# DRAFT - MULTIMEDIA RISK ASSESSMENT TIER II COMPLETION REPORT: BIODIESEL

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## 1.0 EXECUTIVE SUMMARY

This document reports on the results of experimental activities performed in order to address priority knowledge gaps in Tier II of the California multimedia risk assessment of biodiesel blends, as identified in the Tier I assessment of biodiesel as an alternative fuel in California (UC, 2008) and as outlined in the plan for these experiments (Ginn et al., 2009). These experimental investigations include study of toxicity, transport in porous media, and aerobic biodegradation. Further testing (solubility, materials compatibility) identified in the TierII plan were not pursued as a result of time and funding limitations. The summary and results of each of the three experimental suites are as follows.

*Toxicity.* A series of aquatic toxicity tests were conducted on the seven fuel types including ultra-low sulfur diesel (ULSD), neat biofuels derived from animal fat (AF B-100) and soy (Soy B-100) feedstocks as well as 80% ULSD:20% (w/w) mixtures of the two biofuels (AF B-20 and Soy B-20) and the two B-20 mixtures amended with an antioxidant additive (AF B-20A and Soy B-20A). The chronic toxicity test species included three freshwater organisms including a green alga (*Selenastrum capricornutum*), an invertebrate (water flea, *Ceriodaphnia dubia*), and a fish (fathead minnow, *Pimephales promelas*), along with three estuarine organisms including a mollusk (red abalone, *Haliotis rufescens*), an invertebrate (mysid shrimp, *Mysidopsis bahia*) and a fish (topsmelt, *Atherinops affinis*). The water accommodated fraction (WAF) of each fuel was prepared by the slow-stir method and tested using a control and six concentrations of WAF (1, 5, 10, 25, 50, and 100%). The tests closely followed published USEPA protocols with regard to quality assurance (QA) including statistical evaluation of test endpoints, monitoring of water quality conditions in test solutions, and protocol control performance requirements. Statistical evaluation of test results included determination of the NOEC, LOEC, $EC_{25}$ and $EC_{50}$ for each test protocol endpoint. Sensitivity of the test organisms to the fuels was evaluated by comparing toxic units (TUs; $100/EC_{25}$). Each of the tests met all protocol QA requirements and tests that were repeated to assess consistency, closely matched the results of the original test. Results of the tests varied widely depending on fuel type and test species. Tests with ULSD only detected effects on mysid growth (1.0 TU) and water flea reproduction (1.8 TU). None of the AF or Soy fuels or their B-20 mixtures without additive produced detectable effects on mysid, topsmelt or fathead minnow endpoints. However, the both biofuels and their mixtures caused variable effects on algae cell growth (5-21.3 TU), water flea survival and reproduction (<1-21.3 TU) and abalone shell development (3.0 - 35.5 TU). Except for algae, tests with the additized B-20 fuels consistently resulted in substantially greater toxicity than was detected with the unadditized B-20 fuels, suggesting that conducting screening for a less toxic additive may be warranted. Completion of the aquatic toxicity assessment awaits analytical chemistry results.

*Infiltration.* Small scale laboratory infiltration experiments in two-dimensional sandboxes were done to visualize the relative rates of biodiesel infiltration, redistribution, and lens formation on the water table in comparison to that of ULSD. Experimental design involved unsaturated sand as model porous media with ~20cm vertical infiltration of fuels to the saturated zone. Experiments were performed in triplicate for Animal Fat and Soybean based biodiesel, including pure (B-100) and blended (B-20) biodiesel formulations. As a control, Animal Fat B-

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100 with antioxidant was also tested and it showed similar behavior to unadditized Animal Fat B-100. Digital photography was used to record images of fuel behavior in side-by-side tests of biodiesel blend and ULSD. Experiments in each of the four blends (AF B-20, AF B-100, Soy B-20, and Soy B-100) were run to effective steady-state lens formation on the top of the saturated zone (water table) that involved durations ranging from 1.5 to 2 hours, with on average 24 photographs taken per experiment, generating 288 images. (24 snapshots in time x 4 fuel blends x 3 replicates). The experiments found that soy biodiesel blends at both 20 and 100 percents, as well as the animal fat 20 percent blend, do not exhibit any significant differences among the four temporal metrics used to time the infiltration and lens formation, nor among the qualitative unsaturated zone residual or lens shape at steady state, compared to the same metrics for ULSD. However while the animal fat 100 percent blend exhibited mostly the same values of the infiltration timing metrics as ULSD, it showed noticeable increases in the amount of residual that occurred in the unsaturated zone, and it resulted in final lens geometry that was thicker in vertical dimension and less extensive in horizontal dimension than the ULSD lens. This behavior is consistent with the physical properties of animal fate biodiesel that include higher viscosity and interfacial tension than ULSD.

*Biodegradation.* Microcosm experiments were conducted to assess the aerobic aqueous biodegradation potential for solutions in contact with biodiesel fuels, relative to ULSD. Fuels derived from animal fat and soy feedstocks at B-100 and B-20 mixtures (with ULSD making up the complement) were used as source phases with and without antioxidant and biocide additives, with ULSD tested for comparison. Experiments were done in batch (250ml) with 2g of soil inoculum added to 190ml of stock solution with addition of 5 μL of test fuel as substrate. Experiments were performed in a respirometer in which the CO2 production in microcosms was measured during the experiment for duration of 28-30 days. Control experiments using sterilized inoculated solution with substrate were done to examine whether the test substrate is degraded abiotically and to test the adsorption of test substrate onto glass and or inoculum material. Controls with inoculum but no fuel also were prepared to test for CO2 production by microorganisms in absence of substrate. Results show enhanced CO2 production for all biodiesel blends and all additive combinations relative to that for ULSD. With some minor variations among blends (soy vs. animal fat; additized vs. non-additized), the results indicate that the additives effects are not significant on the biodegradation of biodiesel blends, and the blends tested are all more readily biodegrable than ULSD.

*Summary*. Experimental investigations address the knowledge gaps as follows. Tested biodiesel blends exhibit somewhat increased toxicity to subsets of tested species than ULSD, and additized blends increase this toxicity for a smaller subset of tested species. Biodiesel fuel blends show similar infiltration and lens formation to ULSD in unsaturated sandy porous media, with animal fat B-100 exhibiting greater residual in the vadose zone and less spreading of fuel lens on subsurface water table, consistent with increased viscosity and interfacial tension of this fuel. Aerobic biodegradation of biodiesel is faster and more extensive than that of ULSD across a range of fuel blends and included additives.

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## 2.0 BACKGROUND

This document summarizes the results of experiments performed at UC Davis as part of the Tier II Multimedia Risk Assessment of Biodiesel for the State of California. Existing

research on the topic has been collected in UC (2008), the Multimedia Working Group (MMWG) Tier I report (http://www.arb.ca.gov/fuels/diesel/altdiesel/090910biodiesel-tier1-final.pdf, referred to henceforth at the "Tier I report"), and the plan for these experiments is found in the "Experimental Plan for TierII Evaluation of Biodiesel," (www.arb.ca.gov/fuels/multimedia/031209TierIIrev.pdf, (Ginn et al., 2010) referred to henceforth at the "Tier II Plan"). Biodiesel B-100 is defined here as a mono-alkyl ester-based non-petroleum derived diesel substitute meeting ASTM D6751-07be1 (Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels). Biodiesel blends B50, B20, B5 also referred to as "biodiesel" are mixtures of B100 with California Air Resources Board Ultra Low-Sulfur Diesel #2 (ULSD) in indicated proportions, by volume. Biodiesel studied here is primarily fatty-acid methyl esters (FAMEs) resulting from the trans-esterification of oils derived from animal fats or vegetable/seed oils or other feedstocks, and may include residual reactants and products of the transesterification (e.g., methanol, water, etc.)

The purpose of the experiments performed is to fill knowledge gaps pertaining to the fate, transport, biodegradation, and toxicity properties of biodiesel occurring in the environment due to unintended precombustion releases. In all instances the experiments are intended to address *relative* risk as compared to that associated with ULSD. Because of time and funding limitations, the experiments performed are designed to address the highest priority knowledge gaps identified in Tier I and outlined in the TierII plan, and in a simplified and riskwise conservative fashion. The Tier 1 study identified as high priority knowledge gaps, Additives impacts, Subsurface fate & transport properties, Biodegradation in soils and aquifers, production and storage release scenarios, complete air emissions studies (Tier I Report, pages 75, 76). These issues are partly addressed in the experimental plan described here as follows:

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| **Knowledge Gap** | **Approach** |
|---|---|
| ○ Toxicity | Aquatic toxicity experiments |
| ▪ unadditized | tested |
| ▪ cold flow additive | not tested |
| ▪ biocidal additive | not tested |
| ▪ antioxidant additive | tested |
| ○ Fate & transport | "Ant Farm" experiments |
| ○ Biodegradation | Microcosm experiments |
| ▪ unadditized | tested |
| ▪ cold flow additive | not tested |
| ▪ biocidal additive | tested |
| ▪ antioxidant additive | tested |
| ○ Release scenarios | Not tested |
| ○ Air emissions studies | ongoing by CARB |
| ○ Solubility | Not tested |
| ○ Materials Compatibility | Not tested |

Budget and time constraints required restriction of the experimental investigation to incomplete treatment of the knowledge gaps identified, and so the experiments cover the highest priority issues. Thus impacts of cold flow additive, evaluation of release scenarios, aqueous solubility, and materials compatibility are not evaluated in this Tier II study. Toxicity studies are restricted to marine and freshwater toxicity.

## 3.0 TIER II EXPERIMENTAL DESCRIPTIONS

Blend selection is restricted to two feedstocks and two blend ratios, B-20 and B-100, as these represent highest expected use and maximum biodiesel samples respectively. Feedstocks include Soy and Animal fat, as they reflect high potential use and wide bracketing of dominant feedstock chemistry. Additives have been selected by criteria defined in Appendix I of the Tier I report: in summary, antioxidant and biocide additives are hypothesized as those most likely to incur departures from ULSD behavior, so one representative additive from each category is selected. These feedstock and additive selections are also made in order to be consistent with ongoing CARB emissions testing.

The following three suites of tests have been carried out.

1. Aquatic **toxicity** tests were carried out to evaluate the relative toxicity of biodiesel blends potentially released to aquatic environments.
2. Sandbox **infiltration** tests are a visual method for studying fluid transport through unsaturated two-dimensional porous media to contact with a saturated zone resulting in lens formation at the unsaturated-saturated interface.
3. Microcosm study and $CO_2$ evaluation were used to study the rates of biodiesel **biodegradation** under aerobic conditions by soil microbes.

Table 1 shows the experimental matrix reflecting the selection of different additive combinations (columns) for testing with different fuel blends (rows), in experimental suites labeled by letter with identifications in the caption. The selection reflects prioritization of particular additives for association with higher risk impacts such as biocides impacting biodegradation as described in the Appendix 1 of the TierII Plan.

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**Table 1. Tier II Testing Matrix: Experiment codes are T Toxicity, I Infiltration, B Biodegradation**

| **Fuel Preparation** | | | |
|---|---|---|---|
| ULSD | T, I, B | | |
| Soy B-100 | T, B | I, B | B |
| Animal fat B-100 | T, I, B | I, B | B |
| Soy B-20 | T, B | T, I, B | B |
| Animal fat B-20 | T, B | T, I, B | B |
| **Additives** | Reference | Bioextend-30 | Kathon FP 1.5, Bioextend-30 |
| **Additive Type** | No Additive | Antioxidant | Biocide and Antioxidant |

The experimental details for each of the three suites, Aquatic Toxicity, Infiltration, and Biodegradation, are presented in the Appendices A, B, and C, respectively. These sections include particulars of experimental design, experimental permutations (fuel blends/additives, experimental conditions) tested, execution of experiments, and results. Conclusions of the experiments are presented here in terms of the relevance to the filling of the knowledge gaps identified in the TierII plan of the California multimedia risk assessment for biodiesel.

## 4.0 RESULTS AND CONCLUSIONS

### 4.1 Aquatic Toxicity

Aquatic toxicity testing involved ULSD in comparison with Soy and Animal fat B-20 and B-100 unadditized fuels, and Soy and Animal fat B-20 with antioxidant additive. Tests involved three freshwater organisms (green alga, fathead minnow larvae, and water flea) and three estuarine/marine organisms (red abalone, mysid shrimp, and topsmelt fish). Toxicity endpoints for each species are detailed in Appendix A. Toxicity metric in each case is in terms of Toxicity Units, "TUc," defined as the quantity 100/EC50, where EC50 is the relative concentration in percent of substrate (relative to equilibrium solubility concentration of a given fuel in aqueous phase) at which 50 percent of the test species population exhibits an effect. Thus if half of a population shows an effect only at the 100% concentration (that corresponding to equilibrium solubility) then the TUc value = 100/100 = 1. If however half of a population exhibits an effect at the concentration equal to 1% of the equilibrium solubility concentration, then the TUc value = 100/1 = 100. Each fuel/species combination tested involved identical solute preparation, standardized to create an experimentally-defined "equilibrium solute concentration" resulting from timed exposure of an aqueous phase to the ULSD or biodiesel blend. Details are given in Appendix A. The results are as follows.

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- ULSD produced relatively low but detectable toxicity on mysid growth (1.0 TUc) and water flea reproduction (1.8 TUc). No toxicity (< 1.0 TUc) was detected with any of the other species tested.
- Neither of the unadditized Animal Fat or Soy biodiesel test materials produced detectable toxicity to the mysid, topsmelt or fathead minnow.
- Animal Fat B-100, Soy B-100 and their B-20 mixtures caused toxicity to algae cell growth, water flea survival and/or reproduction, and abalone shell development
- Except for algae, the additized biodiesel B-20 test materials were substantially more toxic than the corresponding unadditized material.
- Tests that were repeated for confirmation produced similar results as the original test.
- Analytical chemistry information is needed on the fuel samples collected during the study to elucidate the chemical causes of toxicity and to provide information on the stability of WAF components during the toxicity tests.

### 4.2 Infiltration

Small scale laboratory infiltration experiments in two-dimensional sandboxes with glass walls to allow visualization of dyed fuels were completed in order to allow observation of the relative rates of biodiesel infiltration, redistribution, and lens formation on the water table in comparison to that of ULSD. These experiments were performed at UC Davis in the lab of Professor T. R. Ginn and involved various preliminary experiments to establish standard procedures, and these are detailed in Appendix B. Experiments involved unsaturated sand as

model porous media with ~20cm vertical domain of unsaturated zone above the saturated level of the sand. Dyed fuel samples (a biodiesel blend and a ULSD sample) of identical volumes were simultaneously emplaced in divots in the sand surface at the top of the sandbox, and time-lapse digital photography was used to record infiltration of this ponded source fuel, redistribution and residual formation in the unsaturated zone, and lens formation on the top of the saturated zone. Experiments were performed in triplicate for animal fat and soybean based biodiesel, including pure (B-100) and blended (B-20) biodiesel formulations (as well as animal fat B-100 with antioxidant additive as control). Experiments in each of the four blends (AF B-20, AF B-100, Soy B-20, and Soy B-100) were run to effective steady-state lens formation on the top of the saturated zone (water table) and involved durations ranging from 1.5 to 2 hours, with on average 24 photographs taken per experiment. A complete description of the experiments and a complete catalogue of the images is contained in Hatch (2010), a summary form of which comprises Appendix B.

Visual analyses of these images was done to evaluate four separate time metrics defined in order to time the progress of the infiltration, redistribution, and formation of the lens of biodiesel on the saturated zone surface at the steady-state. These metrics are characteristic times for: elimination of ponded fuel, plume separation from surface, initial commencement of lens spreading on water table, steady-state lens formation on water table. In addition the qualitative characteristics of quantity of residual fuel appearing in the unsaturated zone and of lens shape after steady-state are reported. The experiments show that

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- The antioxidant additive did not affect the infiltration of Animal fat B-100
- Soy biodiesel blends at both 20 and 100 percents, as well as the animal fat 20 percent blend, do not exhibit any significant differences among the four temporal metrics or among the qualitative residual or lens shape metrics compared to ULSD.
- Animal fat 100 percent blend exhibited similar values of the temporal metrics as ULSD, but it showed noticeable increases in the amount of residual that occurred in the unsaturated zone, and it resulted in final lens geometry that was thicker in vertical dimension and less extensive in horizontal dimension than the ULSD lens.

This behavior is consistent with the physical properties of animal fate biodiesel that has higher viscosity and interfacial tension than ULSD. These differences become significantly more pronounced at temperatures below 20 degrees Celsius.

### 4.3 Biodegradation

Aerobic biodegradation is a primary path for natural remediation of unintentional releases of fuel compounds. Although anaerobic conditions may make up a larger fraction of the environmental domain in which fuels may occur, aerobic conditions are typically encountered first in releases, and are selected in the TierII plan as the highest priority knowledge gap for natural remediation of biodiesel. In order to investigate the relative rates of aerobic biodegradation of biodiesel blends and ULSD, microcosm experiments were conducted in

laboratory setting with 250ml batch reactors. Fuels derived from animal fat and soy feedstocks at B-100 and B-20 mixtures (with ULSD making up the complement) were used as source phases, with ULSD tested for comparison. The biodiesel blends included either no additives, an antioxidant additive, or both antioxidant and a biocide additive, at manufacturer-specified concentrations, while the reference ULSD fuel contained no additives. This experimental approach is designed intentionally as a conservative evaluation of the differences in biodegradation potential between petroleum and biomass-derived diesels. Each batch reactor includes190 ml of prepared solution, 2 g soil (Yolo, silty-loam) as bacterial inoculum and addition of 5 μL of test fuel as substrate. Experiments were performed in a respirometer in which the CO2 production in microcosms was measured during the experiment for duration of 28-30 days. Control experiments using sterilized inoculated solution with substrate were done to examine whether the test substrate is degraded abiotically and to test the adsorption of test substrate onto glass and or inoculum material. Controls with inoculum but no fuel also were prepared to test for CO2 production by microorganisms in absence of substrate. Conclusions are as follows.

- Controls reveal no CO2 production in the absence of fuel substrate
- Controls reveal no CO2 production in the absence of soil inoculum
- Respironmeter data show enhanced CO2 production for all biodiesel blends relative to that for ULSD.
- Additives do not impart a significant effect on the aerobic biodegradation of biodiesel blends

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## 5.0 REFERENCES

Hatch, T., 2010, Biodiesel Relative Risk: A Qualitative Approach to Determining the Environmental Fate of Animal Fat and Soy Biodiesels through a Direct Experimental Comparison with ULSD and Screening Model Simulations using HSSM, Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science, Department of Civil and Environmental Engineering, University of California, Davis.

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## A1.0 APPENDIX A: TOXICITY OF BIODIESEL BLENDS AND ULSD TO SELECTED FRESHWATER AND MARINE/ESTUARINE ORGANISMS

## A2.0 BACKGROUND

Biodiesel is a fuel composed of mono-akyl esters of long chain fatty acids derived from biological sources such as animal fat or vegetable oils. It can be used as a pure fuel or as a blend with petroleum diesel, since it is miscible with diesel at all ratios. The most common blend is B20 (20% biodiesel with 80% ultra-low sulfur diesel, ULSD). Since biodiesel is a new fuel, the California air Resources Board must provide a "multimedia risk assessment". As a result, the California Environmental Protection Agency has initiated a 3-tier program conducted by UC Davis and UC Berkeley to assess the multimedia life-cycle impacts, including ecological effects, of biodiesel fuels used in California. One of the data gaps identified by the Tier I assessment (1) is the paucity of aquatic toxicity information on the most common biofuels, from soy and animal feedstocks, along with their most common blend and additive. The impact of biodiesel is assessed as a relative risk compared with ULSD. Accordingly, AQUA-Science was retained by UC Davis (Dr. Michael Johnson, Director of the Ecosystems Analysis Laboratory) to conduct aquatic toxicity testing using a suite of three freshwater and three estuarine/marine organisms. The test organisms are phylogenetically diverse and have published USEPA aquatic toxicity protocols available. AQUA-Science has over 30 years experience in conducting these test protocols and is certified by the Environmental Laboratory Accreditation Program (ELAP; Certificate No. 2205) to conduct chronic toxicity tests with all six organisms selected for this study.

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## A3.0 METHODS AND MATERIALS

### A3.1 Source and Preparation of Biodiesel Test Solutions

The test materials included seven fuel types, including ultra-low sulfur diesel (ULSD), neat biofuels derived from animal fat (AF B-100) and soy (Soy B-100) feedstocks, 80% ULSD:20% (w/w) mixtures of the two biofuels (AF B-20 and Soy B-20), as well as the two B-20 mixtures amended with an antioxidant additive (AF B-20A and Soy B-20A). The test materials were provided by CA Air Resources Board (c/o R. Okamoto) and collected by T. Ginn/UC Davis and . stored in 1-gallon or 1-quart glass amber bottles in the dark at 20 °C with minimal headspace. Samples transferred to the AQUASCI lab were stored in original containers in the dark at 4°C until the water accommodated fractions (WAFs) were prepared. WAFs of the test materials were prepared using a low mixing energy procedure that eliminates the entrainment of particulate oil in the water column and prevents emulsification (2, 3, 4). The test materials were added to the top of a 2-gallon glass aspirator bottle containing the appropriate toxicity test dilution water at a 1:10 fuel-water ratio. The bottle was capped with aluminum foil and stirred using a magnetic stirrer at low speed (~120 rpm using a stir bar of 1.5 cm L x 0.5 cm diameter) without vortex formation. Mixing was conducted at the toxicity test protocol temperature for 18 hours followed by a 2-hour settling period to allow re-coalescence and surfacing of bulk oil particles. The WAF was carefully removed by siphon and stored at toxicity test protocol temperature until use within 24 hours of preparation. Samples of each WAF (100 mL) were taken immediately after

preparation and from the highest concentration in the toxicity test after 24 hours or at test termination (as appropriate) for analytical chemistry.

The fuels and mixtures tested in this study are shown in Table A1.

**Table A1. Fuels used in the Aquatic Toxicity testing**

| *Fuel Type*[a] | *Code* |
|---|---|
| 100% Ultra-Low Sulfur Diesel | ULSD |
| 100% Soy Biodiesel | Soy B-100 |
| 20% Soy Biodiesel + 80% ULSD (w/w) | Soy B-20 |
| 20% Soy + 80% ULSD (w/w) amended with additive[b] | Soy B-20A |
| 100% Animal Fat Biodiesel | AF B-100 |
| 20% Animal Fat Biodiesel + 80% ULSD (w/w) | AF B-20 |
| 20% Animal Fat + 80% ULSD (w/w) amended with additive | AF B-20A |

a Soy and Animal Fat refer to the feed stocks for the fuel
b The additive was Eastman BIOEXTEND™ 30 antioxidant

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## A3.2 Aquatic Toxicity Tests

The suite of aquatic test organisms tested in this study included both freshwater and estuarine/marine species comprising a wide phylogenetic diversity. Freshwater organisms included a green alga (*Selenastrum capricornutum*), a larval fish (fathead minnow, *Pimephales promelas*), and an invertebrate (water flea, *Ceriodaphnia dubia*). These species constitute the USEPA three-species test series that is employed extensively throughout the U.S. to evaluate the toxicity of discharges (treated effluents and storm waters), as well as chemicals that may enter ambient freshwaters (5). The estuarine/marine organisms included a mollusk (red abalone, *Haliotis rufescens*), an invertebrate (mysid shrimp, *Mysidopsis bahia*), and a fish (topsmelt, *Atherinops affinis*). The abalone and topsmelt are species recommended by USEPA when tests are used in assessment of toxicity of effluents and chemicals discharged to West Coast estuarine and marine waters (6), while the mysid shrimp is a standard estuarine/marine species recommended by USEPA (7) for use in toxicity tests with discharges into all estuarine receiving waters. A summary of the test protocol conditions are shown in Table A2.

For continuity, each of the toxicity tests were conducted using the same dilution series: Control (laboratory dilution water amended to protocol specifications), 1, 5, 10, 25, 50 and 100% WAF for each fuel and mixture. Some tests were randomly repeated to check for reproducibility.

**Table A2. Summary of Aquatic Chronic Toxicity Test Protocol Conditions**

| *Category* | *Test Species* | *Test Type* | *Test Endpoints* | *Replicates* | *Temp.* |
|---|---|---|---|---|---|
| Freshwater | Green algae (*S. capricornutum*) | 96-hour static | Cell growth | 10,000 cells/rep 4 reps/conc | 25 ± 1 °C |
| | Water flea (*C. dubia*) | 7-day daily renewal | Survival Reproduction | 1 flea/rep 10 reps/conc | 25 ± 1 °C |
| | Fathead minnow (*P. promelas*) | 7-day daily renewal | Survival Growth | 10 fish/rep 4 reps/conc | 25 ± 1 °C |
| Estuarine/ Marine | Red abalone (*H. rufescens*) | 48-hour static | Normal shell development | 5 reps/conc 2000 embryos/rep | 15 ± 1 °C |
| | Mysid shrimp (*M. bahia)* | 7-day daily renewal | Survival Growth Fecundity | 5 fish/rep 8 reps/conc | 25 ± 1 °C |
| | Topsmelt (*A. affinis*) | 7-day daily renewal | Survival Growth | 5 fish/rep 5 reps/conc | 20 ± 1 °C |

### A3.2.1 Green Algae Chronic Test Procedures

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The 96-hour algae (*S. capricornutum*) toxicity tests were conducted in 4 replicates of 125-mL flasks containing 50-mL of test sample filtrate (0.45 µm). A fifth replicate was used as a surrogate for daily water quality measurements. The flasks, containing algal assay media with EDTA, were inoculated with 1 x $10^4$ cells/mL of a 2-4 day-old culture of *S. capricornutum* (University of Texas Algae Type Collection, Austin, TX) in log phase growth. A sixth replicate was tested without algae inoculate to confirm that indigenous algae were not present. This replicate was also used as a sample blank. Flasks were placed on a shaker table (100 rpm) in an environmental chamber at 25 °C ± 1 °C with continuous lighting (400 ± 40 fc) and were randomized twice daily. After the 96-hour test period, the absorbance was measured with a spectrophotometer at 750 nm (Model DR2800, Hach Co., Loveland, CO). The absorbance units were corrected to cell number using a calibration curve as follows:

$$\text{cell number} = (\text{absorbance units @ 750 nm} \times 13.026) - 0.0328 \qquad (R^2 = 0.9995)$$

Using this conversion, the test was acceptable if the mean algal density in the control flasks was greater than or equal to 1 x $10^5$ cells/mL and the coefficient of variation in the control replicates was ≤20%.

### A3.2.2 Water Flea Chronic Test Procedures

Water flea *(C. dubia*) neonates (< 24 hours old) were obtained from in-house cultures maintained in reverse osmosis- and granular carbon-treated well water amended with dry salts to USEPA moderately hard (EPAHM) specifications. Tests were conducted in 20 mL glass scintillation vials containing 18 mL of test solution, which was renewed daily. There were ten vials per concentration with one *C. dubia* per vial. EPAMH was used as dilution water. Tests were

conducted in an environmental chamber at 25 ± 1 °C with a photoperiod of 16 hours light:8 hours dark. Organisms were fed a mixture of green algae (*S. capricornutum*); University of Texas Algae Type Collection; Austin, TX), blended trout food (Silvercup, Murray, UT), and organic alfalfa obtained locally. Mortality and reproduction endpoints and water quality parameters were monitored daily. The test was terminated after ≥60% of the controls had delivered three broods. The test protocol requires 80% survival and a minimum of 15 neonates per female in the control.

**A3.2.3 Fathead Minnow Chronic Test Procedures**

Fathead minnows (*P. promelas*; < 24 hours old) were obtained from AQUA-Tox Inc. (Hot Springs, AK) via overnight air freight. Exposures were conducted in 500 mL glass beakers containing 200 mL of sample using 10 fish per replicate with 4 replicates per concentration, in a temperature-controlled room at 25 ± 1 °C with a photoperiod of 16 hours light:8 hours dark. Dilution water was reverse osmosis- and granular carbon-treated well water amended with dry salts to EPAMH specifications. Fish were fed *Artemia* sp. nauplii twice daily. Test solutions were renewed and mortality was noted daily. At test termination, fish were killed by immersion in anesthetic (MS-222), pooled by replicate, dried for 6 hours at 100 °C and weighed to an accuracy of 0.01 mg using an electronic balance (Denver Instrument Co., Denver, CO). The test protocol requires a minimum of 80% survival and a minimum weight of 0.25 mg/fish in the control.

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**A3.2.4 Red Abalone Chronic Test Procedures**

Gravid red abalone (*H. rufescens)* were obtained from The Cultured Abalone (Goleta, CA) and acclimated in a recirculating seawater system for ≥48 hours prior to testing. Test samples were brought to protocol salinity (34 ± 2 ppt), using hypersaline brine (HSB) prepared by freezing high quality seawater. Dilution water was EPAMH water amended with HSB to 34 ± 2 ppt. Four male and female abalone were induced to spawn using a hydrogen peroxide solution and gametes were collected separately. Sperm and eggs were combined and 2000 embryos were used for each replicate with five replicates per concentration. Tests were conducted in an environmental chamber at 15 ± 1 °C with a light intensity of 10 µE/$m^2$/sec and a photoperiod of 16 hours light:8 hours dark. After 48 hours, embryos were removed from the replicates, washed with seawater, placed in 20-mL labeled glass vials, and terminated by addition of 750 µL of 37% formalin to each replicate. One hundred embryos from each replicate were examined microscopically and scored for normal shell development. The protocol acceptability requirement is ≥80% normal shell development in the control.

**A3.2.5 Mysid Chronic Test Procedures**

Mysids (*M. bahia*; 7 days old at test initiation) were obtained from Aquatic Bio Systems, Inc. (Fort Collins, CP) via overnight air freight. Mysids were acclimated in EPAHM water amended with dry sea salts (Instant Ocean™, www.marinedepot.com) to 20-30 ± 2 ppt. Testing was conducted in an environmental chamber at 25 ± 1 °C using a 16 hours light:8 hours dark photoperiod. Test containers were 400 mL plastic beakers containing 250 mL of test solution using eight replicates containing five mysids for each test concentration. Mysids were fed

*Artemia* sp. nauplii twice daily. Test solutions were renewed by 80% water replacement and mortality was noted daily. At test termination, mysids were anesthetized in an ice bath, grouped by replicate, dried at 100 °C for 6 hours and weighed to 0.01 mg using an electronic balance (Denver Instrument Co., Denver, CO). The protocol control performance requirements are ≥80% survival and a minimum weight of 0.20 mg/mysid.

### A3.2.6 Topsmelt Chronic Test Procedures

Larval topsmelt (*A. affinis*; 9-12 days old) were obtained from Aquatic Bio Systems, Inc. (Fort Collins, CO) via overnight air freight. Fish were acclimated in EPAMH water amended with dry sea salts to 25 ± 3 ppt. Testing was conducted in an environmental chamber at 20 ± 1 °C using a 16 hours light:8 hours dark photoperiod. Test containers were 600 mL plastic beakers containing 200 mL of test solution using five replicates containing five fish for each test concentration. Fish were fed *Artemia* sp. nauplii twice daily. Test solutions were renewed and mortality was noted daily. At test termination, fish were anesthetized (MS-222), grouped by replicate, dried at 100 °C for 6 hours and weighed to 0.01 mg using an electronic balance (Denver Instrument Co., Denver, CO). The protocol control performance requirements are ≥80% survival and a minimum weight of 0.85 mg/fish.

## A3.3 Water Quality Measurements

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Water quality measurements including temperature, dissolved oxygen (D.O.), pH, alkalinity, hardness, and conductivity or salinity were made on freshly prepared samples. Temperature, D.O. and pH were measured in 24-hour solutions from sample change-out. Temperature was measured in initial and daily test solutions at change-out with a calibrated digital thermometer (Central Co., Friendswood, TX), and was continuously recorded in the environmental chambers using a Dickson circular chart recorder (Model ICT855, Addison, IL). Water quality instrumentation included dissolved oxygen (YSI Model 550A, Yellow Springs, OH), pH (Beckman 240, Fulton, CO), and conductivity (WTW Model 330, Ft. Myers, FL) meters. Alkalinity (Hach Model AL-DT) and hardness (Hach HA-DT) were measured with Hach colorimetric tests (Hach Co., Loveland, CO).

## A3.4 Test Endpoint Determination

Test endpoint calculations were performed using a computer program (ToxCalc v. 5.2.23, TidePool Scientific, McKinleyville, CA) and the results are reported in terms of four metrics, per species-endpoint combination. The metrics are: no-observable-effect-concentration (NOEC), the highest concentration that did not produced statistically significant effects compared with the control; lowest-observable-effect-concentration (LOEC), the lowest concentration that produced a statistically significant effect compared with the control; effects concentration affecting 25% of the test population ($EC_{25}$); effects concentration affecting 50% of the test population ($EC_{50}$); and toxic units (TU) defined as the reciprocal of the $EC_{25}$ x 100. The percent minimum significant difference (PMSD) is the smallest difference between the control and another test treatment that can be determined as statistically different in a given test. Therefore, PMSD is a measure of test sensitivity that is dependent upon the within-test variability. Each of the statistical outputs was checked against the test raw data by the Laboratory Quality Assurance Manager.

## A4.0 RESULTS AND DISCUSSION

Chronic toxicity test results for ULSD and the two biofuels and mixtures are presented by test species.

### A4.1 Algae Chronic Toxicity Test Results

Table A3 and Figure A1 summarize results of the biofuel toxicity tests with green algae. The raw data for this test series is found in Section A1.

**Table A3. Summary of Biodiesel Toxicity Tests with Green Algae (*S. capricornutum*)**

| *Fuel Type* | *Values are % WAF* | | | | *Toxic Units* | *PMSD* |
|---|---|---|---|---|---|---|
| | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | *($100/EC_{25}$)* | *(%)* |
| ULSD | 100 | 100 | > 100 | > 100 | **< 1.0** | 12.1 |
| AF B-100 | 1 | 5 | 8.8 | 26.1 | **11.4** | 9.3 |
| AF B-100[a] | 5 | 10 | 9.3 | 21.9 | **10.8** | 6.6 |
| AF B-20 | 5 | 10 | 13.0 | 28.9 | **7.7** | 6.2 |
| AF B-20[a] | 1 | 5 | 20.1 | > 100 | **5.0** | 6.4 |
| AF B-20A | 50 | 100 | > 100 | > 100 | **< 1.0** | 6.8 |
| Soy B-100 | 1 | 5 | 4.7 | 9.3 | **21.3** | 5.3 |
| Soy B-20 | 5 | 10 | 44.1 | 75.5 | **2.3** | 8.9 |
| Soy B-20A | 25 | 50 | > 100 | > 100 | **< 1.0** | 14.2 |
| Soy B-20A[a] | 50 | 100 | > 100 | > 100 | **< 1.0** | 9.1 |

a Repeat test

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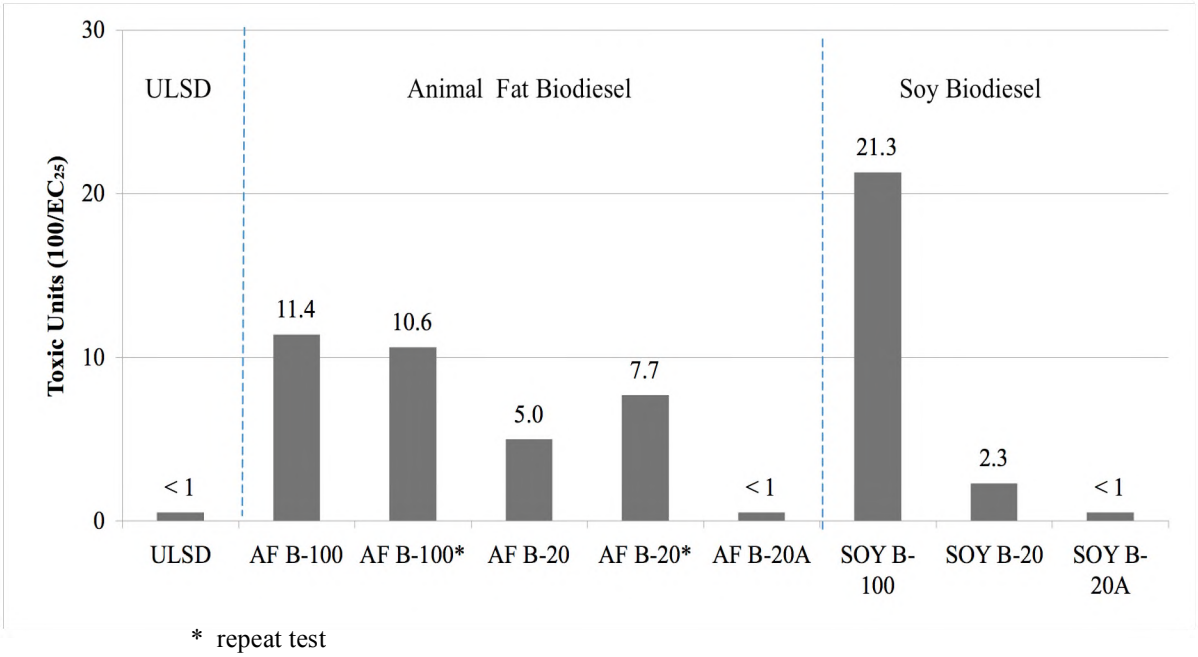
**Figure A1. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Green Algae**




* repeat test

ULSD did not produce a detectable reduction in algal cell growth, e.g., the NOEC=100%. Two tests conducted with AF B-100 resulted in TUc values of 11.4 and 10.6, while two tests conducted with AF B-20 demonstrated less toxicity with values of 5.0 and 7.7. Toxicity tests with the Soy biodiesel resulted in 21.3 TUc for the Soy B-100 and 2.3 TUc for the Soy B-20. The Soy B-20A and the AF B-20A mixtures with the additive did not exhibit toxicity, which was surprising given the increased toxicity imparted by the additive in toxicity tests with all of the other species. Additional tests with the additive and with the B-20 mixtures coupled with the analytical chemistry results would be required to elucidate the causes of these results.

### A4.2 Water Flea (*C. dubia*) Chronic Toxicity Test Results

Table A4 and Figure A2 summarize results of the biofuel toxicity tests with *C. dubia*. The raw data for this test series is found in Section A2.

**Table A4. Summary of Biodiesel Toxicity Tests with Water Flea (*C. dubia*)**

| *Fuel Type* | *Values are % WAF* | | | | | *Toxic Units* | *PMSD* |
|---|---|---|---|---|---|---|---|
| | *Test Endpoint* | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | *($100/EC_{25}$)* | *(%)* |
| ULSD | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 7.9 |
| | Reproduction | 25 | 50 | 54.5 | 71.9 | **1.8** | 22.6 |
| AF B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 19.6 |
| | Reproduction | 100 | > 100 | > 100 | > 100 | **< 1** | 22.7 |
| AF B-20 | Survival | 25 | 50 | 37.5 | > 50 | **2.7** | 16.3 |
| | Reproduction | 10 | 25 | 21.2 | 34.8 | **4.7** | 17.8 |
| AF B-20A | Survival | 1 | 5 | 2.0 | 3.0 | **50** | a |
| | Reproduction | < 1 | < 1 | 1.0 | 2.4 | **100** | 18.1 |
| Soy B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 19.2 |
| | Reproduction | 5 | 10 | 14.7 | 31.8 | **6.8** | 10.6 |
| Soy B-20 | Survival | 1 | 5 | 4.7 | 9.3 | **21** | 5.3 |
| | Reproduction | 5 | 10 | 44.1 | 75.5 | **2.3** | 8.9 |
| Soy B-20A | Survival | 1 | 5 | 2.0 | 3.0 | **50** | 6.5 |
| | Reproduction | 1 | 5 | 0.9 | 2.5 | **111** | 17.8 |

a Cannot be determined

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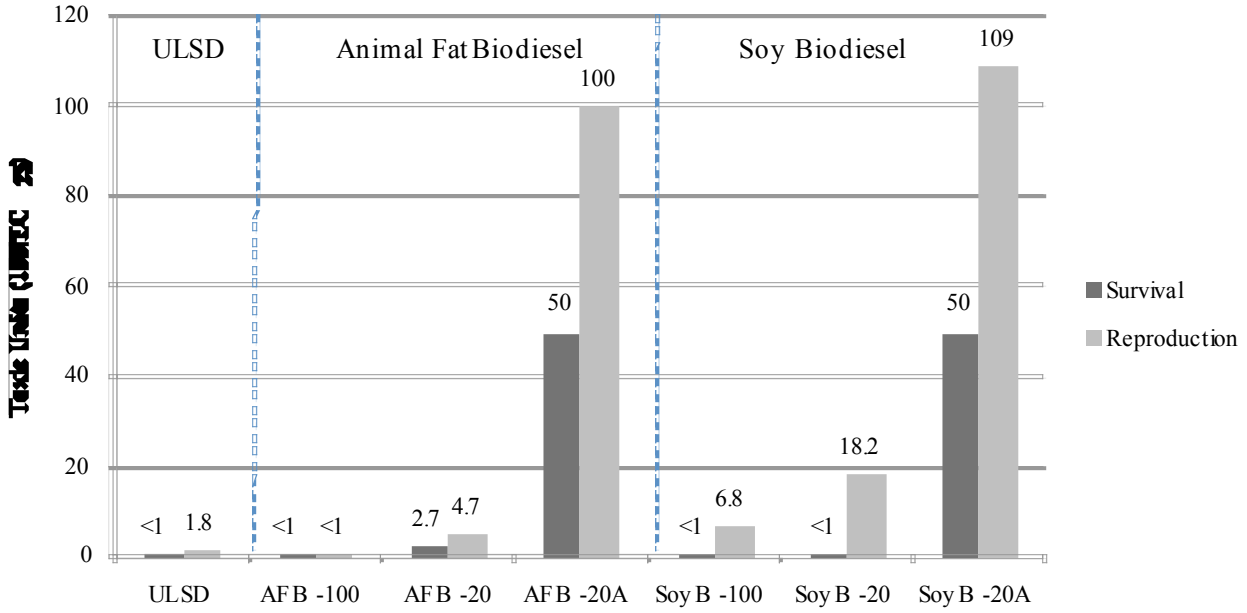
**Figure A2. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to *C. dubia* Survival and Reproduction**

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ULSD produced no effects on water flea survival and relatively low toxicity (1.8 TUc) on reproduction. Similarly, the AF B-100 resulted in no toxicity to both endpoints (< 1 TUc), while the AF B-20 resulted in moderate toxicity to both survival (2.7 TUc) and reproduction (4.7 TUc), which, interestingly, was greater than the toxicity of either of the two individual components (ULSD and AF B-100) that comprise the mixture. A similar pattern was seen with the soy biodiesel materials for the reproductive endpoint. Neither Soy B-100 nor B-20 exhibited effects on survival. Soy B-100 exhibited 6.8 TUc, while Soy B-20 exhibited 18.2 TUc on reproduction. There are obvious interactions between USLD and both biodiesel materials that would require additional toxicity tests on the mixtures to elucidate. Very high toxicity (50 to >100 TUc) was observed on survival and reproduction with both B-20A mixtures (containing additive). Dose-response curves associated with both tests were extremely steep (a large effect resulted from a very small increase in the additive concentration), which suggests that the additive affected a very sensitive and possibly specific receptor in the organisms. Toxicity screening of other additive chemicals to identify less toxic alternatives for use in biodiesel appears warranted.

### A4.3 Fathead Minnow Chronic Toxicity Test Results

Table A5 and Figure A3 summarize results of the biofuel toxicity tests with fathead minnow. The raw data for this test series is found in Section 3.

The fathead minnow survival and growth endpoints were unaffected by ULSD, AF B-100, AF B-20, Soy B-100 and Soy B-20. However, both biodiesel B-20A mixtures resulted in toxicity to both endpoints. AF B-20A exhibited moderately greater toxicity (7.3 TUc and 7.7 TUc) than did the Soy B-20A (3.6 TUc and 3.2 TUc) to the survival and reproduction endpoints, respectively.

**Table A5. Summary of Biodiesel Toxicity Tests with Fathead Minnow (*P. promelas*)**

| *Fuel Type* | *Values are % WAF* | | | | | *Toxic Units* | *PMSD* |
|---|---|---|---|---|---|---|---|
| | *Test Endpoint* | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | *($100/EC_{25}$)* | *(%)* |
| ULSD | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.8 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 14.4 |
| AF B-100 | Survival | 100 | 100 | > 100 | > 100 | **< 1** | 3.8 |
| | Growth | 25 | 50 | > 100 | > 100 | **< 1** | 8.7 |
| AF B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | a |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 12.4 |
| AF B-20[a] | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | a |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 10.7 |
| AF B-20A | Survival | 10 | 25 | 13.7 | 17.4 | **7.3** | 2.5 |
| | Growth | 10 | 25 | 13.0 | 17.0 | **7.7** | 11.0 |
| Soy B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 2.0 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 13.2 |
| Soy B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | b |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 10.7 |
| Soy B-20A | Survival | 10 | 25 | 27.9 | 35.3 | **3.6** | 2.3 |
| | Growth | 10 | > 10 | 30.9 | 37.3 | **3.2** | 11.7 |

a PMSD could not be determined
b Repeat test

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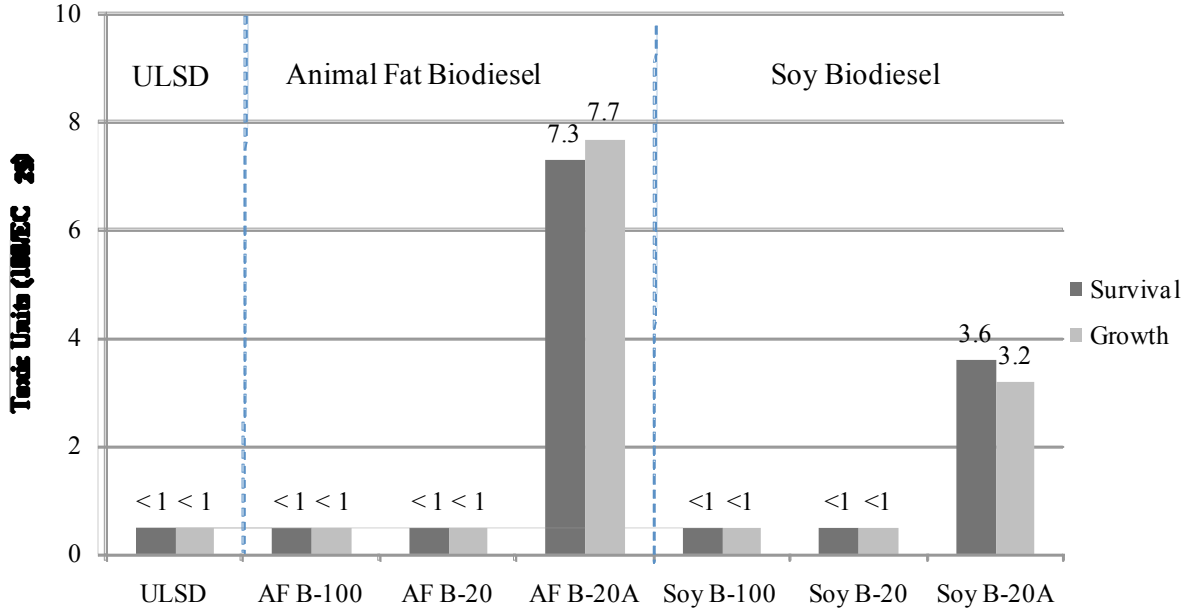
**Figure A3. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Fathead Minnow Survival and Growth**



## A4.4 Abalone Chronic Toxicity Test Results

Table A6 and Figure A4 summarize results of the biofuel toxicity tests with abalone. The raw data for this test series is found in Section A4.

No effects on abalone shell development were detected with ULSD. AF B-100 exhibited somewhat higher toxicity than the Soy B-100 (7.4 TUc and 3.0 TUc, respectively), while the AF B-20 and Soy B-20 mixtures had similar or slightly less toxicity as their respective B-100 fuels (4.8 and 3.1 TUc, respectively), as expected. The additive substantially increased the toxicity of both B-20 mixtures: AF B-20A exhibited 34.5 TUc, a 7-fold increase, while two Soy B-20A tests detected 7.7 TUc and 8.1 TUc, approximately a 3-fold increase.

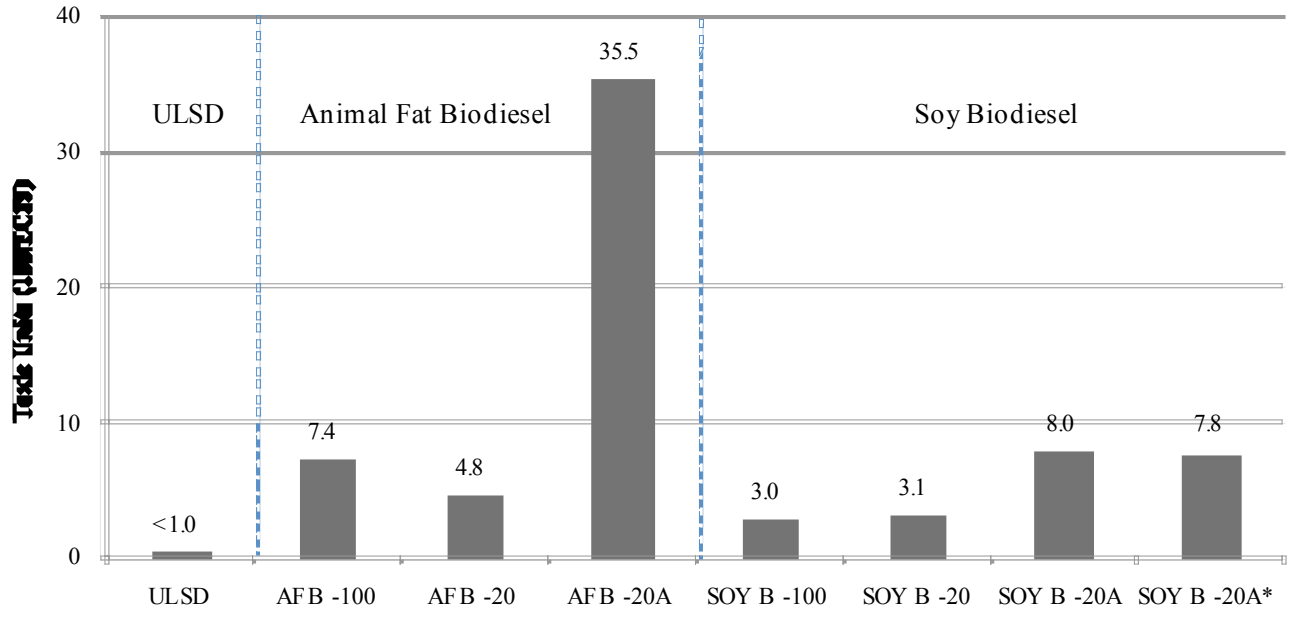
**Table A6. Summary of Biodiesel Toxicity Tests with Abalone (*H. rufescens*)**

| *Fuel Type* | *Values are % WAF* | | | | *Toxic Units (100/$EC_{25}$)* | *PMSD (%)* |
|---|---|---|---|---|---|---|
| | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | | |
| ULSD | 1 | 5 | > 100 | > 100 | **< 1.0** | 4.0 |
| AF B-100 | 10 | 25 | 13.5 | 17.4 | **7.4** | 3.0 |
| AF B-20 | 10 | 25 | 20.6 | 31.0 | **4.9** | 4.6 |
| AF B-20A | 1 | 5 | 2.9 | 5.1 | **34.5** | 4.0 |
| Soy B-100 | 25 | 50 | 33.1 | 42.7 | **3.0** | 4.0 |
| Soy B-20 | 10 | 25 | 32.0 | 41.2 | **3.1** | 4.5 |
| Soy B-20A | < 1 | 1 | 13.0 | 17.0 | **7.7** | 3.5 |
| Soy B-20[a] | 5 | 10 | 12.3 | 16.5 | **8.1** | 4.2 |

a Repeat test

DRAFT

**Figure A4. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Abalone Shell Development**



## A4.5 Mysid Chronic Toxicity Test Results

Table A7 and Figure A5 summarize results of the biofuel toxicity tests with mysid. The raw data for this test series is found in Section A5.

**Table A7. Summary of Biodiesel Toxicity Tests with Mysid (*M. bahia*)**

| *Fuel Type* | *Test Endpoint* | *Values are % WAF* | | | | *Toxic Units (100/$EC_{25}$)* | *PMSD (%)* |
|---|---|---|---|---|---|---|---|
| | | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | | |
| ULSD | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.4 |
| | Growth | 50 | 100 | 99.0 | > 100 | **1.0** | 14.3 |
| AF B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.2 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 17.1 |
| AF B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 4.0 |
| | Growth | 50 | 100 | > 100 | > 100 | **< 1** | 16.4 |
| AF B-20A | Survival | 25 | 50 | 31.5 | 39.6 | **3.2** | 10.0 |
| | Growth | 25 | 50 | 31.4 | 39.6 | **3.2** | 18.6 |
| Soy B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 4.4 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 13.1 |
| Soy B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.8 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 11.4 |
| Soy B-20A | Survival | 100 | >100 | > 100 | > 100 | **< 1** | 15.2 |
| | Growth | 25 | 50 | 56.9 | > 100 | **1.8** | 19.1 |

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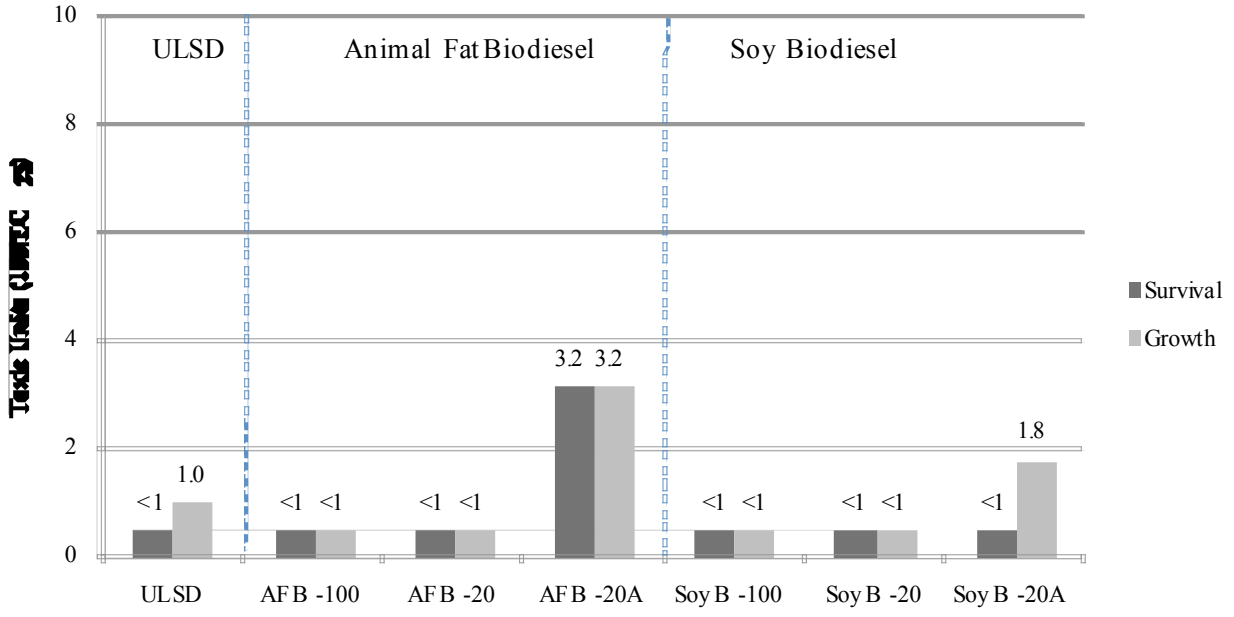
**Figure A5. Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Mysid Survival and Growth**



Effects on the mysid survival and growth endpoints were either absent or very low (< 1 or 1.0 TUc) for the USLD, and all biofuels and mixtures tested except those containing additive. The AF B-20A exhibited 3.2 TUc to both endpoints, while the Soy B-20A produced 1.8 TUc to the growth endpoint.

### A4.6 Topsmelt Chronic Toxicity Test Results

Table A8 and Figure A6 summarize results of the biofuel toxicity tests with topsmelt. The raw data for this test series is found in Section A6.

No effects on either survival or growth were detected with ULSD or either of the biofuels and mixtures that did not contain the additive. The AF B-20A test detected 13.0 TUc on survival and 10.5 TUc on growth, while the Soy B-20A test detected slightly less toxicity with 8.5 TUc on survival and 7.3 TUc on growth.

**Table A8. Summary of Biodiesel Toxicity Tests with Topsmelt (*A. affinis*)**

| *Fuel Type* | *Test Endpoint* | *Values are % WAF* | | | | *Toxic Units* | *PMSD* |
|---|---|---|---|---|---|---|---|
| | | *NOEC (%)* | *LOEC (%)* | *$EC_{25}$ (%)* | *$EC_{50}$ (%)* | *($100/EC_{25}$)* | *(%)* |
| ULSD | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 11.5 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 18.4 |
| AF B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.2 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 16.1 |
| AF B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | 3.1 |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 12.5 |
| AF B-20A | Survival | 5 | 10 | 7.7 | 11.2 | **13.0** | 15.3 |
| | Growth | 5 | 10 | 9.5 | 14.6 | **10.5** | 15.2 |
| Soy B-100 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | a |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 16.0 |
| Soy B-20 | Survival | 100 | > 100 | > 100 | > 100 | **< 1** | a |
| | Growth | 100 | > 100 | > 100 | > 100 | **< 1** | 11.0 |
| Soy B-20A | Survival | 5 | 10 | 11.8 | 16.2 | **8.5** | 6.9 |
| | Growth | 10 | 25 | 13.7 | 17.5 | **7.3** | 15.7 |

a PMSD could not be determined

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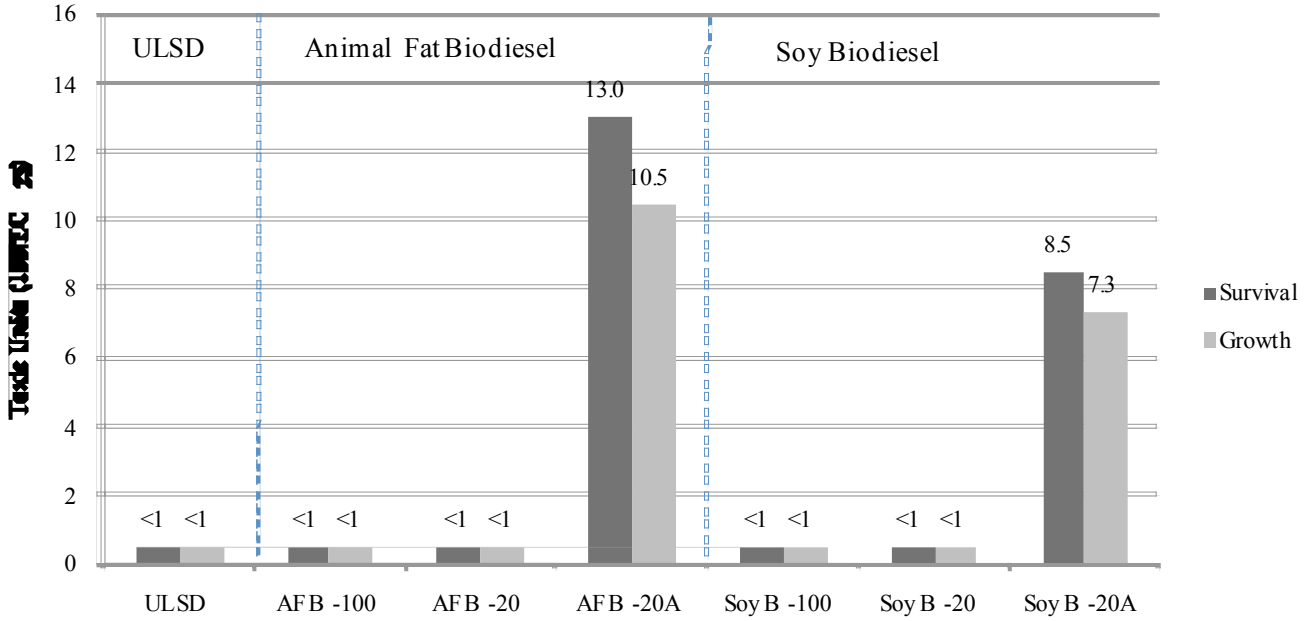
**Figure A6. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Topsmelt Survival and Growth**

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## A5.0 CONCLUSIONS

- ULSD produced relatively low but detectable toxicity on mysid growth (1.0 TUc) and water flea reproduction (1.8 TUc). No toxicity (< 1.0 TUc) was detected with any of the other species tested.
- Neither of the unadditized Animal Fat or Soy biodiesel test materials produced detectable toxicity to the mysid, topsmelt or fathead minnow.
- Animal Fat B-100, Soy B-100 and their B-20 mixtures caused toxicity to algae cell growth, water flea survival and/or reproduction, and abalone shell development
- Except for algae, the additized biodiesel B-20 test materials were substantially more toxic than the corresponding unadditized material.
- Tests that were repeated for confirmation produced similar results as the original test.
- Analytical chemistry information is needed on the fuel samples collected during the study to elucidate the chemical causes of toxicity and to provide information on the stability of WAF components during the toxicity tests.

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6. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. August 1995. USEPA 600/R-95/136.

7. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. October 2002. USEPA 821-R-02-014..

## B1.0 APPENDIX B: RELATIVE RATES OF INFILTRATION OF BIODIESEL BLENDS AND ULSD IN LABORATORY-SCALE SANDBOXES

As part of a multimedia risk assessment of biodiesel, the relative risks associated witih infiltration into the subsurface and eventual fate and transport processes affecting groundwater were identified as a priority knowledge gap (UC, 2008; Ginn et al., 2009). To address this knowledge gap, small scale "sandbox" infiltration experiments, were performed in order to simulate and evaluate the qualitative impacts of biodiesel fate and mobility in the subsurface compared directly to Ultra Low Sulfur Diesel (ULSD). For the purpose of the study two feedstocks were used: Animal Fat and Soybean Oil. Experiments were run with a pure fuel (B100) and a blended fuel (B20) for both feedstocks in a relative setting to afford relative assessmnt of the differences in fuel infiltration into unsaturated porous media, redistribution within the unsaturated zone, and eventual lens formation on the saturated surface.

Biodiesel is made up of multiple fatty-acid methyl esters (FAMEs), all of which have densities lighter than water. The resulting light non-aqueous phase liquid (LNAPL) is expected to float on water and thus to form lens geometries upon infiltration to a ground water table. As LNAPLs infiltrate into the subsurface after a spill, capillary forces cause some of the LNAPL to remain trapped in the pores above the water table. Once the main front of the plume reaches the water table it will start ponding within the capillary fringe just above the water table. The geometry of this lens is important to groundwater contamination because it is from the associated LNAPL/groundwater table interface that soluble components partition into the water phase. With enough LNAPL ponding, the weight of the lens can displace some water from the beneath the lens. As the groundwater flows beneath the lens, more LNAPL is free to partition into the water phase.

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## B2.0 METHODS AND MATERIALS

### B2.1 Source and Preparation of Biodiesel Test Solutions

Infiltration experiments were carried out for 5 different biodiesels blends, including three fuels derived from animal fat and two derived from soybean oil. For both animal fat and soy feedstocks, a pure sample (B-100) and a blended sample (B-20, with ULSD as the blend) all additized with the antioxidant Bioextend as per manufactuer's suggestion was evaluated. The fuels were provided by CA Air Resources Board (c/o R. Okamoto) and collected by T. Ginn/UC Davis and stored in 1-gallon or 1-quart glass amber bottles in the dark at 20 °C with minimal headspace.Each of these four fuel blends were compared in triplicate experiments to CARB #2 ULSD. An additional unadditized animal fat B100 was also tested in triplicate to see if there were any noticable effects on infiltration induced by the additive itself. The resulting suite of experiments is given in Table B1.

Table B1. Suite of blends studied in the sandbox infiltration experiments.

Sandbox Experimental Matrix

| Type | Feedstock | Totals | Additization | | | |
|---|---|---|---|---|---|---|
| | | | None | | Bioextend | |
| | | | # | Quantity | # | Quantity |
| B100 | Animal-fat | 6 | 3 | 50 mL/test | 3 | 50 mL/test |
| B100 | Soy | 3 | | | 3 | 50 mL/test |
| B20 | Animal-fat | 3 | | | 3 | 50 mL/test |
| B20 | Soy | 3 | | | 3 | 50 mL/test |
| ULSD | petroleum | 15 | 15 | 50 mL/test | | |

**Note:** Tests will include side by side comparison between ULSD and Biodiesel within the same antfarm for consistency of sand compaction.

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## B2.2 Sandbox Design

The objective of the sandbox design is to allow visualization of infiltrating fuels in side-by-side (biodiesel blend vs. ULSD) plumes introduced simultaneously. This calls for small scale infiltration domains in unsaturated porous media in two dimensions. The overall design of the sandbox is similar to commonly known vertical glass sandboxes known as "ant-farms." The design criteria for the fate and transport experiments were that it be of a scale where we could run side-by-side tests within the same apparatus to compare the biodiesel and ULSD. Sandbox design targets also easy assembly/disassembly and cleaning for use in multiple experiments with watertight conditions and with hose assembly to allow control of the elevation of the water table within the sandbox. It also needed to be made of non-reactive materials that would last long enough to complete all the experiments while exposed to the ULSD and biodiesel. The preliminary experiments and design testing details pertaining to these and other aspects of the sandboxes are described in detail in Hatch (2010). Only summary aspects of the medium selected, the fuel dye, and the photographic set up are presented here.

In order to provide a standardized medium for comparative assessment of fuel behavior, a uniform medium to coarse sand was selected for the model porous medium since it is easily replicated for future experiments and it would provide a relatively high hydraulic conductivity

for infiltration of the fuels thus reducing the experiment run time while representing a high-risk environment for groundwater contamination. Thus for the experiments, Cemex #30 sandblasting sand was used as the porous media. It was readily available in the local hardware store and provided a size range based on the #30 sieve size.

In order to perform a direct comparison of the fate and transport of biodiesel to ULSD it was necessary that they be done simultaneously. It was also important for the plumes to be far enough apart so that they would not meet and interact prior to reaching the water table.
To accommodate digial photography of the dual infiltrating plumes, a sandbox design was developed using wood to build a three-sided frame, 16 inches by 11 inches, that is used to separate two glass walls of same dimension (Figure B1). Glass is used instead of plexiglass in order to maintain a consistent refractive index in the presence of potentially reactive fuels after replicate use of the sandbox. Clamps are used to hold the sandbox together as these afford ready reassembly. The frame includes internal sealant on the wood components, watertight seals, and hoses with ports in the side panels to allow control of the water table elevation.

**Figure B1: Sandbox in photo booth**

Diesel fuel and biodiesel are not clearly visible compared to water in porous media. To render all fuel blends visible, 0.15 ml of a hydrophobic fuel dye (Solvent Red 26, Kinder Morgan, Inc.) used to dye diesel fuel for agricultural and off road applications was added to the 50 ml fuel samples. Preliminary experiments were done as controls to investigate the impact of this dye concentration on fuel transport effects and none were found (Hatch, 2010).

Digital photography was used to capture time-series of images of the side-by-side dyed fuel infiltration, redistribution, residual formation in the vadose zone, and lens formation on the water table. Each experiment was run for a duration of up to 2.0 hours (until steady state was reached). A mobile photo booth was designed following advice of George Redden of Idaho National Laboratory, an expert in digial photograph of experiments involving flow in porous media. This booth (Figure B1) involves consistent placement of the sandbox, a black velvet drape with fasteners to eliminate external light, and internal lamps placed at angles to the sandbox's outer facing glass window in order to provide controlled lighting without glare. A camera is placed on a tripod within a sealed window of the drape with remote control to allow the experimentalist to take photos at specified times without touching the experimental apparatus.

Visual analyses of the images was done to evaluate four separate time metrics defined in order to time the progress of the infiltration, redistribution, and formation of the lens of biodiesel on the saturated zone surface at the steady-state. These metrics are characteristic times for: elimination of ponded fuel, plume separation from surface, initial commencement of lens spreading on water table, steady-state lens formation on water table. In addition the qualitative characteristics of quantity of residual fuel appearing in the unsaturated zone and of lens shape after steady-state are reported.

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## B3.0 RESULTS AND DISCUSSION

Figure B2 shows the final images for two example fuels, Soy B-20 and Animal Fat B-100. These are selected to reflect the main result of the experiments, that with the exception of Animal Fat B-100, the biodiesel blends do not behave significantly differently from ULSD.formation and mobility of the biodiesel in a qualitative fashion for groundwater contamination. The left-hand panel shows Soy B-20 (with ULSD) and the similarity between the biodiesel and petroleum diesel fuel behavior here is representative of that observed in all fuel blends except for Animal Fat B-100, that shows a greater residual and thicker lens formation than ULSD, as shown in the right-hand panel. The behavior of the additized Animal Fat B-100 was very similar to that of the unadditized Animal Fat B-100.

The four time metrics are shown respectively for each experiment in Figures B3, B4, B5, and B, respectively. These figures show the characteristic times for each initial formation of the U-shaped plume underneath the ponded fuels, the time to separation of the fuel from the surface, the time for initial lens spreading on the water table, and the time for complete lens formation on the water table. These figures reflect identical behavior for each test fuel vs. ULSD in all cases with one minor difference seen for Soy B-100 in Figure B-3. The images themselves show the different qualitative behavior seen for Animal Fat B-100 (e.g., Figure B2).

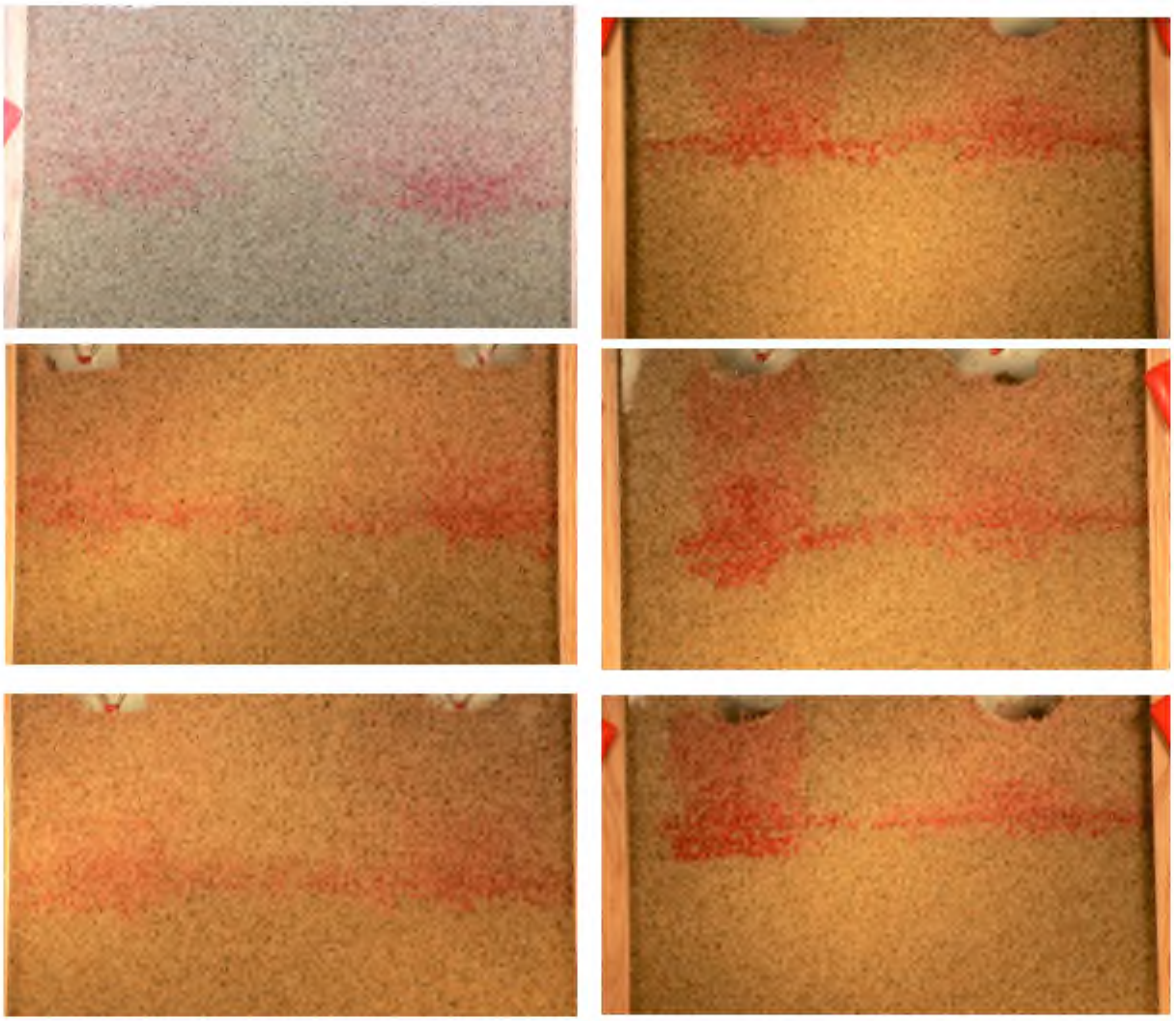

Figure B2. Example final digital images. Left panel: the triplicate images for Soy B-20 at 2 hours; the left hand plume is Soy B-20 and the right-hand plume is ULSD. Right pane: those for the Animal Fat B-100 at 2 hours; the left hand plume is Animal Fat B-100 and the right-hand plume is ULSD. Note the greater color density indicating increased residual of the Animal Fat B-100 in the vadose zone and the thicker lens formation on the water table, with respect to that of ULSD.

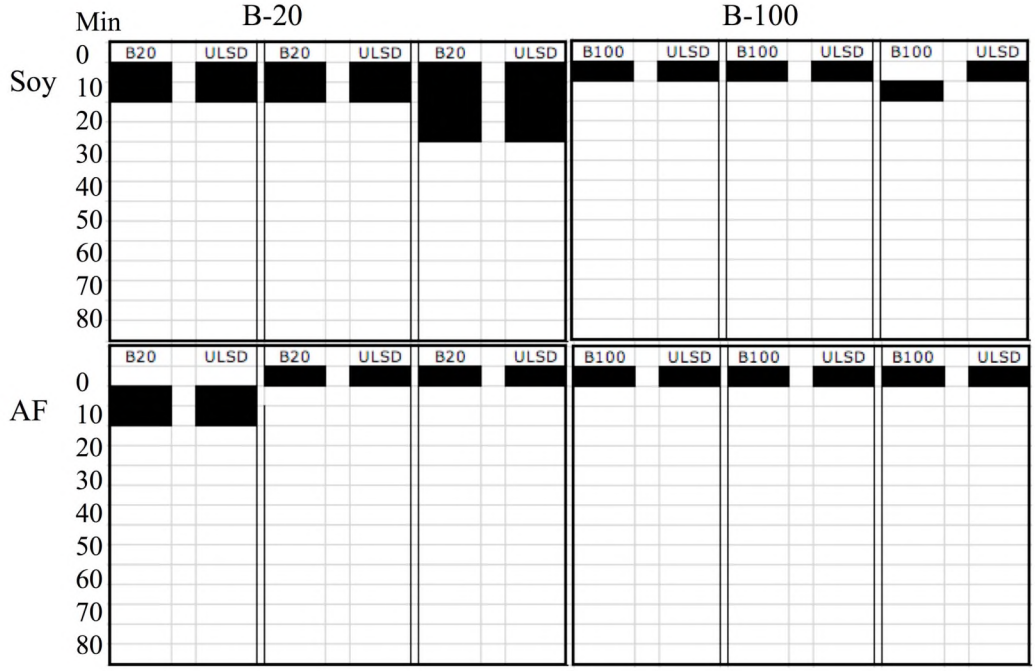


Figure B3: Characteristic times to formation of the U-shaped plume for each of the four blends (Soy B-20, Soy B-100, Animal Fat (AF) B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

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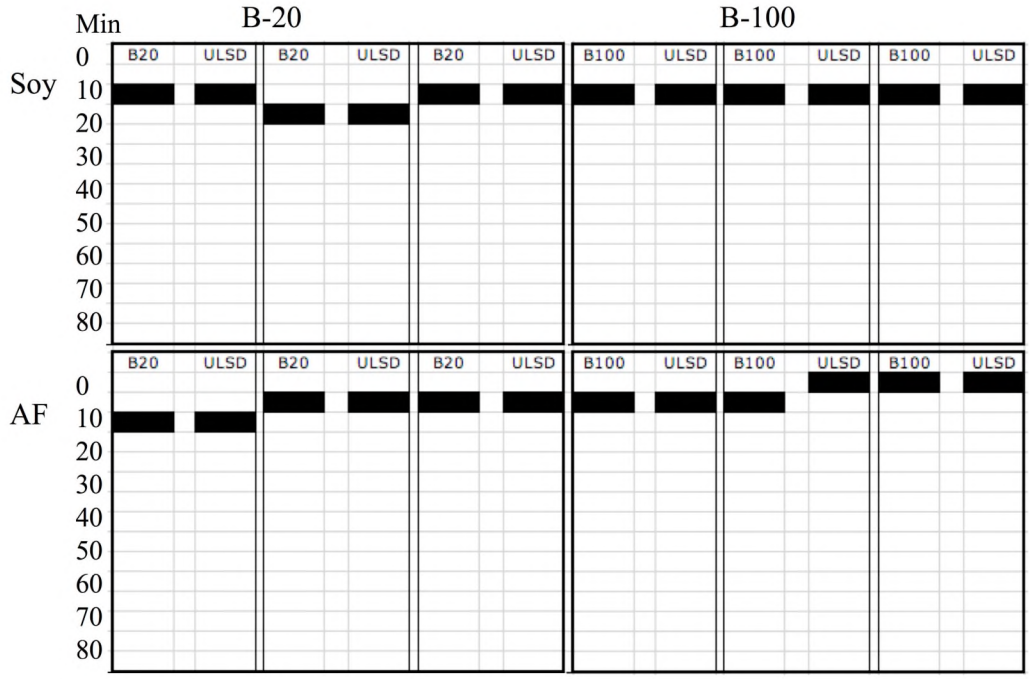


Figure B4: Characteristic times to plume plume separation from the sand surface for each of the four blends (Soy B-20, Soy B-100, Animal Fat (AF) B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

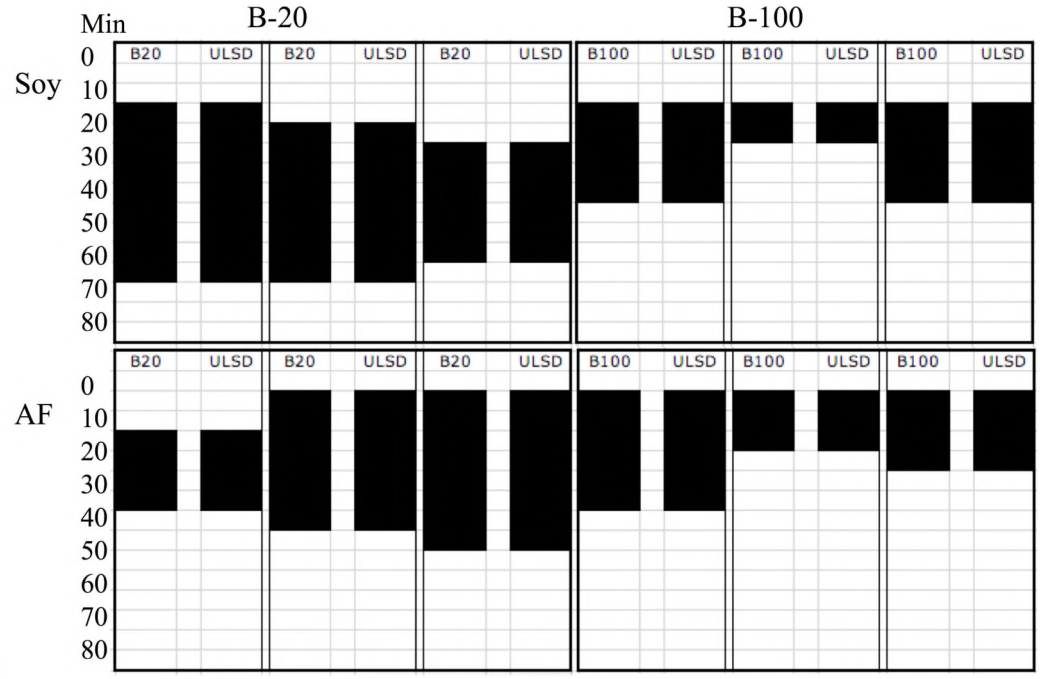


Figure B5: Characteristic times for commencement of lens spreading on the water table for each fuel (Soy B-20, Soy B-100, B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

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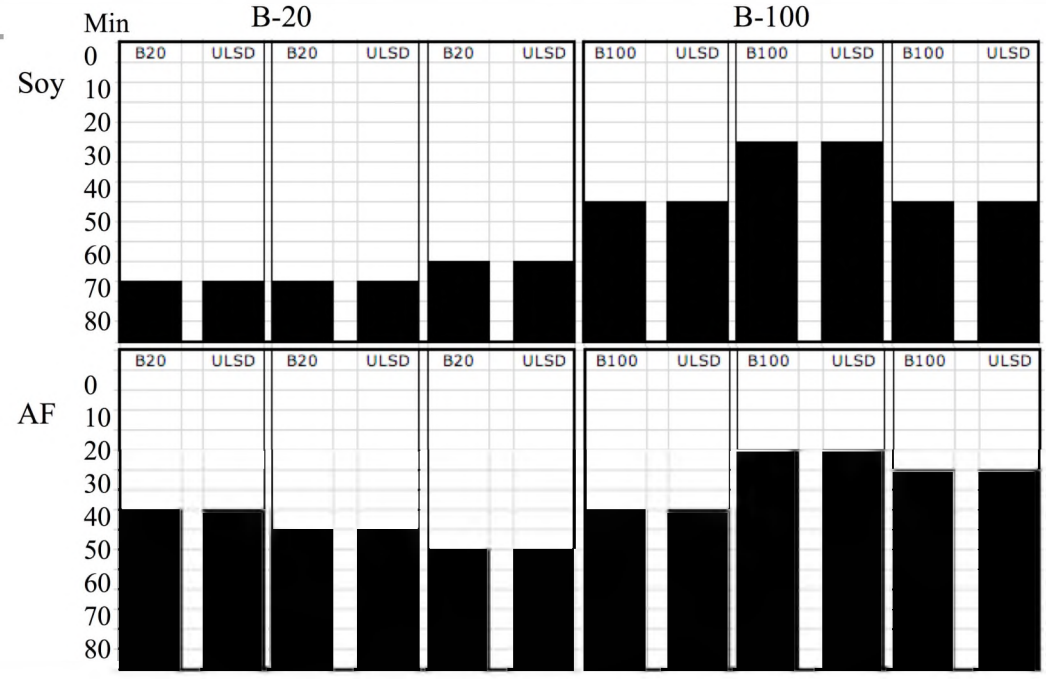


Figure B6: Characteristic times for lens formation on the water table for each of the four blends (Soy B-20, Soy B-100, AF B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

## B3.1 Discussion

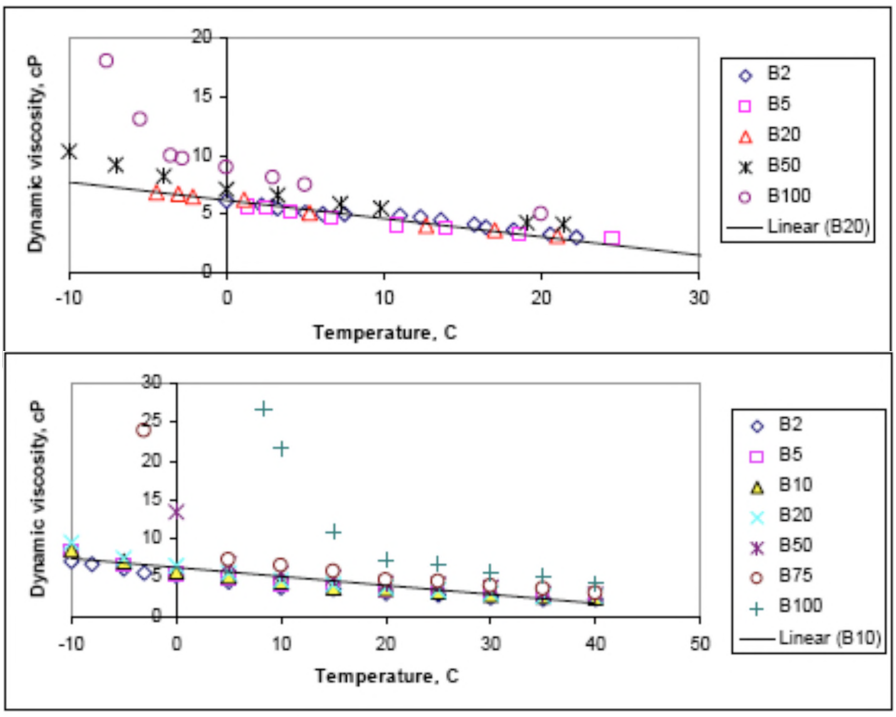


Figure B7. Biodiesel fuel blend (Soy, top; Animal fat, bottom) viscosities for a range of temperatures. Note relatively large increase in Animal Fat B-100 viscosity for temperatures below 20 degrees C. From Yang et al., 2008.

The increased residual and thicker form of the lens formed on the water table for the Animal Fat B-100 fuel may be ascribed to measureable physical propreties of the fuel. Yang et al. (2008) present data for a range of properties of animal fat and soy based biodiesel blends at different mixture fractions with petroleum diesel, from four states.. Important distinguishing characteristics for Animal Fat biodiesel is an increased viscosity and interfacial tension. Figure B7 (from Yang et al., 2008) shows the viscosity values for different fuel blends as a function of temperature: note the enhanced viscosity for animal fat blends. The interfacial tensions reported by Yang et al. (2008) for biodiesel blends from Minnesota are 8.5/12.0 (mN/m) for Soy (B20/B100), and 15.0/19.5 AF (B20/B100), whereas the value for low-sulfur petroleum diesel is 7.4 mN/m. Increased values of these properties lead to increased residual and thicker lenses (e.g. Charbeneau, 2000; Weaver et al., 1994).

## B4.0 CONCLUSIONS

- The antioxidant additive did not affect the infiltration of Animal fat B-100
- Soy biodiesel blends at both 20 and 100 percents, as well as the animal fat 20 percent blend, do not exhibit any significant differences among the four temporal metrics or among the qualitative residual or lens shape metrics compared to ULSD.
- Animal fat 100 percent blend exhibited similar values of the temporal metrics as ULSD, but it showed noticeable increases in the amount of residual that occurred in the

unsaturated zone, and it resulted in final lens geometry that was thicker in vertical dimension and less extensive in horizontal dimension than the ULSD lens.

This behavior is consistent with the physical properties of animal fat based biodiesel that has higher viscosity and interfacial tension than ULSD. These differences become significantly more pronounced at temperatures below 20 degrees Celsius.

## B5.0 REFERENCES


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## C1.0 APPENDIX C: RELATIVE RATES OF AEROBIC BIODEGRADATION OF BIODIESEL BLENDS AND ULSD

Microcosm experiments were conducted to assess the aerobic aqueous biodegradation potential (relative to that of petroleum diesel) for solutions exposed to the test biodiesel fuels. Ultra low sulfur diesel (ULSD) was used as the benchmark. Fuels derived from animal fat and soy feedstocks were tested as source phases as received (B100) or blended with ULSD to a B20 mixture (20% biodiesel). The biodiesel blends were tested in three forms: unamended, , amended (at industry specified amounts) with the antioxidant Bioextend-30, and amended with both Bioextend-30 and the biocide Kathon FP1.5. The reference ULSD fuel contained no additives. This suite of experiments is designed for a risk wise conservative simplified examination of the differences in biodegradation potential between petroleum and biomass-derived diesels.

The requirements for biodegradation testing of new chemicals vary widely among agencies, both in the US and internationally. The most extensive set of biodegradability tests are published by the OECD (a consortium of European agencies, the European Economic Community, the World Health Organization (WHO), and the United Nations). We followed the suite of microcosm experiments described here is designed based on the modified recommended OECD biodegradability test (OECD 2004). The OECD recommendsation, that microcosms be comprised of mineral salts medium, tested substrate, and bacterial inoculation using activated sludge from the aeration tank of a sewage treatment plant. In our microcosm experiments, we inoculated with soil rather than activated sludge for better representation of environmental conditions for biodegradation of spills of diesel and biodiesel.

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Biological activity was assessed by measuring products of measured through respiration. Under aerobic biodegradation, carbon compounds are transformed to biomass and $CO_2$ and the latter can be quantified by standard methods (per EPA 560/6-82-003, PB82-233008). Thus the evolution of $CO_2$ from biodegradation of the substrates as a result of microbial activity was measured in our microcosms using a respirometer (Columbus Instrument, Columbus, OH). Microcosms were incubated at controlled temperature of 25 °C for the recommended 28-30 days test period.

## C2.0 METHODS AND MATERIALS

### C2.1 Fuel Sample and Microcosm Preparation

The test materials included thirteen fuel types, including ultra-low sulfur diesel (ULSD), neat biofuels derived from animal fat (AF B-100) and soy (Soy B-100) feedstocks, 80% ULSD:20% (w/w) mixtures of the two biofuels (AF B-20 and Soy B-20): each of these four biodiesel blends was tested in the three forms, unadditized, additized with an antioxidant (Bioextend) and additized with both the antioxidant and a biocide (as per manufacturer's specifications). The fuels were provided by CA Air Resources Board (c/o R. Okamoto) and collected by T. Ginn/UC

Davis and stored in 1-gallon or 1-quart glass amber bottles in the dark at 20 °C with minimal headspace. The full suite of fuels tested are listed in Table C1 below.

The microcosms were prepared using a 250 mL flask that consists of 190 ml mineral medium, 2 g soil (Yolo, silty-loam) as bacterial inoculum and addition of 5 μL of test fuel as substrate- using micro pipette- that was roughly equivalent of a nominal concentration of 25 ppm (effective massic mass density if the fuel were to be dissolved) for each fuel test. The mineral medium contained the OECD-recommended nutrients $KH_2PO_4$, $K_2HPO_4$, $NaHPO_4$, $NH_4Cl$, $CaCl_2.H2O$, $MgSO_4$, and $FeCl_3.6H_2O$ (OECD 2004). Each treatment microcosm was prepared in three replicates. For each treatment, one abiotic sterile control was prepared using addition of 1% sodium azide. This control was to examine whether the test substrate is degradable in the absence of microorganisms. Three replicates of inoculum blank (no fuel substrate) were also prepared. The inoculums blank was to examine if there is any $CO_2$ production by microorganisms in the absence of fuel substrate.

Table C1 – Arrangement of fuel types and their abbreviation for each set of respirometer experiment

| Experiment | Fuel Type | |
|---|---|---|
| | Description | Abbreviation |
| #1 | Diesel | ULSD |
| | Soy biodiesel 20% blend + bioextend | Soy B-20 A |
| | Animal fat biodiesel 20% blend + bioextend | AF B-20 A |
| | Soy biodiesel 20% blend -no additives | Soy B-20 |
| #2 | Diesel | ULSD |
| | Soy biodiesel 100% - no additives | Soy B-100 |
| | Animal fat biodiesel 20% blend - no additives | AF B-20 |
| | Animal fat biodiesel 100% - no additives | AF B-100 |
| #3 | Diesel | ULSD |
| | Soy biodiesel 20% blend + bioextend + biocide | Soy B-20 AA |
| | Animal fat biodiesel 20% blend + bioextend + biocide | AF B-20 AA |
| | Soy biodiesel 100% + bioextend + biocide | Soy B-100 AA |
| #4 | Diesel | ULSD |
| | Animal fat biodiesel 100% + bioextend + biocide | AF B-100 A A |
| | Animal fat biodiesel 100% + bioextend | AF B-100 |
| | Soy biodiesel 100% + bioextend | Soy B-100 |

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## C2.2 Assessing Biological Activity

The $CO_2$ production in microcosms was automatically measured using a respirometer during the experiment. The carbon content of each fuel was determined by combustion/gas chromatography (Costech ECS4010 elemental analyzer). The carbon content of each fuel type measured by combustion/gas chromatography was reported as percent carbon by weight (percent gram of carbon per gram of fuel). The carbon content of 5 uL, initial fuel test in each microcosm, was calculated using percent carbon content and density of each fuel.

The carbon content of each microcosm is correlated with the accumulated CO2 production to compare the potential biodegradability of each fuel test in regard to diesel.

## C2.3 Respirometer

Aerobic biodegradation of diesel and biodiesel in microcosms was studied monitoring the respiration of microorganisms as indicated by $CO_2$ production. Respiration of the microcosms was measured using a Micro-Oxymax closed circuit respirometer (Columbus Instrument, Columbus, OH). The respirometer was equipped with a single beam, nondispersive, infrared $CO_2$ detector with a range of 0 to 0.8%. The headspace in the microcosms was refreshed with air when $CO_2$ concentrations exceeded ± 0.5%. $CO_2$ measurements were taken every 8-10 hours. The respirometer has 20 chambers (Figure C1) and each experiment comprised of 4 sets of fuel test and 1 set of control blank (no substrate) microcosms. At each experiment diesel fuel was one of the sets for comparison with other test fuels. Table C1 shows the arrangement of each experiment and code used for each fuel type. The duration of each experiment was 28-30 days.

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Figure C1: Respirometer equipment used for aerobic biodegradation monitoring in 29-day tests.

For each microcosm, the total initial carbon was compared to the cumulative carbon evolved as CO2 production. The fraction of initial carbon evolved as CO2 was taken as a measure of the biodegradability of each fuel.

### C2.4 Fuel Carbon Content

Carbon content of each fuel type was determined using combustion/gas chromatography (Costech ECS4010 elemental analyzer).

## C3.0 RESULTS AND DISCUSSION

### C3.1 Initial Carbon Content of Fuel Blends

Initial carbon contents for the fuels tested are shown in Table C2. Because each microcosm receives 5 mL of fuel substrate, the initial carbon is calculated as the mass fraction of carbon in the fuel times the volumetric mass density times 5 mL. The volumetric mass densities (data not shown) range from 0.86 to 1.02 g/mL, and the resulting initial carbon contents (last column of Table C2) range from 3.78 to 4.15 for the biofuel blends compared to 4.54 for the ULSD.

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Table C2: Carbon content of the 12 biodiesel blends and 1 petroleum diesel tested.

| Fuel type | | % Carbon by weight | g C/mL Fuel | Initial **C** content in microcosm (mg) |
|---|---|---|---|---|
| | AF B-100 | 84.7 | 0.81 | 4.066 |
| | AF B-100 A | 76.8 | 0.81 | 4.032 |
| AB100 | AF B-100 AA | 74.9 | 0.76 | 3.782 |
| | Soy B-100 | 78 | 0.79 | 3.939 |
| | Soy B-100 A | 77 | 0.81 | 4.043 |
| SB100 | Soy B-100 AA | 77.2 | 0.77 | 3.860 |
| | AF B-20 | 84.6 | 0.83 | 4.145 |
| | AF B-20 A | 84.2 | 0.78 | 3.915 |
| AB20 | AF B-20 AA | 85.9 | 0.79 | 3.951 |
| | Soy B-20 | 84.2 | 0.80 | 4.000 |
| | Soy B-20 A | 84.1 | 0.78 | 3.911 |
| SB20 | Soy B-20 AA | 71.6 | 0.67 | 3.365 |
| ULSD | | 88.1 | 0.91 | 4.537 |

## C3.2 Biodegradation Results: CO2 production over time for all fuels

Assuming accumulated $CO_2$ in each microcosm is a result of utilizing the fuel carbons by microorganisms aerobically, the total carbon consumption in each microcosm was calculated using the stoichiometry of Equation C1.

$C + O_2 \rightarrow CO_2$ Equation C1

Sterile (no biological activity) and blank (no fuel substrate) microcosms showed no $CO_2$ production. Lack of $CO_2$ production in these controls indicates that any $CO_2$ production in test microcosms is a result of microbial activities and not due to chemical reactions.

The percent degradation of each fuel type was calculated based on the initial carbon content and total carbon oxidation (Table C3). In Experiment number 4, the amount of utilized carbon was measured more than initial carbon content due to malfunction of respirometer during the experimental period.

Table C3 – Percent degradation of different fuel types

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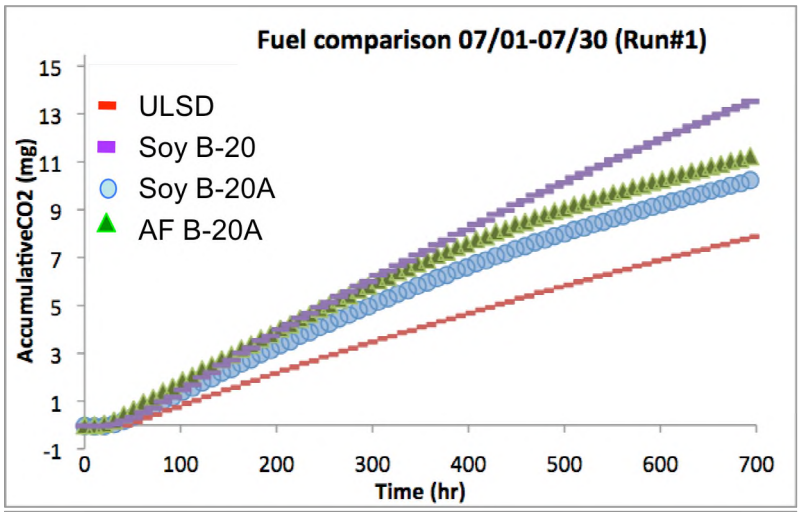
| Experiment | Fuel Type | Accumulated CO2 (mg) | Equivalent oxidized carbon (mg) | Percent degradation |
|---|---|---|---|---|
| #1 | ULSD | 7.87 | 2.15 | 47.40 |
| | Soy B-20 A | 10.23 | 2.80 | 71.48 |
| | AF B-20 A | 11.24 | 3.07 | 78.43 |
| | Soy B-20 | 13.53 | 3.70 | 92.40 |
| #2 | ULSD | 6.37 | 1.74 | 38.36 |
| | Soy B-100 | 9.04 | 2.47 | 62.70 |
| | AF B-20 | 8.83 | 2.41 | 58.18 |
| | AF B-100 | 11.31 | 3.09 | 75.99 |
| #3 | ULSD | 7.43 | 2.03 | 44.74 |
| | Soy B-20 AA | 10.30 | 2.81 | 83.65 |
| | AF B-20 AA | 9.55 | 2.61 | 66.02 |
| | Soy B-100 AA | 9.30 | 2.54 | 65.80 |
| #4 | ULSD | 10.78 | 2.95 | 64.92 |
| | AF B-100 AA | 18.86 | 5.15 | 136.26 |
| | AF B-100 A | 21.89 | 5.98 | 148.32 |
| | Soy B-100 A | 18.56 | 5.07 | 125.42 |

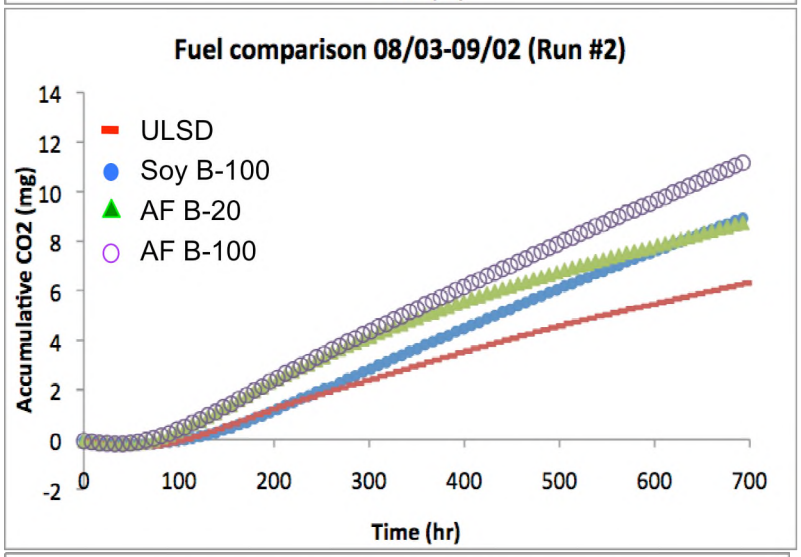
Figure C2 shows the time-dependent accumulation of CO2 in experimental suites 1, 2, and 3, for each fuel tested. These data show a small lag time (20-60 hours) followed by linear to mildly-decreasing accumulation rates with all biodiesel blends exhibiting faster degradation in all cases

than ULSD. Animal fat blends generally show a more rapid production of CO2 at early time, that is followed by a slowing of production so that Soy blend CO2 production in some cases reaches the same cumulative CO2 production.

The mild slowing of the Animal Fat blends may be due to product or other inhibition process. Another potential explanation is that the degrable fraction component in Animal Fat biodiesel is different from that in Soy blends, and more limited. Interestingly the 20% biodiesel blends appear to induce greater CO2 production than the 100% biodiesel fules. Unfortunately the identity of the degraded fraction component is unknown. Further study would involve chemical analyses of the samples selected from various points in time during the biodegradation, to identify degraded and undegraded fractions.

Figure C4 shows a comparison of percent of carbon biodegradation with the different fuel types in microcosm respirometry at the end of the experiments. These results reflect the mixed degrability of Animal Fat



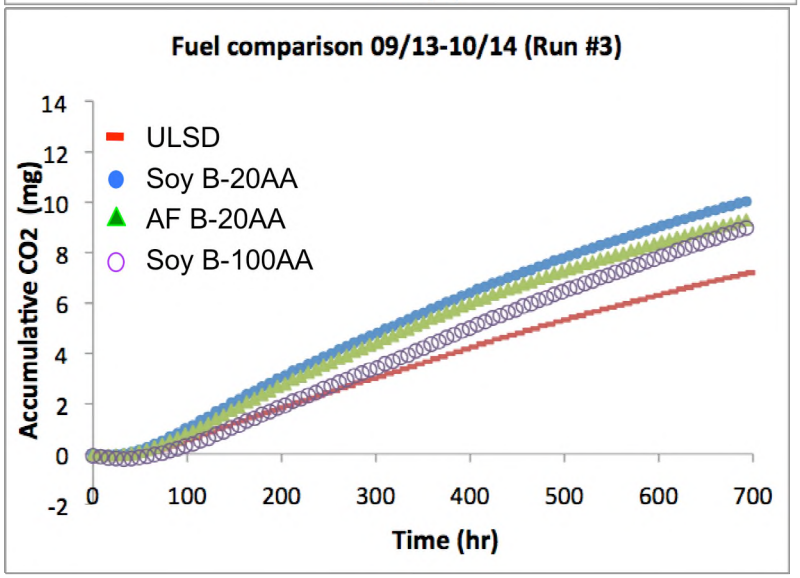




Figure C3. Respirometry data on CO2 production in experimental suites #1 (top), #2 (middle) and #3 (bottom).

vs. Soy biodiesel blends observed at the end of the ~29-day experiments shown in Figure C3.

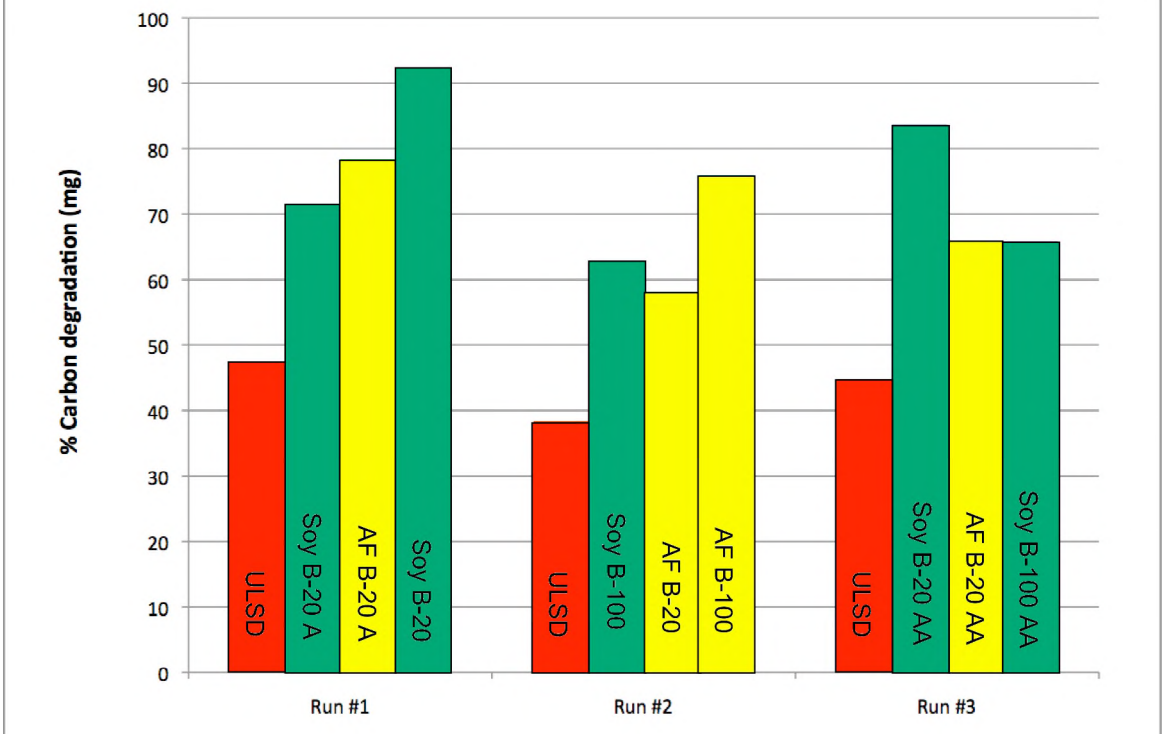



Figure C4: Comparison of percent of carbon biodegradation with different fuel types in microcosm respirometry at the end of the experiments.

The primary implications of these results are that the biodiesel blends of all types and all additive cases are significantly more biodegrable than CARB ULSD#2. Mild variations in rate are seen in the transient data, most clearly the decline in CO2 production rate for Animal Fat blends. Sample chemical analyses would be required to identify organic fractions associated with the degrable and non-degrable fractions. Further study could include different soil inocula, different temperatures, and different moisture contents to represent soil conditions. In our tests only respiration was measured and more information may be obtained by identifying microbial growth in terms of cell number or protein.

## C4.0 CONCLUSIONS

- All biodiesel blends are more readily degrable than the reference ULSD#2
- Additives do not exhibit any clear impact on biodiesel biodegrability
- The 20% biodiesel blends appear to be somewhat more degrable than 100% blends.

## C5.0 REFERENCES

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