spilled plates increased. After week 6, the germination rates in all three biodiesel fuel-spilled plates reached 92-98%. Among the biodiesel fuels, neat rapeseed had the highest germination rate, about 87%.

Table 1.
Seed germination in the fuel-contaminated soils seeded at day 1, week 1, 3, and 6

Time of Seeding (day)	Seed germination (%)				
	Ctrl	Raw Rapeseed Oil	RME	REE	Diesel
1	100	84.0	60.5	51.9	19.8
7	100	76.1	55.4	73.9	62.0
21	100	91.1	82.2	83.3	87.8
42	100	95.4	92.0	97.7	19.5
avg.	100	86.6	72.5	76.7	47.3

The seeds in the 2-D-spilled plate germinated at least 7 days later than those in biodiesel-spilled plates in the first seeding. Moreover, fungus growth was not observed in the diesel-spilled plate until week 4. This is probably the reason why the seed germination in the diesel-spilled plate dropped dramatically after week 4.

These results demonstrate that biodegradation can restore a biodiesel fuel contaminated soil in 4-6 weeks to a degree that it can support plant germination.

Biodegradability Conclusions

1. All the biodiesel fuels are "readily biodegradable" in the aquatic and soil environments. During a 28 day period, average C02 evolution for all the biodiesel reached 84% in the aqueous system and the average substance disappearance amounted to 88% in the soil environment.
2. From the result of C02 evolution, the increase of REE concentration in the blends increased the percentage of ultimate biodegradation of the blends linearly. According to the results of GC analysis, co-metabolism was observed in the primary biodegradation of the 50REE blend. The biodiesel in the blend appeared to promote and increase the extent of biodegradation of petroleum diesel up to twice.
3. Biodegradation can restore a biodiesel fuel spill contaminated soil in 4-6 weeks to a degree that it can support plant germination. However, the seed germination test

showed that biodiesel contaminated soil did have an effect on plant growth for the first 3 weeks due to the rapid growth of microorganisms during the period of fuel degradation.

BOD_5 – COD

Biochemical oxygen demand (BOD_5) is a measure of the dissolved oxygen consumed during the biochemical oxidation of organic matter present in a substance. In the current study, BOD_5 was used as a relative measure of the amount of organic matter subject to microbially mediated oxidative processes present in biodiesel fuel. This may also serve as a relative measure of biodegradability. Chemical oxygen demand (COD) is a measure of the amount of oxygen required to chemically oxidize organic matter in a sample. COD values were used in the study as an independent measure of the total oxidizable organic matter present in the fuels.

EPA methods for BOD_5 and COD were used to assess the biodegradability of rapeseed ethyl ester (REE), rapeseed methyl ester (RME), neat rapeseed oil, soy methyl ester (SME), neat soybean oil, and Phillips 2-D reference fuel.

Biochemical Oxygen Demand (BOD_5) - EPA Method 405.1. The method consists of filling an airtight 300 mL BOD bottle with sample, removing the headspace, and incubating it for 5 days at 20 °C in the dark. Samples are prepared using a non-nutrient buffer and appropriate dilution. Dissolved oxygen is measured initially and following incubation. BOD is computed from the difference between initial and final dissolved oxygen (DO). Replicate analyses were performed in triplicate (method specifies duplicate samples be used, therefore n=6). Reference samples of glucose/glutamic acid solution, and a commercially available WasteWatR™ demand reference were also tested in duplicate. Fuels were tested at their appropriate water accommodated fraction (WAF: the highest concentration at which the test substance is maintained in the aqueous phase of the solution) and WAF values were converted to pure substance for statistical comparison.

Chemical Oxygen Demand (COD): EPA Method 410.1. The test is performed using commercially prepared vials which contain a potassium dichromate solution in sulfuric acid. The sample is introduced into these vials which are then sealed, mixed, and heated. Any "organic" material present reduces the dichromate to chromium ion and is detected spectrophotometrically at 600 nm. A standard solution of potassium biphthalate is used for standard curve concentrations, and WasteWatR™ demand reference is used as the reference sample. Fuels were tested at their appropriate WAF and these values were converted to pure substance for statistical comparison.

Results show BOD_5 values for REE (1.7×10^6 mg/L), RME (1.5×10^6 mg/L), SME (1.7×10^6 mg/L), neat rapeseed oil (1.7×10^6 mg/L) and soybean oil (1.6×10^6 mg/L) were significantly higher (ANOVA, Fisher's protected LSD, $p<0.01$) than the 2-D reference

fuel (0.4 x 10^6 mg/L), Figure 8.

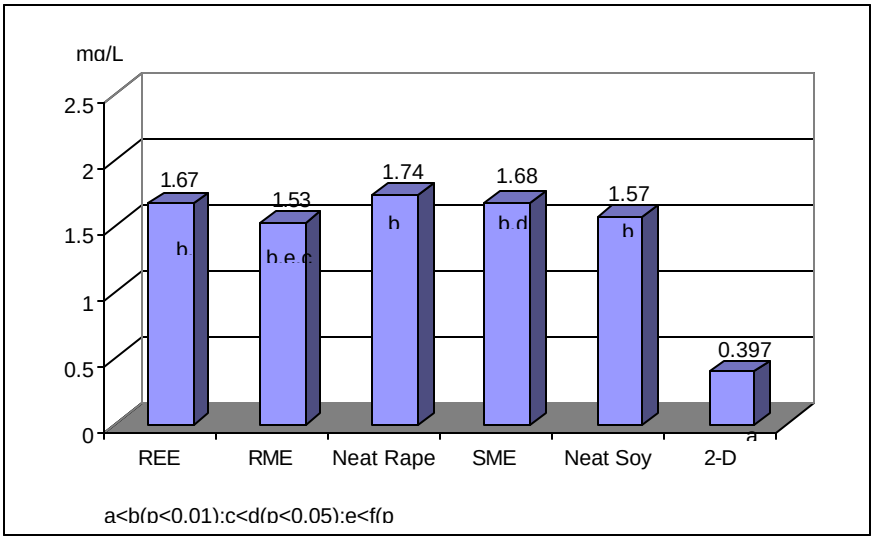

Figure 8. Mean BOD_5 Values (n=6).

Figure 9 shows COD values were similar for all fuels tested. Results indicate biodiesel fuel substances contain significantly more microbially biodegradable organic matter than does 2-D reference fuel.

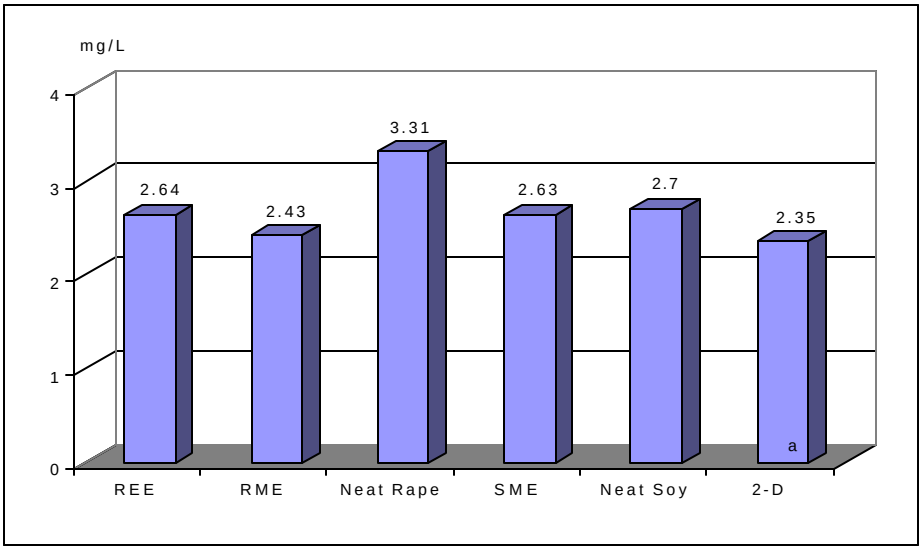

Figure 9. Mean COD Values (n = 3).

Conclusions BOD_5 – COD

No significant difference was expected or observed between COD values for the 2-D control substance and any of the test substances. Due to the chemical nature of the test, a total measure of all oxidizable organic matter is given.

This is in contrast to the BOD_5 test which more appropriately limits oxidative activity to microbial populations. The significant differences ($p<0.01$ and $p<0.05$, respectively) between REE and SME and between REE and RME may reflect slight differences in the amount of organic matter oxidizable by microbial processes. Although the difference is significant, the magnitude of the differences is less than 10%. Therefore, the biodegradability of these substances may be considered similar. The significantly lower BOD_5 value for the 2-D control substance and the large magnitude of the difference (average 122% difference) may be attributed to various factors. The WAF value for 2-D was also noted at 3.8 mg/L, much lower than those of the test substances, which may account for less of the substance in solution and available for microbial oxidative processes. The significantly lower ($p<<0.01$) BOD_5 values indicate the presence of a much smaller amount of microbial biodegradable organic matter in the Phillips 2-D diesel fuel. The lower BOD_5 value may also reflect the microbial toxicity of the diesel fuel, or its components.

Toxicity

This section reports on acute oral and acute dermal toxicity tests and static acute aquatic toxicity tests with rapeseed methyl ester (RME) and rapeseed ethyl ester (REE) and blends of each with diesel fuel. The acute oral toxicity tests were conducted with albino rats and the acute dermal toxicity tests were conducted with albino rabbits at WIL Research Laboratories in Ashland, Ohio. Acute aquatic toxicity tests were performed by CH2M Hill in Corvallis, Oregon with daphnia magna and rainbow trout. Each of these

studies were under contract with the University of Idaho. A separate set of studies with daphnia magna and juvenile rainbow trout were conducted by the Unversity of Idaho.

The LD50 (the point at which 50% have died and 50% are still alive determined by interpolation) values for each of the substances tested was found to be greater than 5,000 mg/kg when administered once orally to rats and greater than 2,000 mg/kg when administered once for 24 hours to the clipped, intact skin of male and female albino rabbits. The LC50 values for acute aquatic toxicity with daphnia magna in mg/L were 3.7 for table salt, 1.43 for 2-D, 23 for RME, 99 for REE and 332 for methyl soy-ate. Duplicate tests with rainbow trout were run with 10 organisms per replicate. LC50 numbers were not reported because of failure to kill a sufficient number of fish at the concentrations tested, even with the diesel fuel. The 20% and 50% blends had scattered losses of fish but none of the tests had less than 85% survival at any concentration after 96 hours.

The toxicology protocol was designed and conducted in compliance with the Environmental Protection Agency (EPA) Guidelines for Registering Pesticides in the U.S. (Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Section 81-1) and the Toxic Substances Control Act (TSCA) Health Effects Test Guidelines, 40 CFR 798.1175. The studies were conducted in compliance with EPA Good Laboratory Practice Regulations (40 CFR Parts 160 and 792) and the Standard Operating Procedures of WIL Research Laboratories, Inc. The study conducted and inspected in accordance with the Good Laboratory Practice Regulations, the Standard Operation Procedures of WIL Research Laboratories, Inc.

Procedures for aquatic toxicity testing are outlined in 40 CFR part 797.1300 (Daphnid acute toxicity test) and part 797.1400 (fish acute toxicity test), and ASTM E 729-88. These procedures include, with the LC50 (median lethal concentration), an EC5O (median effective concentration), and an IC50 (inhibition concentration). All static tests for this study were performed according to: Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Weber C., et al. (1991); EPA/600/4-90/027.

Acute Oral Toxicity Studies - One group of five male and five female rats was administered single doses at a level of 5000 mg/kg for each test substance. The rats were observed for mortality at approximately 1.0, 3.0 and 4.0 hours post-dose on day 0 and twice daily (morning and afternoon) thereafter for 14 days. The rats were observed for clinical observations at approximately 1.0, 3.0 and 4.0 hours post-dose on day 0 and once daily thereafter for 14 days. Body weights were obtained and recorded on study days -1, 0 (initiation), 7 and 14 (termination). Upon termination, all rats were euthanized by carbon dioxide asphyxiation. The major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals.

Acute Dermal Toxicity Studies - One group consisting of five male and five female albino rabbits was administered a single dose (24-hour, semi-occluded exposure) of each

test substance at a dose level of 2000 mg/kg. The rabbits were observed for mortality at approximately 1.0, 3.0 and 4.0 hours post-dose on day 0 and twice daily (morning and afternoon) thereafter for 14 days. The rabbits were observed for clinical observations at approximately 1.0, 3.0 and 4.0 hours post-dose on day 0 and once daily thereafter for 14 days. The application sites were examined for erythema, edema and other dermal findings beginning approximately 30-60 minutes after bandage removal and daily thereafter for thirteen days. The rabbits were clipped to facilitate dermal observations on study days 3, 7, 10 and 14. Body weights were obtained and recorded on study days 0 (initiation), 7 and 14 (termination). Upon termination, the rabbits were euthanized by intravenous injection of sodium pentobarbital. The major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals.

Results: Acute Oral Toxicity Studies

100% RME - There were no deaths, remarkable body weight changes or test material-related gross necropsy findings during the study. Single instances of wet yellow urogenital staining was noted for two female rats at day one. There were no other clinical findings. There were 2 individual clinical observations reported. All animals appeared normal by day two or earlier and throughout the remainder of the study. The LD50 of 100% RME was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

100% REE - There were no deaths, remarkable body weight changes or gross necropsy findings during the study. Single instances of wet yellow urogenital staining was noted for three female rats at day one. There were no other clinical findings. There were 3 individual clinical observations reported. All animals appeared normal by day two or earlier and throughout the remainder of the study. The LD50 of 100% REE was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

50% RME/50% 2-D - There were no deaths, remarkable body weight changes or test material-related gross necropsy findings during the study. Wet and/or dried yellow urogenital and/or ventral abdominal staining were noted for all animals. One animal each had clear ocular discharge or dried red material around the nose. There were no other clinical findings. There were 18 individual clinical observations reported. All animals appeared normal by day 3 or earlier and throughout the remainder of the study. The LD50 of 50% RME/50% 2-D was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

50% REE/50% 2-D - There were no deaths, remarkable body weight changes or test material-related gross necropsy findings during the study. Wet and/or dried yellow urogenital and/or ventral abdominal staining were noted for all animals. Single animals had clear ocular discharge, hypoactivity, hair loss on the dorsal head or dried red material around the nose or forelimb(s). There were no other clinical findings. There were 30 individual clinical observations reported. With the exception of one animal having hair loss on the dorsal head, all animals appeared normal by day 3 or earlier and throughout the remainder of the study. The LD50 of 50% REE/50% 2-D was greater than 5000 mg/kg

when administered once orally via gastric intubation to fasted male and female albino rats.

20% RME/8O% 2-D - There were no deaths, remarkable body weight changes or gross necropsy findings during the study. Wet and/or dried yellow urogenital and/or ventral abdominal staining were noted for all animals. Two male rats each had clear wet matting around the mouth and clear ocular discharge. Findings documented for one animal each included soft stool and hair loss on the hindlimb(s) and ventral abdominal area. There were no other clinical findings. There were 48 individual clinical observations reported. With the exception of hair loss on the hindlimb(s) and ventral abdominal area for one rat, all animals appeared normal by day 8 or earlier and throughout the remainder of the study. The LD50 of 20% RME/80% 2-D was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

20% REE/80% 2-D - There were no deaths, remarkable body weight changes or test material-related gross necropsy findings during the study. Wet yellow urogenital and ventral abdominal staining were noted for all animals. Two animals each had soft stool, dried red material around the nose and hair loss on the hindlimb(s) and/or ventral abdominal area. Findings note for one animal each included clear wet matting around the mouth, clear ocular discharge and desquamation on the urogenital area. There were no other clinical findings. There were 66 individual clinical observations reported. With the exception of hair loss on the hindlimb(s) and/or ventral abdominal area for two rats, all animals appeared normal by day 9 or earlier and throughout the remainder of the study. The LD50 of 20% REE/80% 2-D was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

100% 2-D - There were no deaths, remarkable body weight changes or test material-related gross necropsy findings during the study. Wet and/or dried yellow urogenital and/or ventral abdominal staining were noted for all animals. Additional findings included various hair loss, clear wet matting around the mouth and/or urogenital area, soft stool and hypoactivity. There were no other clinical findings. There were 105 individual clinical observations reported. With the exception of hair loss on the hindlimb(s) and/or urogenital area for four rats, all animals appeared normal by day 9 or earlier and throughout the remainder of the study. The LD5O of 100% 2-D was greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

Results Acute Dermal Toxicity Studies

100% RME - There were no deaths, test material-related clinical findings, body weight changes or gross necropsy findings during the study. The test material induced very slight to slight erythema on all animals and very slight to slight edema on seven animals. All sites had desquamation. Fissuring was noted for one rabbit. There were no other dermal findings. All edema completely subsided by day 12. With the exception of two animals having very slight erythema, all dermal irritation completely subsided by day 14 or earlier. There were 8 individual clinical observations reported. There were 102 very slight erythema occurrences and 8 slight, 32 very slight edema and 7 slight edema, 70

desquamation occurrences and one fissuring occurrence during the 14 day study. The LD50 of 100% RME was greater than 2000 mg/kg when administered once for 24 hours to the clipped, intact skin of male and female albino rabbits. In addition, the 2000 mg/kg dose level was found to be a No Observable Effect Level (NOEL) for systemic toxicity under the conditions of this study.

100% REE – There were no deaths, test material-related clinical findings, body weight changes or gross necropsy findings during the study. The test material induced very slight to slight erythema on all animals and very slight to slight edema on all animals. Desquamation was noted on all sites. There were no other dennal findings. All edema completely subsided by day 10. All dermal irritation completely subsided by day 14 or earlier. There were 5 individual clinical observations reported. There were 98 very slight erythema occurrences and 10 slight, 55 very slight edema and 2 slight edema, and 62 desquamation occurrences during the 14 day study. The LD50 of 100% REE was greater than 2000 mg/kg when administered once for 24 hours to the clipped, intact skin of male and female albino rabbits. In addition, the 2000 mg/kg dose level was at a No Observable Effect Level (NOEL) for systemic toxicity under the conditions of this study.

100% 2-D - There were no deaths, test material-related clinical findings, body weight changes or gross necropsy findings during the study. The test material induced moderate erythema, slight to moderate edema and desquamation on all animals. Two animals had fissuring. There were no other dermal findings. All erythema and edema completely subsided by day 14 or earlier. Desquamation persisted to day 14 for seven animals. There were no individual clinical observations reported. There were 42 very slight, 27 slight and 31 moderate occurrences of erythema; 22 very slight, 28 slight and 6 moderate occurrences of edema; three occurrences of fissuring and 120 desquamation occurrences during the 14 day study. The LD50 of 100% 2-D was greater than 2000 mg/kg when administered once for 24 hours to the clipped, intact skin of male and female albino rabbits. In addition, the 2000 mg/kg dose level was at a No Observable Effect Level (NOEL) for systemic toxicity under the conditions of this study.

Acute Aquatic Toxicity CH2M HILL

The daphnia magna were obtained from CH2M Hill's in house cultures and were less than 24 hours old prior to initiation of the test. All organisms tested were fed and maintained during culturing, acclimation, and testing as prescribed by EPA (1989). The test organisms appeared vigorous and in good condition prior to testing. The daphnia magna were placed below the test surface at test initiation due to the non-soluble nature of the sample.

The juvenile rainbow trout used in the first round of tests were obtained from Thomas Fish Company, Anderson, California, and were 22 days old and 32±2 mm in length. The rainbow trout were acclimated to test conditions (dilution water and temperature) for 10 days prior to test initiation. The rainbow trout used in the second round of tests were obtained from Spring Creek Trout Hatchery, Lewiston, Montana, and were 24 days old and 28±1 mm in length. The rainbow trout were acclimated to test conditions (dilution water and temperature) for 12 days prior to test initiation. All the test organisms appeared

vigorous and in good condition prior to testing.

Test Concentrations

Daphnia Magna - The concentrations tested in definitive test on REE were 33, 167, 833, 4170, and 20800 mg/L of sample and dilution water for the control. The concentrations tested in the definitive test on RME were 67, 333, 1330, 6670, and 26700 mg/L of sample and dilution water for the control. The concentrations tested in the definitive test on D2 were 6.7, 13.3, 33.3, 66.7, and 1333 mg/L of sample and dilution water for control. The concentrations tested in the definitive test on Methyl Soyate were 13.3, 33.3, 66.7, and 6667 mg/L of sample and dilution water for control. The fuel mixture concentrations were run in quadruplicate with five organisms per replicate. Additional concentrations of 1.43 and 3.33 mg/L were set up for D2 with 10 organisms in one chamber. The fuel was stirred into the water before the daphnia magna were introduced into the chamber. There was a sheen of fuel on the top of each chamber.

Rainbow Trout - The concentrations tested for round 1 in the definitive test on D2, 20RME, 20REE and REE were 100, 300, 600, 1200, and 2400 mg/L with dilution water for control. The concentrations tested for round 2 in the definitive test on RME and 50REE were 100, 500, 750, 1000, and 7500 mg/L and the 50RME sample was tested at 100, 500, 600, and 7500 mg/L due to a shortage of the sample.

The rainbow trout bioassays were run in 5-gallon glass aquaria, with a volume of 5 liters water. The samples were run in duplicate with 10 organisms per replicate. The photo period was 16 hours light\8 hours dark. The temperature range was 12±1C. Loading of test organisms was 0.53g wet fish weight per liter in round one, and 0.26g wet fish per liter in round two. Mortality was measured by lack of response to tactile stimulation and lack of respiratory movement. The fuel was stirred into the water before the rainbow trout were introduced into the chamber. There was a sheen of fuel on the top of each chamber.

Results CH2M-Hill Acute Aquatic Toxicity

Daphnia Magna - The raw data is summarized in Table 2 below for 100% REE. Some of the mortality seen in the tests may have been caused by the physical nature of the test substances. The raw data sheets noted when the Daphnia Magna were trapped on the oil sheen at the surface of the test containers. The LC50 for the REE sample was 99 mg/L. Table 2 summarizes the results of the RME sample and a reported LC50 for RME of 23 mg/L. Table 4 summarizes the results of the Methoyl Soyate with a reported LC50 of 332 mg/L.

The methyl and ethyl esters are not water soluble and formed a sheen on the water surface. This sheen could be easily skimmed off, but the daphnia magna get captured in the sheen. Fifty percent of the daphnia magna in common table salt had died at a concentration of 3.7 mg/L. With diesel, 50 percent of them had died at less than 1.43 mg/L and CH2M Hill reported all were dead at this concentration. When this test was first completed, CH2M Hill reported that the LC50 for diesel fuel was less than 6 mg/L because all the daphnia magna had died. They tested four more concentrations less than 6 mg/L and the diesel fuel at all concentrations killed the Daphnia Magna. For the RME the

LC50 was 23 mg/L, and at 26,700 mg/L, 30% of them were still alive. With REE the LC50 was 99 mg/L and 20% were still alive at 20,800 mg/L. With methyl soyate the LC50 was 332 mg/L; however, only 45% were alive at 667 mg/L. This difference between rapeseed esters and SME may be due to the high Erucic acid content of the rapeseed. If one takes the worst case, the 23 mg/L for REE, and compare it to the 1.4 mg/L for diesel fuel, the acute aquatic toxicity is 15 times less. What is even more significant is the 20% and 30% that were still alive at very high concentrations of Biodiesel.

Rainbow Trout - The LC50 for D2 was not determined. This data compares cadmium chloride (CdC1), diesel fuel, and methyl and ethyl esters of rapeseed. The 50RME percent survival summary results were identical to the 100% RME results.
The 48-hour LC50 value and Control Chart limits for the reference toxicant (cadmium chloride) was at a concentration of 2.8g/1 for round one rainbow trout study and 4.6g/l for the round two trout study. The results indicate that the test organisms were within their expected sensitivity range. Comments included in round one test data at 24-hours was a general behavior of twitching and they were swimming on their sides and skittering; at 48-hours their condition was the same as at 24-hours. The trout in the 20REE containers at 100 and 300 mg/L were swimming vertically, at 600 mg/L the trout were on their sides at the bottom, and at 2400 mg/L they were barely moving at the bottom of the tank. The trout in the REE containers were not as active as in the other three test substances. The end condition of survivors was reported as being poor. The only comment in round two was at 48-hours that the fish were dark and swimming vertical at concentrations as low as 500 mg/L in the 50RME and 50REE with the end condition of survivors as being poor.

U of I Static Non-renewal and Flow-Through Acute Aquatic Toxicity Tests-
Twenty Daphnia magna were exposed to each of five concentrations of test/reference substance and a control for 48 hour periods in static and flow-through environments as outlined in EPA TSCA Environmental Effects Test Guidelines @ 40 CFR §797.1300 Daphnid Acute Aquatic Toxicity Test and additional guidelines in EPA Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms Fourth Edition (EPA/600 4-90-027). Mortality data was collected at 24 and 48 hours and EC50 results were calculated using EPA Probit Analysis Program.

The static non-renewal toxicity test is a system in which the test solution and test organisms are placed in the test chamber and kept there for the duration of the test without renewal of the test solution. The insoluble and glassware-coating nature of these test substances required the derivation of a water accommodated fraction (WAF) to minimize mortality occurring by suffocation of the daphnids. Tests were performed at or below the water accommodated fraction levels and pretest mixing for extended periods was necessary to avoid "floating" the test substance on the surface of the test chamber where mortality may be caused by other than toxic effects. The derived WAF was used as the highest concentration and was proportionally diluted to the other concentrations of the analysis. Test chambers were filled with appropriate volumes of dilution water and the test chemical was introduced into each treatment chamber. The test started within 30

minutes after the test chemical had been added and was uniformly distributed in static test chambers. At the initiation of the test, daphnids which had been cultured and acclimated in accordance with the test design were randomly placed into the test chambers. Daphnids in the test chambers were observed periodically during the test, the immobile daphnids removed, and the findings recorded. Dissolved oxygen, pH, temperature, concentration of test chemical, and water quality parameters were measured.

In the Flow-Through tests the test substances were initially mixed at the WAF for a minimum of 20 hours in a 50 liter holding tank under constant stirring in the flow - through test. The stirring action proceeded throughout the duration of the test. The test substance mixture at WAF was drawn from the mixing tank for each cycle. The amount withdrawn from the tank was replaced with each cycle, maintaining an equilibrium mixture at the WAF concentration.

Results of the U of I Static and Flow through tests - 48-hour LC50 values are presented in Figure 10 for static non-renewal and in Figure 11 for flow-through tests. The lowest static 48-hour Daphnia magna EC50, indicative of highest toxicity, was 0.37 mg/L for the 20% REE / 80% 2-D mixture. This was followed by the 2-D Reference Fuel at 1.56 mg/L and SME at 2.13 mg/L. The 50/50 mixture of REE and 2-D showed a 48 hour EC50 of 2.75 mg/L. The highest EC50 were seen with the REE and RME at 4.11 mg/L and 3.07 mg/L, respectively.

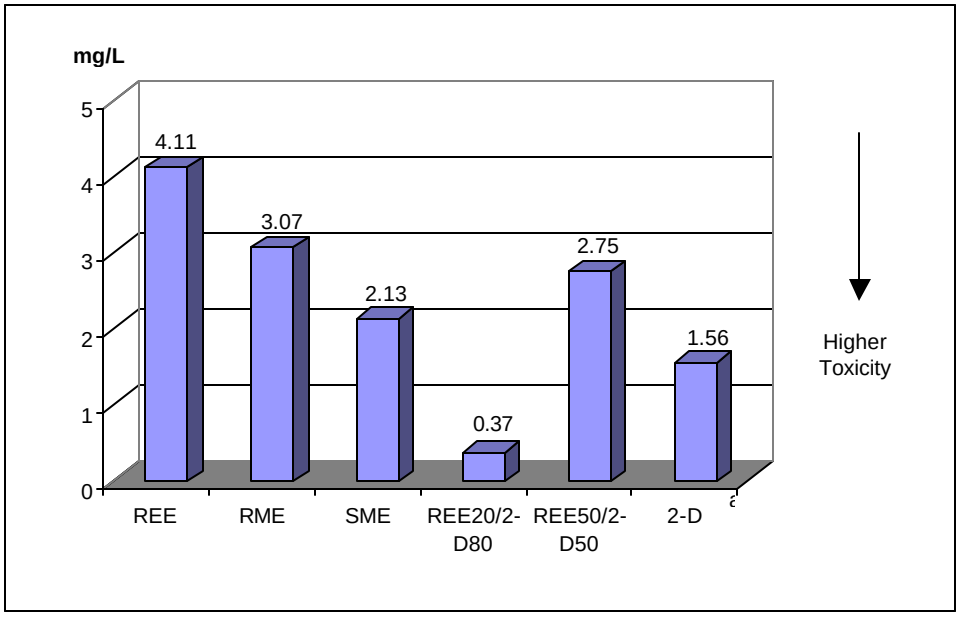

Figure 10. 48 Hour Static, Non-Renewal, Daphnia Magna EC_{50}

The lowest flow-through 48-hour Daphnia magna EC50, indicative of highest toxicity, was 0.19 mg/L for the 2-D Reference fuel. This was followed by the 20REE mixture at 0.21 mg/L and SME at 0.40 mg/L. The 50/50 mixture of REE and 2-D showed an EC50 of 5.12 mg/L. The highest EC50s were seen with the RME and REE at 587 mg/L and 25.2 mg/L, respectively.

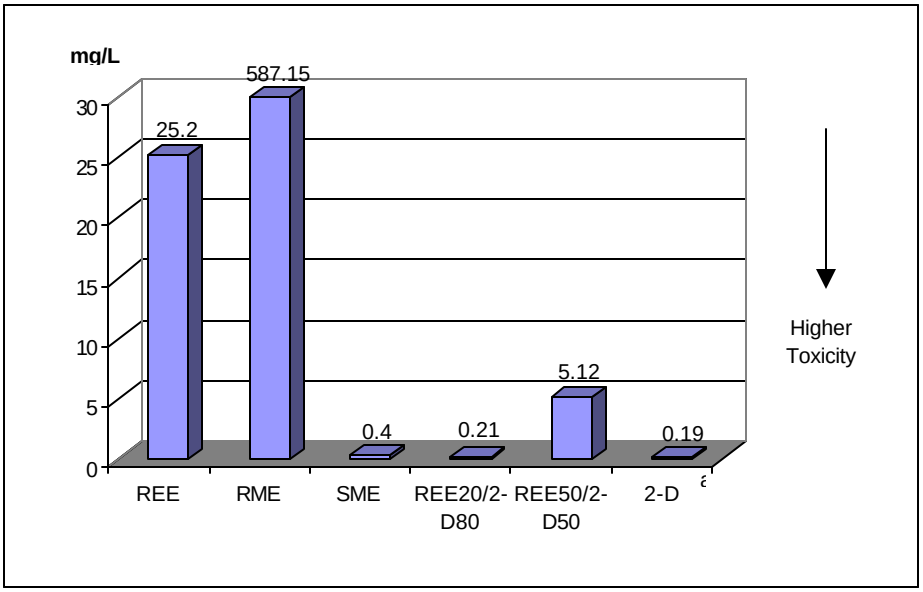

Figure 11. 48 Hour Flow-Through, Daphnia Magna EC_{50}.

Toxicity Conclusions

The toxicity tests show that biodiesel is considerably less toxic than diesel fuel but that one still should avoid ingesting biodiesel or getting it on the skin. Although some adverse effects are noted in the tests with rats and rabbits, none died from either the biodiesel or the diesel fuel. The animals treated with diesel had more injurious clinical observations but some effects were noted for both fuels.

The LD50 of each test substance was greater than 5000 mg/kg (the limit dose) when administered once orally via gastric intubation to fasted male and female albino rats. The occurances of clinical observations increased as the ratio of diesel fuel increased.

The LD50 of 100% REE was greater than 2000 mg/kg (the limit dose) when administered once for 24 hours to the clipped, intact skin of male and female albino rabbits. In addition, the 2000 mg/kg dose level was found to be a No Observable Effect Level (NOEL) for systemic toxicity under the conditions of this study for the three fuels tested.

The 100% RME fuel was the least severe in the acute oral toxicity study and the 100% REE was the least severe in the acute dermal toxicity study.

Biodiesel is not as toxic to Daphnia Magna as NaC1. When compared to the reference

toxicant (sodium chloride) diesel fuel was 2.6 times more toxic, RME was 6.2 times less toxic, REE was 26 times less, and SME 89 times less toxic. When compared to number two diesel fuel RME is 16 times less toxic, REE is 69 times less toxic, and SME was 237 times less toxic. CH2M Hill repeated the toxicity study with rainbow trout at the water accommodated fraction (WAF) and below to produce a LC50. A LC50 was not produced at or below the WAF using rainbow trout.

In the U of I tests, the least toxic of the test substances in the flow-through test was REE, followed by RME and the 50/50 mixture of REE and 2-D. The 2-D reference substance had the lowest EC50 value at 24 and 48 hours. This was followed at both times by the 20/80 mixture of REE and 2-D, and the SME test substance.

In both static and flow-through tests, the rapeseed based fuels, REE and RME, displayed the highest EC50 values, signifying them to be less toxic than the other test substances. The EC50 values for the other vegetable oil based fuel, SME, are lower than the rapeseed fuels, significance untested. It should be noted that the results of the static test for SME failed the Chi-square test for heterogeneity using a Probit model.

The REE and 2-D mixture results are follow as expected in that for both static and flow-through analysis, the 20/80 mixture showed higher EC50 values than the 50/50 mixture. This agrees with the results of the other tests which indicate a higher toxicity (lower EC50) for a higher percentage of 2-D in the mixture and a lower toxicity (higher EC50) with increasing REE percentage.

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