California Biodiesel Multimedia Evaluation

Tier II Report on Aquatic Toxicity, Biodegradation, and Subsurface Transport Experiments

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Executive Summary

This document reports on the results of experimental activities performed to address and rank 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, 2009) 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 Tier II plan were not pursued as a result of time and funding limitations.

Additionally, a Tier II Biodiesel Air Emissions Characterization and NO_x Mitigation Study was coordinated by California Air Resources Board (CARB) in conjunction with researchers from the University of California Riverside (UCR), the University of California Davis (UCD), and others including Arizona State University (ASU). The results of this study are reported in Durbin, et al., 2011.

The summary and results of each of the toxicity, transport in porous media, and aerobic biodegradation experimental suites are as follows.

Aquatic Toxicity Tests

A series of aquatic toxicity tests were conducted on the seven fuel types including ultra-low sulfur diesel (ULSD), neat 100% biofuels derived from animal fat (AF B-100) and soy (Soy B-100) feed stocks as well as 80% ULSD:20% (w/w) mixtures of the two biofuels (AF B-20 and Soy B-20) and 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 no-observable-effect-concentration (NOEC), lowest-observable-effect-concentration (LOEC), Effects Concentration (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; $100/EC_{25}$, For example if 25% of the population shows effects at 50WAF, then the TU is 100/50=2. On the other hand if 25% of the population shows effects at 1WAF, then the TU is 100/1=100. This way, TU is an increasing measure of toxicity). 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 B-100 fuels or their B-20 mixtures without antioxidant additive produced detectable effects on mysid, topsmelt or fathead minnow endpoints. However, both B-100 biofuels and their B-20 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.

The Lawrence Berkeley National Laboratory (LBNL) Environmental Energy Technologies Division provided chemical analyses of the biodiesel/diesel components present in the WAFs prepared in a similar manner to those used during toxicity testing. Sample chemical analyses were not taken during toxicity testing.

LBNL developed and applied a stir bar sorptive extraction (SBSE) method followed by thermal desorption gas chromatography/mass spectrometry (TD-GCMS) analysis to identify and quantify the chemical composition of the aqueous-phase solutions for four different biofuels and ULSD under four different WAF preparations. Insufficient ULSD sample volume led to an analysis of the four biofuels under four WAF preparations, for a total of 16 analyses.

The fuels analyzed included all the biodiesel mixtures used during toxicity testing (AF B-100, Soy B-100, AF B-20, Soy B-20). Since unadditized ULSD was not available, all the resulting fuel mixtures were additized. In addition, the same four salinity and temperature conditions used during the toxicity testing were used during the preparation of the WAFs eventually analyzed.

The chemical analyses did not unambiguously reveal any causative compound for the toxicity, and further testing is required to confirm the identity of compounds or combination of compounds responsible for the toxic response.

Infiltration Experiments

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, AF B-100 with antioxidant was also tested and it showed similar behavior to unadditized AF 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-100, AF B-20, Soy B-100, and Soy B-20) 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 B-100, Soy B-20, as well as AF B-20, 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 AF B-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 Experiments

Microcosm experiments were conducted to assess the aerobic aqueous biodegradation potential for solutions in contact with biodiesel fuels, relative to ULSD. Fuels mixtures used were AF B-

100, AF B-20, Soy B-100, Soy B-20, and ULSD. These mixtures 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.

Biodiesel Tier II Summary

Experimental investigations address the knowledge gaps as follows:

- Tested biodiesel blends exhibit somewhat increased toxicity to subsets of tested species compared to ULSD, and additized blends increase this toxicity for a smaller subset of tested species. Future testing addressing the potential toxicity of additives including chemical analysis of exposure medium may be needed.
- Biodiesel fuel blends show similar infiltration and lens formation to ULSD in unsaturated sandy porous media, with AF 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.

Remaining Tier II Uncertainties

- Additional testing addressing the potential toxicity of additives including chemical analysis of exposure medium is needed.
- Of the three groups of additives only blends with antioxidants, and biocidal additives (biodegradation experiments only) were studied. Cold flow additives were not studied in any of the performed experiments. The impact of cold flow additives on aquatic toxicity and biodegradation needs to be studied.
- Infiltration experiments with biocidal and cold flow additives were not performed. Additional test may be needed as those additives may have different impact on the biodiesel infiltration.

1. Background

This document summarizes the results of experiments performed at Davis and Lawrence Berkeley National Laboratory (LBNL) 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 (2009), the Multimedia Working Group (MMWG) Tier I report (referred to henceforth at the "Tier I report")[1], and the plan for these experiments is found in the "Experimental Plan for Tier II Evaluation of Biodiesel," (Ginn et al., 2010)[2] 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 D6751-12 (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.

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

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

[1] http://www.arb.ca.gov/fuels/diesel/altdiesel/090910biodiesel-tier1-final.pdf
[2] www.arb.ca.gov/fuels/multimedia/031209TierIIrev.pdf

Tier II plan, and in a simplified and riskwise conservative fashion. The Tier I 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:

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, esturine, and freshwater toxicity.

Additionally, a Tier II Biodiesel Air Emissions Characterization and NO_x Mitigation Study was coordinated by California Air Resources Board (CARB) in conjunction with researchers from the University of California Riverside (UCR), the University of California Davis (UCD), and others including Arizona State University (ASU). The results of this study are reported in Durbin, et al., 2011.

2. Tier II Experimental Descriptions

Blend selection is restricted to two feedstocks and two blend ratios, B-20 and B-100, as these represent the 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. Chemical analyses of separately prepared water accomodated fractions was performed in an attempt to identify the chemical compounds associated toxic responses.
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 Tier II Plan.

Table 1. Tier II Testing Matrix:

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

[a] Experimental codes are T = Toxicity, A = Analyses, I = Infiltration, B = Biodegradation.

The experimental details for each of the three experimental suites, Aquatic Toxicity with Chemical Analyses, Infiltration, and Biodegradation, are presented in the Appendices A and B, C, and D, 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 Tier II plan of the California multimedia risk assessment for biodiesel.

3. Tier II Results and Conclusions

3.1. Aquatic Toxicity Experiments

Aquatic toxicity testing involved ULSD compared to Soy and Animal Fat (AF) B-20 and B-100 unadditized fuels, and Soy and AF B-20 with an 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 II-A. Toxicity metric in each case includes both the 25 and 50% Effects Concentrations (EC_{25}, EC_{50}) as reported in Appendix II-A. For instance, EC_{25} is the relative concentration in percent of substrate (relative to equilibrium solubility concentration of a given fuel in aqueous phase) at which 25 percent of the test species population exhibits an effect. Also reported are Toxicity Units, "TU," defined as the quantity $100/EC_{25}$. Thus, if one-quarter of a population shows an effect only at the 100% concentration (that corresponding to equilibrium solubility) then the TU value = 100/100 = 1. If however one-quarter 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 II-A. The results are as follows.

- 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.
- Tests that were repeated for confirmation produced similar results as the original test.
- Except for algae, the additized biodiesel B-20 test materials were substantially more toxic than the corresponding unadditized material. Figure 1 illustrates the frequency and magnitude of the toxic response to the additized AF B20a and Soy B20a exposures, as Toxicity Unit (TU) response for all species and all endpoints except for that of Green Algae that showed a different trend (reduced toxicity with additive). Note that the vertical axis is on a logarithmic scale for TU. Maximum toxicity was achieved for all species (except for that of Green Algae) in their exposure to AF and/or Soy B20a (with additive). This toxicity was pronounced (greater than or equal to 50 TU) for *C. dubia* and Abalone.

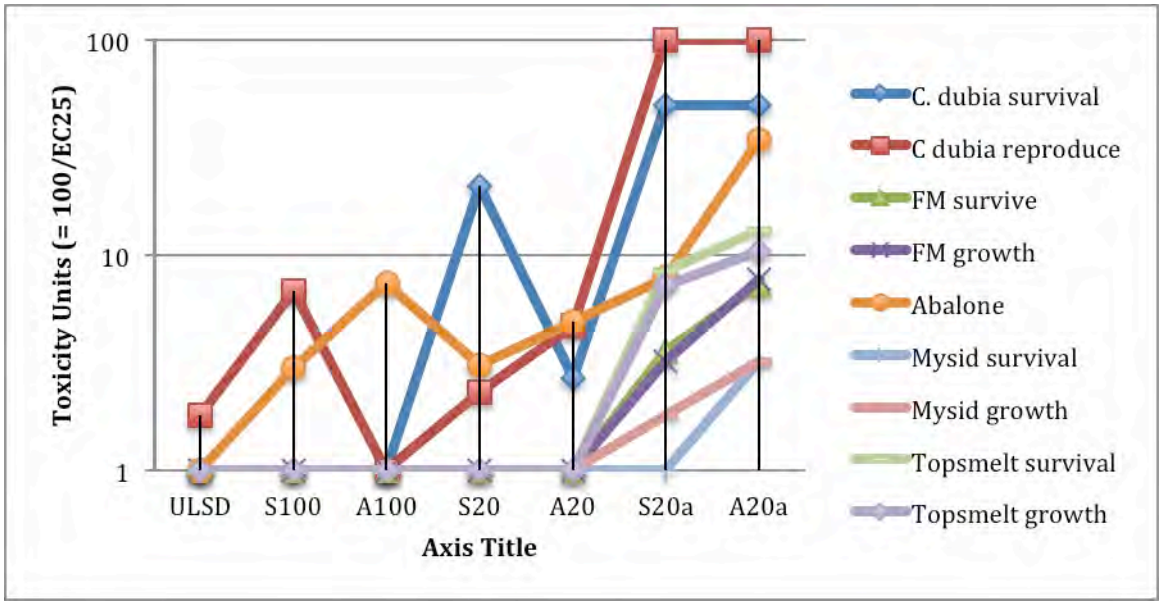

Figure 1. Toxicity scores (as Toxicity Units, = 100/ EC_{25}), for the different endpoints (e.g., survival, reproduction, growth) of 5 of the 6 species tested, as a function of fuel blend to which species was exposed. The graph is absent Green Algae that showed different behavior than the trend observed here.

3.2. Chemical Analyses of Selected Water Accomodated Fractions

The LBNL Environmental Energy Technologies Division provided chemical analyses of the biodiesel/diesel components present in the WAFs prepared in a similar manner to those used during toxicity testing. Samples for chemical analysis were not taken during toxicity testing. LBNL developed and applied a stir bar sorptive extraction (SBSE) method followed by thermal desorption gas chromatography/mass spectrometry (TD-GCMS) analysis to identify and quantify the chemical composition of the aqueous-phase solutions for four different biofuels and ULSD under four different WAF preparations. Insufficient ULSD sample volume led to an analysis of the four biofuels under four WAF preparations, for a total of 16 analyses.

The fuels analyzed included all the biodiesel mixtures used during toxicity testing (AF B-100, Soy B-100, AF B-20, Soy B-20). Since unadditized ULSD was not available, all the resulting fuel mixtures were additized. As noted above the most toxic cases for *all* species with the exception of Green Algae corresponded to exposure to 20% blends with additive. Therefore we analyzed the four WAFs after exposure to AF B20a and Soy B20a. To also evaluate occurrence of additive in the 100% biofuel cases we analyzed the four WAFs after exposure to AF B100a and Soy B100a as well.

In addition, the same four salinity and temperature conditions used during the toxicity testing were used during the preparation of the WAFs eventually analyzed. Conditions used (mixing temperature and salinity) of these solutions are given in Table II-B-1 of Appendix II-B.

The measured chemical concentrations for each of the fuel WAFs are listed in Tables B8 – B11 of Appendix II-B for Soy-B100a, Soy-B20a, AF-B100a and AF-B20a, respectively. The antioxidant fuel additives acetic acid, butyl ester and 1,4-Benzenediol, 2-(1,1-dimethylethyl), also known as TBHQ, were identified in the majority of the samples. However, the

concentrations were highly variable. We presume that the addition of the additive to the original fuel was consistent so the variability was likely due to either the WAF mixing conditions or the extraction conditions. The additive butyl acetate was lowest in the WAF-04 sample, which had the highest salinity so the solubility may be affected by pH. Without further testing one cannot rule out the extraction as a source of the variability for either of the measured additives.

Despite the variability, the concentrations of acetic acid butyl ester additive do in fact increase in all four WAFs from Soy B20a exposures to AF B20a exposures, and this is consistent with the increase in toxicity for the majority of species/endpoints between Soy B20a and AF B20a exposures (see Figure 1, right-hand side). However the measured concentrations of this additive are generally below 50 ug/l, whereas the concentrations associated with toxicities (EC50) reported for various species in the Materials Safety Data Sheet for this compound are in the 10's-100's of mg/l range. TBHQ did not appear increasing from Soy B20a to AF b20a exposed WAFs and concentrations overall were rather low.

The only other compounds exhibiting increased concentrations associated with Soy B20a to AF B20a WAFs include some petroleum diesel compounds and some FAMEs, both at low or suspect concentrations. Both of the animal fat biofuel WAF-01 (low salinity) mixtures (AF B-100, AF B-20) had significantly higher concentrations of FAMEs and the Soy-B100 WAF-01 also had somewhat elevated FAME. Sample contamination was suspected in the form of oil droplets present in the AF-B100 WAF-01 (greyed out values in Table 10) but this was not noticed in the other WAF-01 samples. Comparing the average results for the duplicate AF-B20 WAF-01measurements to the previous measurement used in the range finding pre-experiment calibration found that the later measurements seem to have been contaminated with FAME. Both the initial measurement from the range finding and the average of the replicate measurements are reported in Table 11 but the results with high FAME are likely due to contamination. The low level of FAME in the Soy-B20 WAF_01 rules out contamination in the source water used to mix the WAF. Further testing would be needed to determine if the mixing conditions used for the WAF_01 samples resulted in elevated FAME in the Soy-B100 relative to the Soy-B20 or if the difference was due to contamination during mixing.

Only one alkane (2,2,3,3-tetramethyl-Butane) was measured in the WAFs and it was also detected at elevated levels in the blanks, including the HPLC water and in the direct analysis. The fact that the alkane was in the diluted fuel which was not extracted with stir-bar indicates that the methanol used in the dilution may have been the source.

In summary, the chemical analyses failed to identify unequivocally a source of the toxicity observed. Hypotheses that may explain the observations include a co-solvency effect associated with a compound in the Soy B20a and AF B20a exposed WAFs that facilitates higher aqueous concentration of a petroleum diesel compound, enhanced (cross-) toxicity associated with the acetic acid, butyl ester additive in combination with another (or more) FAME or petroleum diesel component. Further toxicity experiments that include chemical analysis of exposure media may be useful.

3.3. Infiltration Experiments

Small-scale laboratory infiltration experiments in two-dimensional sandboxes with glass walls to allow visualization of dyed fuels were completed 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 II-C. 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 a 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 II-C.

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

- The antioxidant additive did not affect the infiltration of AF B-100
- Soy biodiesel blends at both 20 and 100 percent, as well as the AF 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.

3.4. Biodegradation Experiments

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 Tier II 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 an

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, 2g soil (Yolo, silty-loam) as bacterial inoculum and addition of 5μL 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

3.5. Biodiesel Tier II Summary

Experimental investigations address the knowledge gaps as follows:

- Tested biodiesel blends exhibit somewhat increased toxicity to subsets of tested species compared to 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 AF 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.

3.6. Remaining Tier II Uncertainties

- Additional testing addressing the potential toxicity of additives including chemical analysis of exposure medium is needed.
- Of the three groups of additives only blends with antioxidants, and biocidal additives (biodegradation experiments only) were studied. Cold flow additives were not studied in any of the performed experiments. The impact of cold flow additives on aquatic toxicity and biodegradation needs to be studied.
- Infiltration experiments with biocidal and cold flow additives were not performed. Additional test may be needed as those additives may have different impact on the biodiesel infiltration.

4. Tier II References

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5. Tier II Appendices

6. Appendix II-A: Toxicity of Biodiesel Blends And ULSD to Selected Freshwater and Marine/Estuarine Organisms

Background

Biodiesel is a fuel composed of monoakyl 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.

Methods and Materials

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 II-A-1.

Table II-A-1. 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

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 II-A-2.

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 II-A-2. 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

Green Algae Chronic Test Procedures

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:

cell number = (absorbance units @ 750 nm x 13.026) - 0.0328 (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%.

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.

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.

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.

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.

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.

Water Quality Measurements

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).

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.

Results and Discussion

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

Algae Chronic Toxicity Test Results

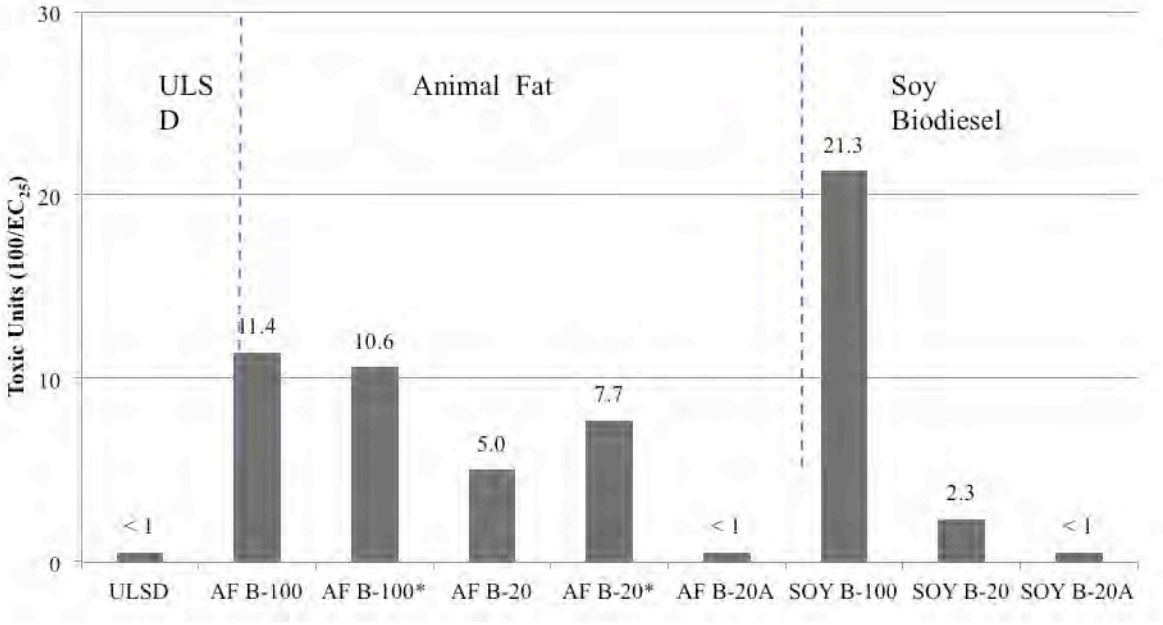
Table II-A-3 and Figure II-A-1 summarize results of the biofuel toxicity tests with green algae.

Table II-A-3. Summary of Biodiesel Toxicity Tests with Green Algae (*S. capricornutum*)

Fuel Type	*Values are % WAF*				*Toxic Units*	*PMSD*
	NOEC[a] (%)	*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[c]	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] No-observable-effect-concentration
[b] Lowest-observable-effect-concentration
[c] Repeat test

Figure II-A-1.Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Green



* 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.

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

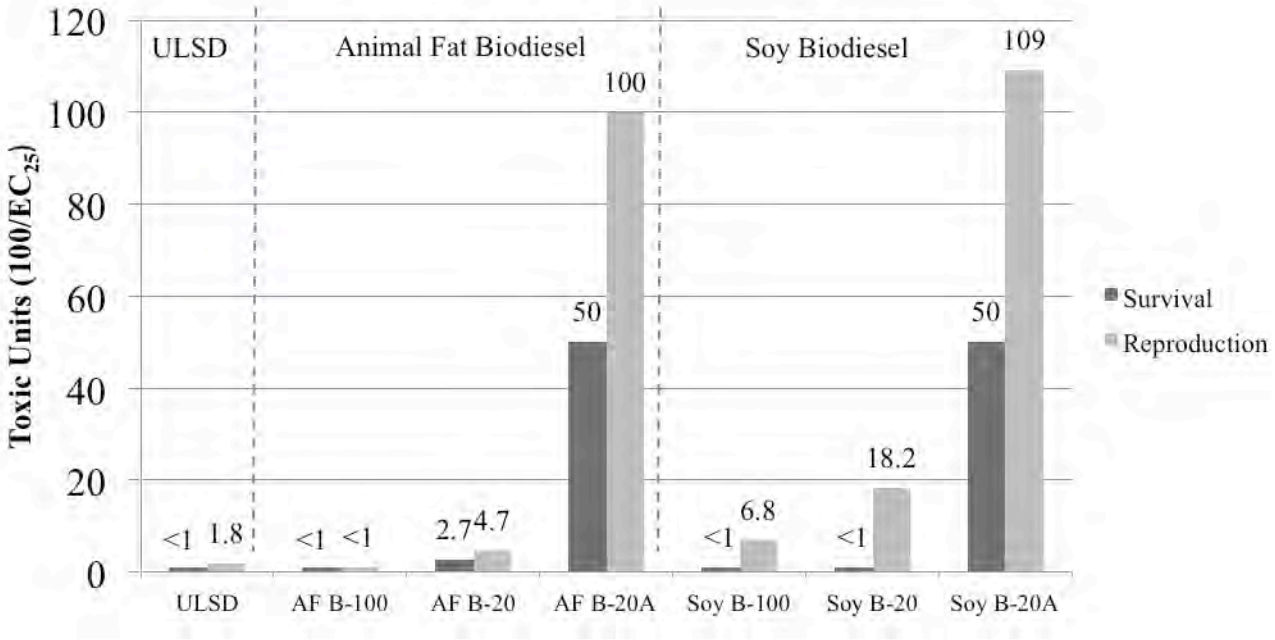
Table II-A-4 and Figure II-A-2 summarize results of the biofuel toxicity tests with *C. dubia*. The raw data for this test series is found in Section A2.

Table II-A-4. 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

Figure II-A-2.Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to *C. dubia* Survival and Reproduction.



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.

Fathead Minnow Chronic Toxicity Test Results

Table II-A-5 and Figure II-A-3 summarize results of the biofuel toxicity tests with fathead minnow. The raw data for this test series is found in Section 3.

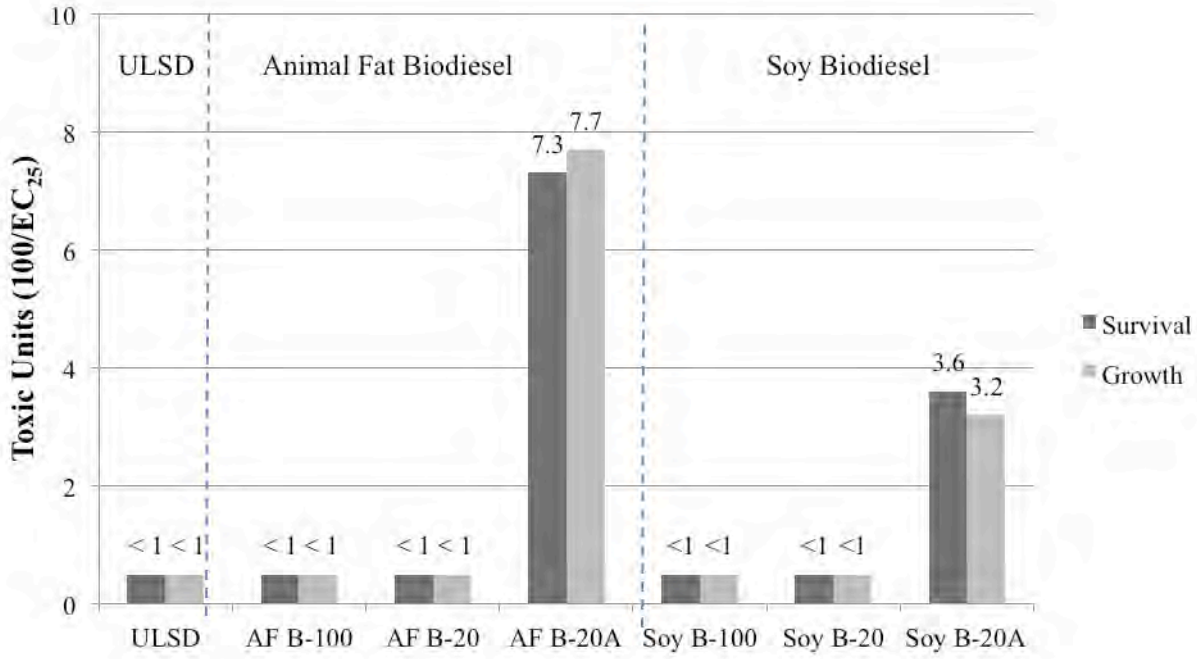
The fathead minnow survival and growth endpoints were unaffected by ULSD, AF B-100, AF B-20, Soy B-100 and Soy B-20. However, both biodiesel B-20A mixtures resulted in toxicity to both endpoints. AF B-20A exhibited moderately greater toxicity (7.3 TUc and 7.7 TUc) than did the Soy B-20A (3.6 TUc and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

Table II-A-5. 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

Figure II-A-3.Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Fathead Minnow Survival and Growth



Abalone Chronic Toxicity Test Results

Table II-A-6 and Figure II-A-4 summarize results of the biofuel toxicity tests with abalone. The raw data for this test series is found in Section A4.

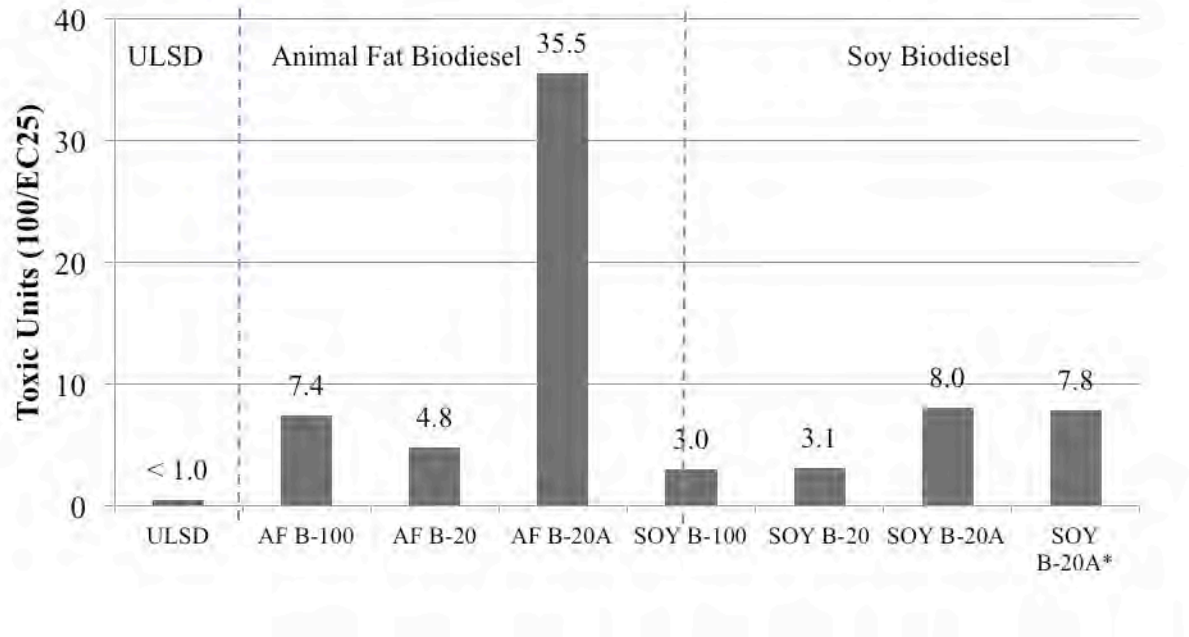
No effects on abalone shell development were detected with ULSD. AF B-100 exhibited somewhat higher toxicity than the Soy B-100 (7.4 TUc and 3.0 TUc, respectively), while the AF B-20 and Soy B-20 mixtures had similar or slightly less toxicity as their respective B-100 fuels (4.8 and 3.1 TUc, respectively), 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 II-A-6. 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

Figure II-A-4.Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Abalone Shell Development



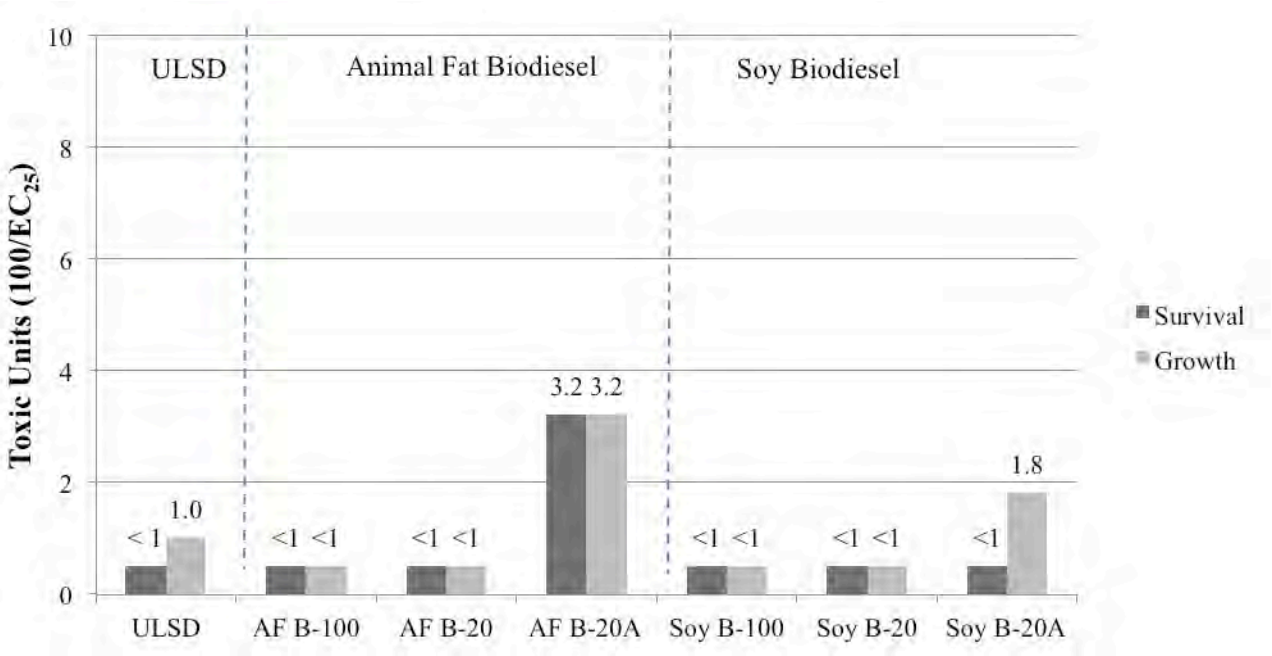
Mysid Chronic Toxicity Test Results

Table II-A-7 and Figure II-A-5 summarize results of the biofuel toxicity tests with mysid. The raw data for this test series is found in Section A5.

Table II-A-7. 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

Figure II-A-5. 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.

Topsmelt Chronic Toxicity Test Results

Table II-A-8 and Figure II-A-6 summarize results of the biofuel toxicity tests with topsmelt. The raw data for this test series is found in Section A6.

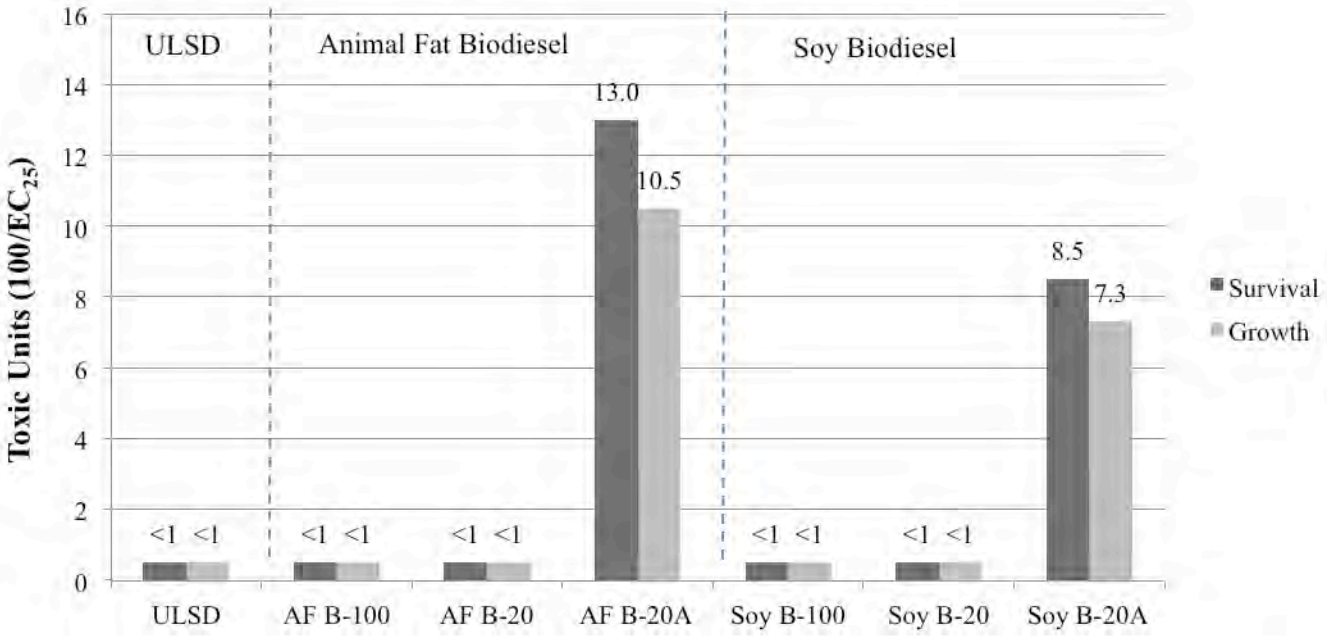
No effects on either survival or growth were detected with ULSD or either of the biofuels and mixtures that did not contain the additive. The AF B-20A test detected 13.0 TUc on survival and 10.5 TUc on growth, while the Soy B-20A test detected slightly less toxicity with 8.5 TUc on survival and 7.3 TUc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

Table II-A-8. 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

Figure II-A-6. Chronic Toxicity of Ultra-Low Sulfur Diesel (ULSD) and Biodiesel to Topsmelt Survival and Growth



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. Appendix II-B provides such information for the WAF made with additized biodiesel blends AF-B100, AF-B20, Soy-B100, and Soy-B20. The results are only partly conclusive, as more work is needed to refine the WAF preparation and techinques. See main body of report and Appendic B for summary conclusions.

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7. Appendix II-B: Chemical Analysis of the Water Accommodated Fractions of Biofuels Using Stir Bar Sorptive Extraction

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ABSTRACT

Biofuels are diesel-equivalent fuels derived from the transesterification of the triglycerides that come from animal- or plant-based biological sources. The resulting fatty acid methyl esters (FAME) can be used in their pure form or mixed with additives and different proportions of diesel to prepare fuel formulations. Biofuels have a number of potential advantages over petroleum-based fuels. For example, biofuels come from renewable sources, may produce lower net greenhouse gas emissions, and have been shown to readily degrade in the environment. However, information about the activity of biodiesel when released into the environment is limited, in particular, its fate in aquatic systems and its effects on aquatic organisms. Biofuel formulations are complex mixtures containing a large number of aliphatic and aromatic hydrocarbons and fatty acid methyl esters. When biofuel comes into contact with water, the solubility and partition coefficients of the individual chemical constituents in the fuels and the salinity and temperature of the water dictate the ultimate composition of the biofuel chemicals in the aqueous phase. It is the aqueous phase composition that is most relevant to aquatic toxicity tests and chemical fate studies.

In this project, we prepare aqueous phase solutions of biofuel formulations for conditions (temperature and salinity) representing four different ecosystems. The aqueous solutions, referred to as water accommodating fractions (WAF), were prepared to represent different ecosystems for standard toxicity test protocols, varying both the salinity of the water and the mixing temperature. We develop and apply a stir bar sorptive extraction (SBSE) method followed by thermal desorption gas chromatography/mass spectrometry (TD-GCMS) analysis to identify and quantify the composition of the aqueous-phase solutions for four different biofuels. The fuels include animal- and plant-based biofuels in pure 100% biodiesel (B100) and 80% diesel/20% biodiesel (B20) formulations.

Although the composition of the fuels are dominated by aliphatic hydrocarbons and/or fatty acid methyl esters, the composition of the WAF was typically dominated by branched aromatics including alky-benzenes, alkyl-indenes/indanes and alkyl-naphthalenes. WAF composition and concentrations are reported for the different fuels and mixing scenarios and the effects of salinity and temperature are discussed.

INTRODUCTION

The world's current dependence on fossil fuels presents inherent dangers and concerns. Given that the sources of petroleum currently being exploited today are naturally finite, research into alternative sources of fuel is increasing rapidly. Biodiesel has emerged as a a potentially important new fuel in an ongoing effort to transition from the use of petroleum-based fuels to renewable fuels. Biodiesels are diesel-equivalent fuels made from methanol transesterification of triglycerides derived from biological sources (Demirbas, 2009). Common biological sources include plant-based oils like soybean, sunflower, rapeseed, canola, and cotton, as well as animal fats and lard (Singh & Singh, 2010). Aside from the fact that it can be made from renewable sources, biodiesel also boasts a number of environmentally friendly attributes not shared with petroleum diesel, such as biodegradability (DeMello et al., 2007; Prince et al, 2008), as well as being carbon neutral and helping to decrease net greenhouse gas emissions (Coronado et al., 2009). In addition, some researchers have studied the potential of biodiesel as a bioremediation agent in helping to clean up oil spills (Fernandez-Alvarez et al, 2007). However, information on the aquatic environmental fate and toxicity of biodiesel is limited.

Leme et al. (2011) found that both diesel and biodiesel blends have cytotoxic effects on human cells, something they attributed to the presence of polycyclic aromatic hydrocarbons (PAHs). Researchers at the University of California, Davis are studying the environmental fate, biodegradability and aquatic toxicity of biofuel blends in support of the California multimedia risk assessment of biodiesel blends (Ginn et al., 2009; UC, 2009). Common to all of these studies is the need for knowledge of the composition and concentration of biofuel constituents in the aqueous phase solutions being tested.

Analysts have used gas chromatography/mass spectrometry (GC/MS) to identify chemicals present in various sample matrices. However, when dealing with organic compounds present in trace amounts, an extraction and enrichment step needs to occur before chromatographic separation. In recent studies, scientists have relied on the use of solvent extractions followed by a pre-concentration step to prepare samples for GC/MS analysis (Deasi, et al., 2010; Hansen, et al., 2011; Rodrigues, et al., 2010). However, traditional solvent extractions contain several drawbacks, such as being labor intensive, expensive, as well as producing high amounts of organic chemical waste (Sabik, Jeannot, & Rondeau, 2000). Solid-phase extraction (SPE) is an alternative that uses less organic solvents and has been used to successfully analyse WAFs (Lewis, Pook, & Galloway, 2008; Gonzalez-Doncel, Gonzalez, Fernandez-Torija, Navas, & Tarazona, 2008), however both solvent extraction and SPE are best suited for semi-volatile compounds due to the differences in boiling points that the analytes and the solvents must possess and the need for solvent evaporation prior to analysis (Roy, Vuillemin, & Guyomarch, 2005).

An alternative solvent free method for extracting organic compounds from aqueous solutions is stir-bar sorptive extraction (SBSE) followed by thermal desorption and GCMS analysis (Baltussen, Sandra, David, & Cramers, 1999). SBSE exploits a compound's hydrophilic and hydrophobic interactions with a polydimethylsiloxane (PDMS) coating on a glass covered stir-bar that is thermally desorbed and cryofocused directly into the GC inlet providing a simple and highly sensitive method for sampling organic chemicals in water. In an earlier phase of this project, we optimized conditions for analyzing water-accommodated fractions of biofuel using SBSE (McCreary Jr., 2010). We expand on that work here and apply the method to 16 different

fuel/WAF mixtures representing the range of biofuels and aquatic ecosystems. The goal of this study is to identify and quantify biodiesel constituents in WAF mixtures prepared with soy- and animal fat biofuels in B100 and B20 formulations. The WAF mixtures were prepared with temperatures and salinity representing fresh-, estuarine- and sea-water that are relevant to standard aquatic toxicity studies (see Appendix II-A) performed as part of the Tier II multimedia risk assessment for biofuels (Ginn et al., 2010).

MATERIALS AND METHODS

Materials

The biofuel used in this study was collected by University of California Davis researchers directly from storage barrels at the California Air Resources Board storage facility in Sacramento, CA (Stockton facility). The fuels include 100% animal fat biofuel (AF-B100), 100% soy biofuel (Soy-B100) and blends prepared with 20% biofuel to 80% ultra-low sulfur diesel (w/w) resulting in an AF-B20 and Soy-B20. All fuels were labeled indicating that the fuels included additives. The headspace in the storage barrels had been purged with nitrogen. The fuel was transferred directly from the storage barrels to 1-gallon amber glass jars filled to the top to minimize headspace in the jars and delivered to LBNL for testing. The jars were stored at room temperature and fuel was used within 1 week of receiving.

The water mixtures that were used to prepare WAF were prepared by Aquasci, Inc. (Davis, CA) and were used during toxicity testing. The samples to be analyzed were collected by UCD researchers during the toxicity testing for delivery to LBNL. The different salinity test waters used during the toxicity tests were prepared as described in Ginn et.al (2011). The fresh water was reverse osmosis and granular carbon filtered well water with dry salts added to achieve USEPA moderately hard (EPAMH) specifications. The EPAMH water was further amended with either dry salts (25 ppt) or hyper-saline brine (33 ppt) to prepare estuarine and marine waters, respectively. The waters were stored in 1-gallon polyethylene jugs and delivered to LBNL along with the test fuels.

Chromatography, Pesticide Residue Analysis, and Spectrophotometry-grade methanol (Burdick & Jackson, Muskegon, MI) was used in this study. An internal standard was prepared using deuterium labeled dimethyl phthalate in methanol (100 ng/μL, AccuStandard, New Haven, CT). Extractions were carried out with 10 mm glass covered magnetic stir bars coated with a 0.5 mm layer of polydimethylsiloxane (PDMS), commercially sold under the name Twister™ (Gerstel, Mulheim a/d Ruhr, Germany). Before initial use, the stir-bars were conditioned in dedicated 6 mm diameter glass thermal desorption tubes at 300°C for 2 hours in a tube conditioning oven (TC2, Gerstel, Mulheim a/d Ruhr, Germany) under a constant flow of Helium 100 mL/min). After conditioning and between uses, the stir-bars were stored in the thermal desorption tube sealed in poly propylene tubes with Teflon end caps.

Preparation of Water Accommodated Fraction (WAF)

The WAF was prepared according to a low-energy mixing procedure (Singer, et al., 2000; Schluep, Imboden, Galli, & Zeyer, 2001) that was developed to prevent oil/water emulsification or oil droplets from getting into the water phase. Mixing temperatures and salinities for the different WAF are outlined in **Error! Reference source not found.**II-B-1.

The WAFs were prepared in clean 250 ml beakers. A small piece of Teflon tubing was fitted with a luer attachment and connected to the wall of each beaker so that the bottom of the tube rested near the bottom of the beaker and the luer fitting extended above the edge of the beaker. The tube apparatus allowed for the removal of the aqueous phase by syringe, after the WAF was prepared, without disturbing the organic (fuel) layer on the surface. For mixing, the test water (200 mL) was added to each beaker along with a small magnetic stir bar (approximately 2 cm long). The fuel (20 mL) was then added to the surface of the water by pipetting gently down the side of the beaker to prevent mixing of the fuel and water. The mouth of the beaker was covered tightly with a piece of foil to limit volatilization of the fuel components during preparation of the WAF. The fuel/water solution was stirred at 120 rpm for 18 hours in a temperature controlled environment set to the appropriate temperature. After the 18 hour stirring period, the beakers were removed from the temperature controlled environment and allowed to sit at room temperature for 2 hours.

The WAF was removed from the beaker by syringe using the Teflon tubing. The first 10 mL of water was transferred to waste. This removed water in the tubing. The remainder of the aqueous layer was then transferred from the beaker, being careful not to disturb the fuel layer. The WAF samples were stored in detergent washed, 250 mL amber glass jars with Teflon-lined caps at room temperature until extraction. **Error! Reference source not found.** summarizes the mixing conditions for each fuel/water combination.

Stir Bar Sorptive Extraction (SBSE)

Range finding experiments were run as part of the method development. The range finding experiments included 1) direct injections of fuels in water followed by SBSE and 2) mixing samples with increasing amounts of WAF (0 mL, 1mL, 10 mL and 20 mL) diluted in a final volume of 40 mL water. The results found that the composition of the WAF was significantly different from the direct fuel spikes and that a 10 mL aliquot of WAF provided good detection of fuel constituents across all fuels without over-loading the analytical instrument.

WAF samples were prepared for extraction by first transferring 10 mL of each WAF from the glass jars to 40 mL glass screw-top vials. Methanol (4 mL) was added to the WAF to achieve a final concentration of 10% MeOH in the final extract volume (Leon, Alvarez, Cobollo, Munoz, & Valor, 2003; Prieto, et al., 2010). The internal standard was added to the vial and the contents were topped off with HPLC water to eliminate headspace resulting in a total extract volume of 40 mL. A preconditioned stir-bar was added to each sample and the vials were capped and stirred for four hours at 1500 rpm at room temperature. After extraction, the stir-bars were removed from the sample solutions using a Kimwipe covered magnet. The stir-bars were rinsed with HPLC water, dried on a clean Kimwipe, and returned to the thermal desorption tube for chemical analysis.

Analytical Instrumentation

Stir-bars were thermally desorbed using a thermodesorption auto-sampler (Model TDSA2; Gerstel), a thermodesorption oven (Model TDS3, Gerstel) and a cooled injection system (Model CIS4; Gerstel). The cooled injection system was fitted with a glass-bead-packed glass liner. Stir-bar desorption was run in splitless mode at a starting temperature of 25 ^{o}C with a 0.5 minute delay followed by a 60 ^{o}C/min ramp to 300 ^{o}C and a 2 minute hold time with the transfer line temperature at 290 ^{o}C and the desorption flow at 20 mL/min (solvent vent mode). The cryogenic trap was held at -100 ^{o}C throughout desorption and then heated within 0.2 minutes to 290 ^{o}C at a

rate of 12 ^{o}C/s, followed by a 2.3 minute hold time then a second temperature ramp to 300 ^{o}C at a rate of 1 ^{o}C/s and held for 2.9 minutes. The inlet was in solvent vent mode throughout desorption until 0.00 minutes (start of injection) then flow was changed to 6.0 mL/min from 0.0 to 3.0 minutes resulting in a 5:1 split injection. After injection (3.0 minutes), the vent flow was returned to 20 mL/min to purge the inlet during the secondary temperature ramp period. Compounds were resolved on a GC (Series 6890Plus; Agilent Technologies) equipped with a 30 meter long by 0.25 mm diameter HP-5 capillary column with 0.25 μm film thickness. The initial oven temperature was 10 ^{o}C held for 5.0 minutes then ramped to 200 ^{o}C at 5 ^{o}C/min then to 280 ^{o}C at 8 ^{o}C/min holding for 5 minutes. The helium flow through the column was constant at 1.2 mL/min (initial pressure 49.5 kPa, 39 cm/sec). The resolved analytes were detected using electron impact MS (5973; Agilent Technologies) operated in scan mode with mass range from 34.0 to 500 amu. The MS temperature settings were 260 ^{o}C, 230 ^{o}C and 150 ^{o}C for the transfer line, MS source and MS quad, respectively.

Identification and Quantification of WAF Constituents

The large numbers of compounds in diesel and biofuel samples make it impractical to identify and quantify all the compounds using retention times and calibration curves that are based on pure standards. In this section, we describe a semi-quantitative approach for the GCMS analysis to identify and quantify compounds using a mass spectral library search and a modified toluene equivalent mass calibration. Toluene equivalent mass has long been used in reporting total volatile organic compounds (TVOC) (Hodgson, 1995). To use toluene equivalent mass for individual compounds, the peaks in the total ion chromatogram (TIC) must be well resolved so that the area under the chromatographic response for the specific compound can be related to the mass of toluene using a toluene response factor. However, for complex chromatograms that have large numbers of unresolved or partially resolved peaks, identifying the area under the TIC that is related to a specific chemical is more difficult. For these chemicals, it is better to use a dominant and/or unique fragment ion chromatogram in the mass spectra, referred to here as the extracted ion chromatogram (EIC).

To identify target compounds for the analytical method we first analyzed a 1000:1 dilution of each fuel in MeOH directly injected (2 μL) into the instrument with the analysis conditions described above except that a Gerstel septumless sampling head with 5:1 split was used to introduce sample onto the column. Each of the four fuels was analyzed in this way to determine their composition. Next, the 1000:1 dilution for each fuel was spiked into 40 mL of EPAMH water amended with 10% MeOH and extracted by SBSE (as part of the range finding experiment). Both the AF-B100 and Soy-B100 had a small number of dominant fatty acid methyl esters (FAME) but the AF-B100 had a larger number of minor FAME. Both neat fuels had been mixed with the same stock diesel so we concluded that the AF-B20 sample provided the widest variety of target chemicals for developing the method. The AF-B20 WAF created in the EPAMH water was extracted using the SBSE to identify the chemical composition of the WAF and to determine the relationship between EIC for individual chemicals and the response factor for toluene.

We identified 127 chemicals in the AF-B20 WAF using a mass spectral library search with the NIST08 database. For each chemical, we recorded both the EIC and the TIC. The chemicals in the WAF SBSE were assigned to one of five categories including 1) alkyl-benzene, 2) alkyl-naphthalene, 3) FAME, 4) alkane and 5) other. For each chemical (x), where we were able to

determine both an EIC and TIC, we calculated the EIC_x/TIC_x ratio. For chemicals that were not well resolved and the TIC could not be determined, we assigned them the average ratio for the particular chemical category.

Specific chemicals were selected as surrogates for the different chemical categories and then a calibration was prepared by spiking the surrogate compounds into water for SBSE analysis. The surrogate compounds and their concentrations are listed in Table II-B-33. We assume that the TIC response factor (instrument response per unit mass of chemical) for the surrogate compounds is equal to the TIC response for all chemicals in the surrogate class. With this assumption, the average response factor for each surrogate category (EI_s) was normalized to the individual chemicals (EI_x) by

$$EI_s \times \frac{TI}{EI_s} \times \frac{EI_x}{TI} = EI_x$$

The EI_x values were then entered into the calibration table within the ChemStation® software for each concentration in the quantification method and the relative response factor determined by forcing the two point calibration curve through zero.

RESULTS AND DISCUSSION

Composition of the raw fuels

Chromatograms from the raw fuel analysis are shown in Figure II-B-1. The large peaks starting at about 2600 seconds are the FAME and the smaller peaks that show up earlier in the in the B20 chromatograms are from the diesel fuel. The major FAME peaks include the hexadecanoic acid methyl ester and isomers of octadecanoic acid methyl ester. Although the AF-B100 was also dominated by two major FAME peaks, there were a larger number of minor FAMEs (lower carbon number) in the animal fat biofuel than in the soy biofuel. This can be seen by the relative size of the major FAME peaks in the two B100 chromatograms in Figure II-B-1. The diesel fuel chromatogram is shown in Figure II-B-2. Diesel fuel consists of approximately 75% saturated hydrocarbons and 25% aromatic hydrocarbons (ATSDR 1995), which was consistent with our analysis.

The direct spike of the 1000:1 MeOH:biofuel dilution (v:v) into EPAMH water followed by SBSE extraction resulted in a similar chemical fingerprint with the FAME and saturated hydrocarbons dominating the chromatogram and the aromatic hydrocarbons making up a smaller fraction of the measured chemicals. A 4 μL spike was added to each of the three salinity waters defined in Table II-B-1 and analyzed by SBSE along with an HPLC water blank. The results are shown in the overlay in Figure II-B-3. The large evenly spaced peaks in the figure are siloxanes from the stir-bar coating and are not included in the quantification method. The saturated hydrocarbons were also excluded from the quantification method because saturated hydrocarbons are not present in WAF as discussed later but we did quantify the FAME and aromatic hydrocarbon fraction in the spiked samples to evaluate the precision of the SBSE method. The precision of the internal standard was 13% (coefficient of variation of the three spike samples) without a clear trend in response of internal standard with changes in water salinity. However, the sum of the aromatic hydrocarbon and FAME chemicals in the 40 mL water spiked with 4 μL of the 1000:1 dilution of AF-B20 did show a decreasing trend as a function of increasing salinity. The EPAMH water concentration after the spike was 0.3 ppm

(sum of aromatics and FAMEs) while the highest salinity water had a concentration of 0.2 ppm representing a drop of approximately 2% in concentration with each unit increase in salinity (r^2 = 0.99). It was not clear why the increasing salinity would reduce the capacity of the stir-bar but future work should consider bringing the pH to neutral in saline waters prior to extraction. Nevertheless, a 2% variation in the spike samples is a reasonable precision for the SBSE of biofuel in water.

Composition of the WAF

After evaluating the fuel composition using direct injections, and the SBSE efficiency using spiked water samples, a range finding experiment was performed using increasing fractions of the AF-B20 WAF in EPAMH water diluted with HPLC water (final volume 40 mL). The resulting chromatograms for the dilutions are shown in Figure II-B-4. A 10 mL dilution of WAF in 40 mL final aqueous phase volume was determined to be appropriate for the SBSE analysis. An important observation with the WAF, compared to the direct fuel analysis and the analysis of fuel spiked in water is that the chemical composition in the WAF was dominated by aromatic hydrocarbons (alkyl-benzene, alkyl-indene/indane and alkyl-naphthalene). The saturated hydrocarbons and the FAME in the direct fuel and the spiked fuel were either not present in the WAF or at very low concentrations. This is highlighted in Figure II-B-5 that zooms in on the region of the chromatogram where FAME elutes and overlays the chromatograms from the direct injection, the spike and the WAF for AF-B20.

The 50% dilution AF-B20 WAF chromatogram was used to identify the initial set of target compounds in the WAFs. The mass spectra from each peak were used to search in the NIST08 Mass Spectral Database using the ChemStation® Enhanced Data Analysis software. After constructing the initial target chemical list using the AF-B20 chromatogram, the spiked fuel extract was used to identify lower concentration FAME peaks. The other WAF samples were then carefully screened using the target compound list and any additional peaks not identified previously were added to the target compound list. The final list of compounds found in the soy and animal fat biofuel WAF are given in Table II-B-4. It is important to note that the library search cannot distinguish between chemical isomers so we included chromatographic retention time in Table II-B-4 to facilitate future identification using pure standards. Also listed in Table II-B-44 are the ratios for the mass spectral fragment ion or extracted ion for the individual chemical (EI) and the total ion for the chemical (TI) which was used in the quantification method to normalize the response of the individual chemicals to that of the surrogate compounds (Table II-B-3) used in the calibration.

Precision of SBSE measurements

Sixteen WAF mixtures plus three water blanks from the test waters were each analyzed one time by SBSE. The AF-B100 and AF-B20 WAF were analyzed a second time to characterize the repeatability of the analysis. The precision of the internal standard was assessed across all analyses and the results are shown for the different WAF mixing conditions and the different fuels in Table II-B-5. The overall precision of the internal standard (n = 21) was 30%. We did not find the same trend in the internal standard response in the WAF samples that we found in the spiked samples. In this case, the EPAMH water (WAF_01) tended to have the lower internal standard response. The WAF_01 samples also had a higher coefficient of variation across all measurements and the AF-B100 WAF_01 had particularly poor precision (CV = 43%). On inspection, we found that the AF-B100 WAF_01 sample had oil droplets in the WAF indicating

contamination with raw fuel. The raw fuel contamination results in excessively high instrument response for a large number of chemicals that can reduce the detector response for the internal standard, particularly when large amounts of co-eluting compounds are present. The coefficient of variation for the AF-B100 samples drops from 43% to 20% when we exclude the contaminated AF-B100 WAF_01 samples.

The duplicate SBSE analysis for the AF-B100 and AF-B20 WAFs were used to assess precision of the measurements. The results for each chemical (ng) from the duplicate samples were first used to estimate the relative precision of the measurements. If relative precision (difference between measurements divided by the average of the measurements) is low then it always indicates that precision is good but if the relative precision is high, then it is important to check the absolute precision. Often, when the concentration measured is exceedingly low then a very small difference in replicate measurements can result in a large relative precision variability. We excluded all values that had absolute precision less than 20 ng and the resulting precision is listed for all compounds in each sample pair in Table II-B-6. The median precision across all sample pairs was approximately 15%.

Composition and quantification of blank source waters

The three test waters and an HPLC grade blank water were analyzed as 36 mL of water with 4 mL of MeOH to determine blank concentrations. The concentrations of each compound in the blank water are listed in Table II-B-7. When there were two or more water blanks that had detectable levels of a given chemical, we calculated three times the blank level (listed in the last column of Table II-B-7) and subtracted that from the subsequent measurements. If only one of the water samples had detectible levels of a compound, then we subtracted that value from the subsequent results. The first blank water (EPAMH or W_01) and the HPLC water both had slightly elevated levels of a number of hydrocarbons and FAME which may indicate instrument carry-over because both these samples were run in series after either a spike sample or after a WAF sample. The other two blank waters were run in series after the first blank and these had very low levels of hydrocarbon and FAME. Even with the possible carry-over between analyses, the chemical concentrations measured in the blank waters were low compared to the actual samples so no additional troubleshooting was done to determine the source of chemicals in the blank waters.

Quantification of WAF Constituents

The measured chemical concentrations for each of the fuel WAFs are listed in Tables II-B-8 thru II-B-11 for Soy-B100, Soy-B20, AF-B100 and AF-B20, respectively. Both of the animal fat biofuel WAF_01 mixtures had significantly higher concentrations of FAMEs and the Soy-B100 also had somewhat elevated FAME. We already noted contamination in the form of oil droplets present in the AF-B100 WAF_01 (greyed out values in Table II-B-10) but we did not notice visible oil droplets in the other WAF_01 samples. Comparing the average results for the duplicate AF-B20 WAF_01measurements to the previous measurement used in the range finding experiment found that the later measurements seem to have been contaminated with FAME. Both the initial measurement from the range finding and the average of the replicate measurements are reported in Table II-B-11 but the results with high FAME are likely due to contamination. The low level of FAME in the Soy-B20 WAF_01 rules out contamination in the source water used to mix the WAF. Further testing would be needed to determine if the mixing

conditions used for the WAF_01 samples resulted in elevated FAME in the Soy-B100 relative to the Soy-B20 or if the difference was due to contamination during mixing.

Only one alkane (2,2,3,3-tetramethyl- Butane) was measured in the WAF and it was also detected at elevated levels in the blanks, including the HPLC water and in the direct analysis. The fact that the alkane was in the diluted fuel which was not extracted using a stir-bar indicates that the methanol used in the dilution may have been the source. The antioxidant fuel additives acetic acid, butyl ester (synonym – butyl acetate) and 1,4-Benzenediol, 2-(1,1-dimethylethyl) (synonym – *tert*-Butylhydroquinone, TBHQ) were also identified in the majority of the samples. However, the concentrations were highly variable. We can assume that the addition of the additive to the original fuel was consistent so the variability was likely due to either the WAF mixing conditions or the extraction conditions. The butyl acetate was lowest in the WAF_04 which had the highest salinity so the solubility may be affected by pH but without further testing we cannot rule out the extraction as a source of the variability for either of the measured additives.

The overall trend in concentrations of the aromatic hydrocarbons indicates that the salinity and the temperature may both have an effect on the solubility of the aromatic hydrocarbons in the fuels. In particular, the highest salinity water had the lowest concentration for FAME, aromatic hydrocarbons and the additives. The lowest salinity water had the highest and most variable FAME concentrations. Additional measurements are needed to characterize the temperature and salinity effect on solubility.

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Table II-B-1: Stock water and mixing temperature for preparing representative WAF for toxicity assays

Water Mix	Organism	Base Water	Mixing Temp (°C)	Salinity (ppt)
W_01	Cerio/Alg/FM	EPAMH[2]	25	0
W_02[1]	Mysid	EPAMH + DS[3]	26	25
W_03[1]	Top smelt	EPAMH + DS	20	25
W_04	Abalone	EPAMH + HB[4]	15	33

[1] The water used to prepare W_02 and W_03 are from the same initial salinity mixture but the WAF is prepared under different temperature as indicated by "Mixing Temp". [2]EPAMH is moderately hard reconstituted water based on USEPA specifications. [3]DS is dry salts. [4]HB is hyper saline brine

Table II-B-2: Mixing volumes for preparation of WAF

	Fuel (mL) All samples include additives				Water (mL) and Mixing Temperature (C) [see Table 1 for details]			
SampleName	$SoyB_{100}$	AFB_{100}	$SoyB_{20}$	AFB_{20}	W_01	W_02	W_03	W_04
Soy-B_{100}_01	20				200			
Soy-B_{100}_02	20					200		
Soy-B_{100}_03	20						200	
Soy-B_{100}_04	20							200
AF-B_{100}_01		20			200			
AF-B_{100}_02		20				200		
AF-B_{100}_03		20					200	
AF-B_{100}_04		20						200
Soy-B_{20}_01			20		200			
Soy-B_{20}_02			20			200		
Soy-B_{20}_03			20				200	
Soy-B_{20}_04			20					200
AF-B_{20}_01				20	200			
AF-B_{20}_02				20		200		
AF-B_{20}_03				20			200	
AF-B_{20}_04				20				200
Blank_01					200			
Blank_02-03						200	200	
Blank_04								200

Table II-B-3: Surrogate compounds in standard mixes used to quantify samples

	Class	Surrogate	Calibration concentrations	
			Low (ppb)	High (ppb)
1	Mono-aromatic	*o*-Xylene	5.0	39.7
		m/p-Xylene	5.1	40.7
		1,2,4-trimethylbenzene	5.2	41.8
		1,2,3-trimethylbenzene	5.2	41.8
2	Poly-aromatic	Naphthalene	4.8	38.0
3	FAME	methyl- Palmitate	10	50
		methyl- Oleate & Linolenate	10	50
		methyl- Stearate	10	50
4	Alkanes	*n*-Undecane	5.1	41.0
		n-Dodecane	5.1	41.2

Table II-B-4: Target chemical identified in Biofuel WAF and Extracted Ion / Total Ion Ratios

Compound Name	Retention Time (min)	Chemical Class	EI/TI[1]
Dimethyl phthalate-3,4,5,6-d 4	33.25	ISTD	0.25
Benzene	6.29	mono-aromatic	0.69
Butane, 2,2,3,3-tetramethyl-	7.19	alkane	0.52
Toluene	10.92	mono-aromatic	0.47
Acetic acid, butyl ester	13.15	ester	0.38
Ethylbenzene	14.79	mono-aromatic	0.47
m-Xylene	15.17	mono-aromatic	0.38
p-Xylene	15.22	mono-aromatic	0.37
o-Xylene	16.03	mono-aromatic	0.39
Oxime-, methoxy-phenyl-	16.45	mono-aromatic	0.47
Benzene, (1-methylethyl)-	17.16	mono-aromatic	0.48
Hexanoic acid, methyl ester	17.29	FAME	0.31
Benzene, propyl-	18.26	mono-aromatic	0.52
Benzene, 1-ethyl-2-methyl-	18.55	mono-aromatic	0.44
Benzene, 1-ethyl-4-methyl-	18.66	mono-aromatic	0.45
Benzene, 1,2,3-trimethyl-	18.86	mono-aromatic	0.38[2]
Benzene, 1,3,5-trimethyl-	19.15	mono-aromatic	0.44
Benzene, 1,2,3-trimethyl-	19.74	mono-aromatic	0.39
Benzene, (1-methylpropyl)-	20.19	mono-aromatic	0.48
Benzene, 1-methyl-2-(1-methylethyl)-	20.57	mono-aromatic	0.44
Benzene, 1,2,4-trimethyl-	20.66	mono-aromatic	0.41
Benzene, 1-methyl-2-(1-methylethyl)-	20.75	mono-aromatic	0.34
Indane	21.15	indane[3]	0.38
Benzene, 1,3-diethyl-	21.49	mono-aromatic	0.25
Benzene, 1-methyl-3-propyl-	21.61	mono-aromatic	0.45
Benzene, 1,2,3,4-tetramethyl-	21.79	mono-aromatic	0.27
Benzene, 1-methyl-4-(1-methylethyl)-	21.83	mono-aromatic	0.40
Benzene, 1-methyl-4-propyl-	22.08	mono-aromatic	0.48
Benzene, 2-ethyl-1,4-dimethyl-	22.42	mono-aromatic	0.41
Benzene, 1-ethyl-2,4-dimethyl-	22.52	mono-aromatic	0.46
Benzene, 4-ethenyl-1,2-dimethyl-	22.66	mono-aromatic	0.34
Benzene, 1-ethyl-2,4-dimethyl-	22.72	mono-aromatic	0.41
Benzene, 1-ethenyl-4-ethyl-	22.80	mono-aromatic	0.40
Benzene, 1-ethyl-2,4-dimethyl-	22.86	mono-aromatic	0.41
Benzene, 1-methyl-4-(1-methylpropyl)-	23.22	mono-aromatic	0.30
Benzene, 1-ethyl-2,3-dimethyl-	23.34	mono-aromatic	0.37
Benzene, 1-methyl-4-(1-methylpropyl)-	23.49	mono-aromatic	0.39
Benzene, 1,2,3,4-tetramethyl-	23.75	mono-aromatic	0.38
Benzene, 1,2,3,4-tetramethyl-	23.86	mono-aromatic	0.39
Benzene, 1-methyl-4-(1-methylpropyl)-	24.28	mono-aromatic	0.41
Benzene, (2-methyl-1-butenyl)-	24.44	mono-aromatic	0.38
Indan, 1-methyl-	24.54	indane	0.27
1H-Indene, 2,3-dihydro-4-methyl-	24.81	indene[3]	0.38
Benzene, 1,2,4,5-tetramethyl-	24.85	mono-aromatic	0.38
Benzene, 1-methyl-4-(1-methylpropyl)-	25.03	mono-aromatic	0.38
Benzene, 1-methyl-4-(1-methylpropyl)-	25.19	mono-aromatic	0.38

Compound Name	Retention Time (min)	Chemical Class	EI/TI[1]
Naphthalene, 1,2,3,4-tetrahydro-	25.27	poly-aromatic	0.26
Benzene, 2-ethyl-1,3-dimethyl-	25.49	mono-aromatic	0.51
1H-Indene,2,3-dihydro-2,2-dimethyl-	25.89	indene	0.38*
1H-Indene,2,3-dihydro-2,2-dimethyl-	25.97	indene	0.34
Naphthalene	26.10	poly-aromatic	0.21*
1H-Indene, 2,3-dihydro-1,6-dimethyl-	26.22	indene	0.38*
Benzene, 1,3-dimethyl-5-(1-methylethyl)	26.48	mono-aromatic	0.38*
Benzene, 1,3-dimethyl-5-(1-methylethyl)	26.74	mono-aromatic	0.41
Naphthalene, 1,2,3,4-tetrahydro-2-methy	26.89	poly-aromatic	0.22
Naphthalene, 1,2,3,4-tetrahydro-1-methy	27.10	poly-aromatic	0.33
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethyl	27.28	poly-aromatic	0.21
Benzocycloheptene	27.68	mono-aromatic	0.38*
1H-Indene, 2,3-dihydro-4,7-dimethyl-	27.80	indene	0.34
1H-Indene, 2,3-dihydro-4,7-dimethyl-	28.15	indene	0.32
Naphthalene, 1,2,3,4-tetrahydro-6-methyl	28.34	poly-aromatic	0.21*
Naphthalene, 1,2,3,4-tetrahydro-1,5-dimethyl	28.53	poly-aromatic	0.21
Phenol, 2-(2-methyl-2-propenyl)-	28.60	mono-aromatic	0.21
1H-Indene, 2,3-dihydro-4,7-dimethyl-	28.70	indene	0.36
Naphthalene, 1,2,3,4-tetrahydro-1,5-dimethyl	28.91	poly-aromatic	0.21*
Naphthalene, 1,2,3,4-tetrahydro-5-methyl	29.05	poly-aromatic	0.21*
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-	29.19	indene	0.29
Naphthalene, 1-methyl-	29.30	poly-aromatic	0.31
Decanoic acid, methyl ester	29.70	FAME	0.11*
Naphthalene, 1-methyl-	29.74	poly-aromatic	0.21*
Naphthalene, 1,2,3,4-tetrahydro-1,4-dimethyl	29.84	poly-aromatic	0.14
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	29.98	indene	0.34
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro	30.14	poly-aromatic	0.13
Naphthalene, 1,2,3,4-tetrahydro-1,5-dimethyl	30.25	poly-aromatic	0.14
Naphthalene, 1,2,3,4-tetrahydro-1,4-dimethyl	30.36	poly-aromatic	0.15
(1,4-Dimethylpent-2-enyl)benzene	30.40	mono-aromatic	0.12
Naphthalene, 1,2,3,4-tetrahydro-1,4-dimethyl	30.47	poly-aromatic	0.16
Naphthalene, 1,2,3,4-tetrahydro-1,5-dimethyl	30.69	poly-aromatic	0.32
Ethanol, 2-(2-butoxyethoxy)-, acetate	30.80	glycolether	0.11*
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	30.85	poly-aromatic	0.21*
Methyl 4-oxododecanoate	31.08	FAME	0.11
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	31.19	poly-aromatic	0.20
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-	31.38	poly-aromatic	0.21*
Biphenyl	31.58	poly-aromatic	0.40
Naphthalene, 1,2,3,4-tetrahydro-5,7-dimethyl	31.74	poly-aromatic	0.28
Naphthalene, 1,2,3,4-tetrahydro-5,7-dimethyl	31.83	poly-aromatic	0.23
Diphenylmethane	31.95	poly-aromatic	0.18
Naphthalene, 1,4-dimethyl-	32.27	poly-aromatic	0.21*
Naphthalene, 1,2,3,4-tetrahydro-6,7-dimethyl	32.51	poly-aromatic	0.21*
Naphthalene, 1,7-dimethyl-	32.58	poly-aromatic	0.21*
Naphthalene, 1,2,3,4-tetrahydro-5,6-dimethyl	32.66	poly-aromatic	0.21*
Naphthalene, 2,6-dimethyl-	32.71	poly-aromatic	0.21*
Nonanoic acid, 9-oxo-, methyl ester	32.81	poly-aromatic	0.10
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	32.93	poly-aromatic	0.23

Compound Name	Retention Time (min)	Chemical Class	EI/TI[1]
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	33.07	poly-aromatic	0.11
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	33.19	poly-aromatic	0.21*
Naphthalene, 1,2-dimethyl-	33.49	poly-aromatic	0.24
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t	33.64	poly-aromatic	0.14
1,4-Dimethyl-2-cyclopentylbenzene	33.95	mono-aromatic	0.18
1,1'-Biphenyl, 4-methyl-	34.26	poly-aromatic	0.25
1,1'-Biphenyl, 4-methyl-	34.51	poly-aromatic	0.17
Dodecanoic acid, methyl ester	34.83	FAME	0.21
Naphthalene, 1,4,6-trimethyl-	35.14	poly-aromatic	0.21*
Naphthalene, 1,6,7-trimethyl-	35.19	poly-aromatic	0.21*
Naphthalene, 1,6,7-trimethyl-	35.37	poly-aromatic	0.23
Nonanedioic acid, dimethyl ester	35.51	FAME	0.08
Naphthalene, 2,3,6-trimethyl-	35.71	poly-aromatic	0.23
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	35.80	mono-aromatic	0.15
4,4'-Dimethylbiphenyl	36.77	poly-aromatic	0.21*
Naphthalene, 1-(2-propenyl)-	37.04	poly-aromatic	0.15
Methyl myristoleate	39.18	FAME	0.07
Methyl tetradecanoate	39.45	FAME	0.19
4,4'-Dimethylbiphenyl	39.50	poly-aromatic	0.21*
Pentadecanoic acid, methyl ester	41.58	FAME	0.20
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydro	42.04	poly-aromatic	0.09
9-Hexadecenoic acid, methyl ester, (Z)-	43.15	FAME	0.05
Methyl palmitoleate	43.22	FAME	0.03
Hexadecanoic acid, methyl ester	43.70	FAME	0.16
cis-10-Heptadecenoic acid, methyl ester	44.97	FAME	0.04
Heptadecanoic acid, methyl ester	45.34	FAME	0.17
9,12-Octadecadienoic acid (Z,Z)-, methy ester	46.45	FAME	0.07
Octadecenoic acid, ME (Isomers #3-4)	46.60	FAME	0.04
Octadecanoic acid, ME(Isomer #5)	46.59	FAME	0.03
Octadecanoic acid, methyl ester	46.91	FAME	0.15
Pyrene, 4,5-dihydro-	46.36	poly-aromatic	0.21*
Pyrene	47.34	poly-aromatic	0.21*
cis-11,14-Eicosadienoic acid, methyl ester	49.05	FAME	0.04

[1]The extracted ion to total ion ratio is used to convert the chemical response of the surrogate compounds (listed in Table 3) to response for the specific chemical in Table 4.
[2]Where a well resolved peak was not achieved and we could not determine TIC for a given compound, the average EI/TI ratio (listed with a * superscript) for the class of chemicals was used.
[3]When a TIC could not be measured for indane or indene, the average EI/TI ratio for the mono-aromatic was used.

Table II-B-5. Precision of internal standard area response for different WAF and Fuels

	Count	Average Area	CV
WAF_01	6	6.93E+05	35%
WAF_02	6	1.19E+06	18%
WAF_03	6	1.26E+06	16%
WAF_04	6	1.38E+06	15%
Soy-B100	4	1.29E+06	25%
Soy-B20	4	1.28E+06	15%
AF-B100	8	1.11E+06	43%
AF-B100 (excluding WAF_01)	6	1.33E+06	20%
AF-B20	8	9.96E+05	20%
blank test waters	3	1.57E+06	17%
Overall	21	1.18E+06	30%

Table II-B-6: Relative precision of sample pairs excluding pairs with absolute precision less than 10 ng

	AF-B100 sample pairs in WAF				AF-B20 sample pairs in WAF			
Compound name	01	02	03	04	01	02	03	04
Benzene	5%				5%	9%	19%	17%
Butane, 2,2,3,3-tetramethyl-	115 %	33%	56%	21%	69%	76%	87%	
Toluene					26%	0%	49%	10%
Acetic acid, butyl ester	16%	16%	12%	77%	1%	31%	25%	59%
Ethylbenzene	8%				10%	22%	5%	34%
m-Xylene	5%				5%	23%	1%	28%
p-Xylene	4%				11%	20%	5%	33%
o-Xylene	3%				9%	14%	5%	26%
Oxime-, methoxy-phenyl-_	8%			17%	5%		17%	5%
Benzene, (1-methylethyl)-					25%	36%	17%	48%
Hexanoic acid, methyl ester	23%	54%	29%	73%	12%	38%	36%	66%
Benzene, propyl-	1%				29%	36%	21%	50%
Benzene, 1-ethyl-2-methyl-	6%				25%	27%	14%	41%
Benzene, 1-ethyl-4-methyl-	8%				26%	29%	16%	43%
Benzene, 1,2,3-trimethyl-					24%	28%	14%	40%
Benzene, 1,3,5-trimethyl-	3%				23%	24%	12%	36%
Benzene, 1,2,3-trimethyl-	2%				21%	21%	10%	34%
Benzene, (1-methylpropyl)-							39%	
Benzene, 1-methyl-2-(1-methylethyl)-					33%	37%	24%	53%
Benzene, 1,2,4-trimethyl-	3%	10%			20%	15%	8%	28%
Benzene, 1-methyl-2-(1-methylethyl)-					32%	40%	27%	53%
Indane	15%	10%			17%	11%	7%	25%
Benzene, 1,3-diethyl-					30%	32%	22%	47%
Benzene, 1-methyl-3-propyl-	13%				33%	38%	26%	52%
Benzene, 1,2,3,4-tetramethyl-	11%	9%			31%	35%	24%	51%
Benzene, 1-methyl-4-(1-methylethyl)-	8%				29%	30%	20%	46%
Benzene, 1-methyl-4-propyl-	3%				30%	32%	22%	47%
Benzene, 2-ethyl-1,4-dimethyl-	2%				26%	25%	17%	41%
Benzene, 1-ethyl-2,4-dimethyl-	4%				26%	25%	17%	41%
Benzene, 4-ethenyl-1,2-dimethyl-					24%	21%	14%	35%
Benzene, 1-ethyl-2,4-dimethyl-	7%			3%	25%	25%	17%	41%
Benzene, 1-ethenyl-4-ethyl-	10%				22%	19%	12%	32%
Benzene, 1-ethyl-2,4-dimethyl-					18%	18%	15%	37%
Benzene, 1-methyl-4-(1-methylpropyl)-	17%				32%	44%	32%	62%
Benzene, 1-ethyl-2,3-dimethyl-					22%	18%	13%	34%
Benzene, 1-methyl-4-(1-methylpropyl)-					30%	44%		58%
Benzene, 1,2,3,4-tetramethyl-	14%				22%	18%	13%	34%
Benzene, 1,2,3,4-tetramethyl-	4%				21%	17%	12%	32%
Benzene, 1-methyl-4-(1-methylpropyl)-					28%			57%
Benzene, (2-methyl-1-butenyl)-					25%			55%
Indan, 1-methyl-	14%	101%			18%	14%	9%	26%
1H-Indene, 2,3-dihydro-4-methyl-	8%	100%		7%	18%	10%	7%	23%
Benzene, 1,2,4,5-tetramethyl-	7%	107%			19%	13%	9%	26%

	AF-B100 sample pairs in WAF				AF-B20 sample pairs in WAF			
Compound name	01	02	03	04	01	02	03	04
Benzene, 1-methyl-4-(1-methylpropyl)-					26%	32%	22%	47%
Benzene, 1-methyl-4-(1-methylpropyl)-					19%	30%	22%	48%
Naphthalene, 1,2,3,4-tetrahydro-	9%	102%			17%	10%	6%	20%
Benzene, 2-ethyl-1,3-dimethyl-					24%	30%		47%
1H-Indene,2,3-dihydro-2,2-dimethyl-	8%	5%	1%	2%	3%	10%	10%	14%
1H-Indene,2,3-dihydro-2,2-dimethyl-		112%			21%	19%	13%	34%
Naphthalene			9%	6%	15%	6%	5%	14%
1H-Indene, 2,3-dihydro-1,6-dimethyl-					20%	17%	12%	31%
Benzene, 1,3-dimethyl-5-(1-methylethyl)				14%	20%	19%	14%	34%
Benzene, 1,3-dimethyl-5-(1-methylethyl)					20%	15%	11%	
Naphthalene, 1,2,3,4-tetrahydro-2-methy	17%				20%	17%	11%	30%
Naphthalene, 1,2,3,4-tetrahydro-1-methy	8%				18%	13%	9%	25%
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy	11%				18%	15%	11%	28%
Benzocycloheptene					123%	13%	10%	19%
1H-Indene, 2,3-dihydro-4,7-dimethyl-		117%			19%	9%	8%	27%
1H-Indene, 2,3-dihydro-4,7-dimethyl-	15%	120%			15%	8%	7%	22%
Naphthalene, 1,2,3,4-tetrahydro-6-methy		113%			16%	9%	8%	22%
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim					16%		15%	42%
Phenol, 2-(2-methyl-2-propenyl)-					14%	11%	8%	21%
1H-Indene, 2,3-dihydro-4,7-dimethyl-					15%	7%	7%	22%
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim					16%	15%	10%	
Naphthalene, 1,2,3,4-tetrahydro-5-methy	18%	115%			15%	6%	6%	18%
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-					14%	14%	9%	31%
Naphthalene, 1-methyl-	15%				13%	4%	5%	14%
Decanoic acid, methyl ester	28%		1%		26%	14%		
Naphthalene, 1-methyl-					13%	2%	6%	14%
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	30%				15%	13%	11%	29%
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	12%				14%	12%	10%	27%
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro					13%	14%	16%	
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim					14%	15%	17%	
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim					31%	40%	15%	76%
(1,4-Dimethylpent-2-enyl)benzene					5%	7%	10%	25%
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim					23%	13%	19%	30%
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim					11%	7%	8%	26%
Ethanol, 2-(2-butoxyethoxy)-, acetate	7%	16%	13%	62%	4%	26%	19%	45%
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro					17%	9%	10%	25%
Methyl 4-oxododecanoate	13%	24%	20%	50%	7%	26%	24%	38%
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro					10%	6%	2%	34%
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-					12%	6%	9%	19%
Biphenyl	3%				13%	3%	4%	14%
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim					11%	5%	6%	22%
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim					10%	5%	8%	23%
Diphenylmethane					11%	3%	6%	16%
Naphthalene, 1,4-dimethyl-					9%	2%	6%	13%
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim					11%	6%	7%	18%
Naphthalene, 1,7-dimethyl-					10%	3%	5%	15%
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim					10%	6%	7%	21%
Naphthalene, 2,6-dimethyl-					9%	4%	6%	17%

	AF-B100 sample pairs in WAF				AF-B20 sample pairs in WAF			
Compound name	01	02	03	04	01	02	03	04
Nonanoic acid, 9-oxo-, methyl ester	31%	31%	41%		1%	51%	51%	
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t					2%	9%	16%	
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t					7%	8%	10%	26%
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t					9%	14%	14%	
Naphthalene, 1,2-dimethyl-					38%	0%	5%	16%
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t					3%	4%	12%	28%
1,4-Dimethyl-2-cyclopentylbenzene					8%	8%	12%	28%
1,1'-Biphenyl, 4-methyl-	5%				9%	3%	5%	13%
1,1'-Biphenyl, 4-methyl-					8%	2%	5%	14%
Dodecanoic acid, methyl ester	46%	16%			13%			
Naphthalene, 1,4,6-trimethyl-					9%	4%	7%	14%
Naphthalene, 1,6,7-trimethyl-					5%	4%	6%	18%
Naphthalene, 1,6,7-trimethyl-					5%	5%	5%	16%
Nonanedioic acid, dimethyl ester	9%	34%	35%		11%	63%	51%	
Naphthalene, 2,3,6-trimethyl-					5%	3%	5%	18%
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	77%	14%	17%	11%	8%	18%	22%	27%
4,4'-Dimethylbiphenyl					3%	3%	4%	15%
Naphthalene, 1-(2-propenyl)-					6%	3%	5%	13%
Methyl myristoleate	51%				7%			
Methyl tetradecanoate	61%				6%			
4,4'-Dimethylbiphenyl					7%	5%	2%	7%
Pentadecanoic acid, methyl ester	56%				3%			
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr	10%	6%	16%		4%	32%	27%	
9-Hexadecenoic acid, methyl ester, (Z)-	88%				5%			
Methyl palmitoleate	59%				2%			
Hexadecanoic acid, methyl ester	49%	47%	62%	17%	2%	79%	12%	42%
cis-10-Heptadecenoic acid, methyl ester	37%				2%			
Heptadecanoic acid, methyl ester	36%				5%			
9,12-Octadecadienoic acid (Z,Z)-, methy	43%	59%			1%			
Octadecenoic acid, ME (Isomers #3-4)		57%		10%	9%			
Octadecanoic acid, ME(Isomer #5)		36%			107%			
Octadecanoic acid, methyl ester		53%	74%	17%		75%		
Pyrene, 4,5-dihydro-					2%	5%	2%	13%
Pyrene					5%	5%	2%	6%
cis-11,14-Eicosadienoic acid, methyl ester	44%				38%			

Table II-B-7: Blank concentrations (μg/L or ppb) of each compound in each water

Compound name	W_01	W_02/03	W_04	HPLC	3XSTDEV
Benzene	0.02	0.02	0.02	0.06	0.05
Butane, 2,2,3,3-tetramethyl-	0.64	1.29	1.15	17.07	24.09
Toluene	0.08	0.03	0.03	0.20	0.23
Acetic acid, butyl ester					
Ethylbenzene	0.22			0.04	0.38
m-Xylene	0.49	0.03	0.03	0.07	0.68
p-Xylene	0.24			0.08	0.36
o-Xylene	0.31			0.05	0.56
Oxime-, methoxy-phenyl-_	0.23	0.17	0.26	0.25	0.11
Benzene, (1-methylethyl)-	0.04				0.04
Hexanoic acid, methyl ester					
Benzene, propyl-	0.26			0.02	0.50
Benzene, 1-ethyl-2-methyl-	0.76			0.07	1.46
Benzene, 1-ethyl-4-methyl-	0.47			0.04	0.92
Benzene, 1,2,3-trimethyl-	0.32		0.10	0.06	0.42
Benzene, 1,3,5-trimethyl-	0.38			0.03	0.73
Benzene, 1,2,3-trimethyl-	1.31		0.03	0.10	2.16
Benzene, (1-methylpropyl)-					
Benzene, 1-methyl-2-(1-methylethyl)-	0.07				0.07
Benzene, 1,2,4-trimethyl-	0.31			0.03	0.59
Benzene, 1-methyl-2-(1-methylethyl)-	0.07			0.02	0.11
Indane	0.13			0.01	0.25
Benzene, 1,3-diethyl-	0.14				0.14
Benzene, 1-methyl-3-propyl-	0.38			0.03	0.75
Benzene, 1,2,3,4-tetramethyl-	0.38			0.03	0.73
Benzene, 1-methyl-4-(1-methylethyl)-	0.34			0.03	0.67
Benzene, 1-methyl-4-propyl-	0.30			0.02	0.59
Benzene, 2-ethyl-1,4-dimethyl-	0.25			0.03	0.47
Benzene, 1-ethyl-2,4-dimethyl-	0.34			0.03	0.66
Benzene, 4-ethenyl-1,2-dimethyl-	0.07				0.07
Benzene, 1-ethyl-2,4-dimethyl-	0.43			0.03	0.85
Benzene, 1-ethenyl-4-ethyl-	0.11			0.01	0.22
Benzene, 1-ethyl-2,4-dimethyl-	0.02				0.02
Benzene, 1-methyl-4-(1-methylpropyl)-	0.24				0.24
Benzene, 1-ethyl-2,3-dimethyl-	0.15				0.15
Benzene, 1-methyl-4-(1-methylpropyl)-	0.09				0.09
Benzene, 1,2,3,4-tetramethyl-	0.20				0.20
Benzene, 1,2,3,4-tetramethyl-	0.27				0.27
Benzene, 1-methyl-4-(1-methylpropyl)-	0.10				0.10
Benzene, (2-methyl-1-butenyl)-	0.03				0.03
Indan, 1-methyl-	0.39			0.02	0.78
1H-Indene, 2,3-dihydro-4-methyl-	0.38			0.03	0.75
Benzene, 1,2,4,5-tetramethyl-	0.26			0.02	0.51
Benzene, 1-methyl-4-(1-methylpropyl)-	0.27			0.02	0.53
Benzene, 1-methyl-4-(1-methylpropyl)-	0.23			0.01	0.45
Naphthalene, 1,2,3,4-tetrahydro-	0.35			0.03	0.68

Compound name	W_01	W_02/03	W_04	HPLC	3XSTDEV
Benzene, 2-ethyl-1,3-dimethyl-	0.11				0.11
1H-Indene,2,3-dihydro-2,2-dimethyl-					
1H-Indene,2,3-dihydro-2,2-dimethyl-	0.33				0.33
Naphthalene	0.10	0.03		0.05	0.10
1H-Indene, 2,3-dihydro-1,6-dimethyl-	0.17				0.17
Benzene, 1,3-dimethyl-5-(1-methylethyl)	0.07				0.07
Benzene, 1,3-dimethyl-5-(1-methylethyl)	0.02				0.02
Naphthalene, 1,2,3,4-tetrahydro-2-methy	0.23			0.01	0.46
Naphthalene, 1,2,3,4-tetrahydro-1-methy	0.10				0.10
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy	0.17				0.17
Benzocycloheptene					
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.23			0.01	0.47
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.18				0.18
Naphthalene, 1,2,3,4-tetrahydro-6-methy	0.54			0.05	1.05
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.18				0.18
Phenol, 2-(2-methyl-2-propenyl)-	0.21				0.21
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.10				0.10
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.03				0.03
Naphthalene, 1,2,3,4-tetrahydro-5-methy	0.29			0.02	0.57
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-	0.08				0.08
Naphthalene, 1-methyl-	0.14			0.03	0.23
Decanoic acid, methyl ester					
Naphthalene, 1-methyl-	0.07			0.03	0.08
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	0.55				0.55
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	0.16				0.16
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro					
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.04				0.04
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	0.10				0.10
(1,4-Dimethylpent-2-enyl)benzene					
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	0.11				0.11
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.06				0.06
Ethanol, 2-(2-butoxyethoxy)-, acetate					
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	0.17				0.17
Methyl 4-oxododecanoate					
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	0.06				0.06
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-					
Biphenyl	0.24			0.04	0.44
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	0.25				0.25
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	0.13				0.13
Diphenylmethane	0.12				0.12
Naphthalene, 1,4-dimethyl-	0.07				0.07
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim	0.10				0.10
Naphthalene, 1,7-dimethyl-	0.10				0.10
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim	0.13				0.13
Naphthalene, 2,6-dimethyl-	0.09				0.09
Nonanoic acid, 9-oxo-, methyl ester					
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	0.11				0.11
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	0.45				0.45
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	0.09				0.09

Compound name	W_01	W_02/03	W_04	HPLC	3XSTDEV
Naphthalene, 1,2-dimethyl-					
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t	0.11				0.11
1,4-Dimethyl-2-cyclopentylbenzene	0.13				0.13
1,1'-Biphenyl, 4-methyl-	0.26			0.03	0.49
1,1'-Biphenyl, 4-methyl-	0.18			0.04	0.29
Dodecanoic acid, methyl ester					
Naphthalene, 1,4,6-trimethyl-	0.08				0.08
Naphthalene, 1,6,7-trimethyl-	0.07				0.07
Naphthalene, 1,6,7-trimethyl-	0.08				0.08
Nonanedioic acid, dimethyl ester					
Naphthalene, 2,3,6-trimethyl-	0.05				0.05
1,4-Benzenediol, 2-(1,1-dimethylethyl)-					
4,4'-Dimethylbiphenyl	0.08				0.08
Naphthalene, 1-(2-propenyl)-					
Methyl myristoleate					
Methyl tetradecanoate					
4,4'-Dimethylbiphenyl					
Pentadecanoic acid, methyl ester					
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr					
9-Hexadecenoic acid, methyl ester, (Z)-					
Methyl palmitoleate					
Hexadecanoic acid, methyl ester				0.32	0.32
cis-10-Heptadecenoic acid, methyl ester					
Heptadecanoic acid, methyl ester					
9,12-Octadecadienoic acid (Z,Z)-, methy					
Octadecenoic acid, ME (Isomers #3-4)					
Octadecanoic acid, ME(Isomer #5)					
Octadecanoic acid, methyl ester				0.28	0.28
Pyrene, 4,5-dihydro-					
Pyrene					
cis-11,14 Eicosadienoic acid, methyl ester					

Table II-B-8: Soy-B100 WAF concentrations (μg/L or ppb) with blank subtracted

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Benzene	0.2	0.1	0.1	0.1
Butane, 2,2,3,3-tetramethyl-	24.5	7.0		
Toluene	1.0	1.5	0.2	
Acetic acid, butyl ester	110.6	63.6	77.7	1.0
Ethylbenzene	0.3	1.1		
m-Xylene	0.3	2.4		
p-Xylene	0.1	1.1		
o-Xylene	0.4	1.5		
Oxime-, methoxy-phenyl-_	0.8	0.4	0.3	0.5
Benzene, (1-methylethyl)-				
Hexanoic acid, methyl ester	0.8	0.2		
Benzene, propyl-		0.4		
Benzene, 1-ethyl-2-methyl-		1.5		
Benzene, 1-ethyl-4-methyl-		0.8		
Benzene, 1,2,3-trimethyl-	0.4	0.8		
Benzene, 1,3,5-trimethyl-		0.9		
Benzene, 1,2,3-trimethyl-		3.2		
Benzene, (1-methylpropyl)-				
Benzene, 1-methyl-2-(1-methylethyl)-		0.1		
Benzene, 1,2,4-trimethyl-		0.9		
Benzene, 1-methyl-2-(1-methylethyl)-		0.1		
Indane	0.1	0.5		
Benzene, 1,3-diethyl-		0.3		
Benzene, 1-methyl-3-propyl-		0.3		
Benzene, 1,2,3,4-tetramethyl-		0.3		
Benzene, 1-methyl-4-(1-methylethyl)-		0.4		
Benzene, 1-methyl-4-propyl-		0.3		
Benzene, 2-ethyl-1,4-dimethyl-		0.4		
Benzene, 1-ethyl-2,4-dimethyl-		0.4		
Benzene, 4-ethenyl-1,2-dimethyl-		0.2		
Benzene, 1-ethyl-2,4-dimethyl-		0.5		
Benzene, 1-ethenyl-4-ethyl-		0.3		
Benzene, 1-ethyl-2,4-dimethyl-		0.1	0.1	
Benzene, 1-methyl-4-(1-methylpropyl)-		0.2		
Benzene, 1-ethyl-2,3-dimethyl-		0.4		
Benzene, 1-methyl-4-(1-methylpropyl)-		0.1		
Benzene, 1,2,3,4-tetramethyl-		0.5		
Benzene, 1,2,3,4-tetramethyl-		0.7		
Benzene, 1-methyl-4-(1-methylpropyl)-		0.1		
Benzene, (2-methyl-1-butenyl)-		0.3		
Indan, 1-methyl-		0.9		
1H-Indene, 2,3-dihydro-4-methyl-		1.0		
Benzene, 1,2,4,5-tetramethyl-		0.5		
Benzene, 1-methyl-4-(1-methylpropyl)-		0.1		
Benzene, 1-methyl-4-(1-methylpropyl)-		0.0		
Naphthalene, 1,2,3,4-tetrahydro-		1.0		

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Benzene, 2-ethyl-1,3-dimethyl-		0.1		
1H-Indene,2,3-dihydro-2,2-dimethyl-	12.6	11.6	11.3	8.9
1H-Indene,2,3-dihydro-2,2-dimethyl-	0.4	0.6	0.4	0.2
Naphthalene	0.1	0.4		0.1
1H-Indene, 2,3-dihydro-1,6-dimethyl-		0.4		
Benzene, 1,3-dimethyl-5-(1-methylethyl)		0.2	0.1	0.2
Benzene, 1,3-dimethyl-5-(1-methylethyl)		0.2		
Naphthalene, 1,2,3,4-tetrahydro-2-methy				
Naphthalene, 1,2,3,4-tetrahydro-1-methy		0.3		
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy		0.4		
Benzocycloheptene				
1H-Indene, 2,3-dihydro-4,7-dimethyl-		0.4		
1H-Indene, 2,3-dihydro-4,7-dimethyl-		0.5		
Naphthalene, 1,2,3,4-tetrahydro-6-methy		1.0		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim		0.2		
Phenol, 2-(2-methyl-2-propenyl)-		0.2		
1H-Indene, 2,3-dihydro-4,7-dimethyl-		0.3		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim		0.5		
Naphthalene, 1,2,3,4-tetrahydro-5-methy		0.6		
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-		0.2		
Naphthalene, 1-methyl-		0.5		
Decanoic acid, methyl ester				
Naphthalene, 1-methyl-		0.3		
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim		0.9		
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-		0.4		
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro				
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim				
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim		0.3		
(1,4-Dimethylpent-2-enyl)benzene				
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim		0.3		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim		0.2		
Ethanol, 2-(2-butoxyethoxy)-, acetate	34.7	21.0	27.8	1.3
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro		0.5		
Methyl 4-oxododecanoate				
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro				
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-				
Biphenyl		0.8		
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim		0.8		
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim		0.3		
Diphenylmethane		0.4		
Naphthalene, 1,4-dimethyl-		0.3		
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim		0.2		
Naphthalene, 1,7-dimethyl-		0.4		
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim		0.3		
Naphthalene, 2,6-dimethyl-		0.3		
Nonanoic acid, 9-oxo-, methyl ester		1.1	0.9	
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t				
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t		1.0		
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t		0.1		

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Naphthalene, 1,2-dimethyl-				
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t		0.1		
1,4-Dimethyl-2-cyclopentylbenzene		0.2		
1,1'-Biphenyl, 4-methyl-		0.7		
1,1'-Biphenyl, 4-methyl-		0.4		
Dodecanoic acid, methyl ester				
Naphthalene, 1,4,6-trimethyl-				
Naphthalene, 1,6,7-trimethyl-		0.2		
Naphthalene, 1,6,7-trimethyl-		0.3		
Nonanedioic acid, dimethyl ester	4.7	0.8	0.6	
Naphthalene, 2,3,6-trimethyl-		0.2		
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	53.3	14.4	12.6	16.4
4,4'-Dimethylbiphenyl		0.2		
Naphthalene, 1-(2-propenyl)-				
Methyl myristoleate				
Methyl tetradecanoate	1.4			
4,4'-Dimethylbiphenyl				
Pentadecanoic acid, methyl ester				
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr				
9-Hexadecenoic acid, methyl ester, (Z)-				
Methyl palmitoleate				
Hexadecanoic acid, methyl ester	127.1	2.4		2.0
cis-10-Heptadecenoic acid, methyl ester				
Heptadecanoic acid, methyl ester				
9,12-Octadecadienoic acid (Z,Z)-, methy	1094.6	73.0		
Octadecenoic acid, ME (Isomers #3-4)	456.9	22.9		
Octadecanoic acid, ME(Isomer #5)	23.8	0.4		
Octadecanoic acid, methyl ester	59.8	0.9		0.6
Pyrene, 4,5-dihydro-				
Pyrene		0.2		
cis-11,14 Eicosadienoic acid, methyl ester				

Table II-B-9: Soy-B20 WAF concentrations (μg/L or ppb) with blank subtracted

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Benzene	0.7	0.4	0.2	0.2
Butane, 2,2,3,3-tetramethyl-				
Toluene	92.0	57.2	27.2	1.6
Acetic acid, butyl ester		13.4	18.0	
Ethylbenzene	115.3	70.0	31.5	
m-Xylene	200.9	120.2	53.5	86.7
p-Xylene	83.7	50.4	22.5	22.7
o-Xylene	186.0	117.4	60.8	88.9
Oxime-, methoxy-phenyl-_	0.5	0.8	0.8	0.7
Benzene, (1-methylethyl)-	16.2	8.4	3.4	1.7
Hexanoic acid, methyl ester				
Benzene, propyl-	56.4	30.9	12.2	1.6
Benzene, 1-ethyl-2-methyl-	186.7	107.5	48.6	82.5
Benzene, 1-ethyl-4-methyl-	96.9	54.6	23.6	28.7
Benzene, 1,2,3-trimethyl-	64.8	36.9	16.9	30.8
Benzene, 1,3,5-trimethyl-	140.8	86.1	42.4	69.3
Benzene, 1,2,3-trimethyl-	319.2	190.9	96.2	149.0
Benzene, (1-methylpropyl)-	19.8			
Benzene, 1-methyl-2-(1-methylethyl)-	16.1	8.6	3.4	7.2
Benzene, 1,2,4-trimethyl-	131.2	83.7	46.8	68.5
Benzene, 1-methyl-2-(1-methylethyl)-	10.7	5.8	2.2	4.4
Indane	87.1	58.1	35.2	25.3
Benzene, 1,3-diethyl-	26.8	14.4	6.1	11.0
Benzene, 1-methyl-3-propyl-	49.3	25.5	9.7	18.2
Benzene, 1,2,3,4-tetramethyl-	47.3	24.5	9.3	10.1
Benzene, 1-methyl-4-(1-methylethyl)-	63.1	34.4	15.0	25.6
Benzene, 1-methyl-4-propyl-	56.5	31.6	13.3	24.5
Benzene, 2-ethyl-1,4-dimethyl-	54.4	31.1	14.9	25.5
Benzene, 1-ethyl-2,4-dimethyl-	58.3	32.9	15.5	26.7
Benzene, 4-ethenyl-1,2-dimethyl-	22.0	13.5	6.9	5.5
Benzene, 1-ethyl-2,4-dimethyl-	68.2	38.1	18.4	29.9
Benzene, 1-ethenyl-4-ethyl-	53.7	33.7	17.9	26.0
Benzene, 1-ethyl-2,4-dimethyl-	9.8	5.9	3.1	4.5
Benzene, 1-methyl-4-(1-methylpropyl)-	28.9	14.2	4.7	11.6
Benzene, 1-ethyl-2,3-dimethyl-	39.2	23.6	12.7	19.0
Benzene, 1-methyl-4-(1-methylpropyl)-	8.3	4.0	1.2	3.1
Benzene, 1,2,3,4-tetramethyl-	33.9	19.9	10.6	15.9
Benzene, 1,2,3,4-tetramethyl-	58.6	34.8	19.0	27.8
Benzene, 1-methyl-4-(1-methylpropyl)-	8.3	3.9	1.4	2.2
Benzene, (2-methyl-1-butenyl)-	6.2	3.6	1.6	2.8
Indan, 1-methyl-	103.5	63.8	37.8	50.0
1H-Indene, 2,3-dihydro-4-methyl-	154.2	99.2	60.5	77.8
Benzene, 1,2,4,5-tetramethyl-	69.7	42.8	25.6	34.1
Benzene, 1-methyl-4-(1-methylpropyl)-	27.9	14.3	6.0	11.7
Benzene, 1-methyl-4-(1-methylpropyl)-	16.6	8.4	3.8	7.4
Naphthalene, 1,2,3,4-tetrahydro-	173.2	113.1	70.8	74.9

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Benzene, 2-ethyl-1,3-dimethyl-	8.4	4.1	1.9	3.3
1H-Indene,2,3-dihydro-2,2-dimethyl-	2.1	2.6	2.4	0.1
1H-Indene,2,3-dihydro-2,2-dimethyl-	63.2	36.8	19.8	28.7
Naphthalene	41.6	29.2	20.5	14.6
1H-Indene, 2,3-dihydro-1,6-dimethyl-	41.3	24.6	13.6	19.1
Benzene, 1,3-dimethyl-5-(1-methylethyl)	9.7	5.1	2.9	4.3
Benzene, 1,3-dimethyl-5-(1-methylethyl)	5.3	3.0	1.8	2.5
Naphthalene, 1,2,3,4-tetrahydro-2-methy	43.8	26.0	14.4	19.2
Naphthalene, 1,2,3,4-tetrahydro-1-methy	38.2	23.9	14.4	18.5
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy	36.1	21.0	12.9	16.4
Benzocycloheptene	1.2	0.7	0.4	0.6
1H-Indene, 2,3-dihydro-4,7-dimethyl-	44.3	25.4	15.6	20.5
1H-Indene, 2,3-dihydro-4,7-dimethyl-	46.5	28.1	17.9	22.0
Naphthalene, 1,2,3,4-tetrahydro-6-methy	105.0	62.9	39.5	48.8
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	9.7	4.7	2.3	4.3
Phenol, 2-(2-methyl-2-propenyl)-	10.3	5.9	3.8	4.7
1H-Indene, 2,3-dihydro-4,7-dimethyl-	26.0	15.8	10.3	12.5
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	5.6	3.1	1.8	2.5
Naphthalene, 1,2,3,4-tetrahydro-5-methy	85.2	52.8	34.8	41.1
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-	13.5	7.5	4.7	6.0
Naphthalene, 1-methyl-	28.4	18.8	13.7	12.9
Decanoic acid, methyl ester	0.3	0.2		0.1
Naphthalene, 1-methyl-	27.2	18.2	13.2	13.6
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	51.8	28.1	17.1	23.5
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	27.4	12.0	7.4	9.9
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro	4.1	2.5	1.6	2.0
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	5.4	3.0	1.9	2.7
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	13.5	14.4	9.2	10.9
(1,4-Dimethylpent-2-enyl)benzene	9.1	6.4	4.1	5.3
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	13.4	6.8	4.9	6.5
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	14.4	8.5	5.6	6.9
Ethanol, 2-(2-butoxyethoxy)-, acetate	6.8	5.1	8.2	
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	18.4	10.1	6.5	8.2
Methyl 4-oxododecanoate				
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	8.6	5.6	3.3	4.9
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-	3.4	2.0	1.3	1.6
Biphenyl	54.6	34.7	26.6	21.6
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	30.3	17.1	11.7	14.1
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	13.6	7.8	5.4	6.6
Diphenylmethane	24.6	14.8	10.4	10.8
Naphthalene, 1,4-dimethyl-	7.8	4.5	3.3	3.5
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim	14.2	8.1	5.6	6.9
Naphthalene, 1,7-dimethyl-	19.0	11.8	8.5	8.6
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim	17.2	9.5	6.9	8.0
Naphthalene, 2,6-dimethyl-	12.9	7.9	5.8	5.8
Nonanoic acid, 9-oxo-, methyl ester				
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	5.5	2.9	2.0	2.9
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	25.7	13.3	9.4	13.0
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	3.4	1.6	1.1	1.6

Compound name	WAF_01	WAF_02	WAF_03	WAF_04
Naphthalene, 1,2-dimethyl-	2.7	1.7	1.2	1.2
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t	8.5	4.2	3.1	4.6
1,4-Dimethyl-2-cyclopentylbenzene	7.9	4.0	2.9	4.0
1,1'-Biphenyl, 4-methyl-	33.0	19.7	15.0	13.8
1,1'-Biphenyl, 4-methyl-	18.8	11.1	8.7	6.7
Dodecanoic acid, methyl ester				
Naphthalene, 1,4,6-trimethyl-	3.4	2.3	1.7	1.6
Naphthalene, 1,6,7-trimethyl-	6.9	3.8	3.0	3.1
Naphthalene, 1,6,7-trimethyl-	6.8	3.8	2.9	2.9
Nonanedioic acid, dimethyl ester				
Naphthalene, 2,3,6-trimethyl-	6.3	3.6	2.7	2.9
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	18.4	4.7	4.6	7.7
4,4'-Dimethylbiphenyl	4.5	2.5	2.1	2.1
Naphthalene, 1-(2-propenyl)-	9.1	5.8	4.4	4.1
Methyl myristoleate				
Methyl tetradecanoate				
4,4'-Dimethylbiphenyl	3.4	2.2	1.6	1.7
Pentadecanoic acid, methyl ester				
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr				
9-Hexadecenoic acid, methyl ester, (Z)-				
Methyl palmitoleate				
Hexadecanoic acid, methyl ester				
cis-10-Heptadecenoic acid, methyl ester				
Heptadecanoic acid, methyl ester				
9,12-Octadecadienoic acid (Z,Z)-, methy	13.1			
Octadecenoic acid, ME (Isomers #3-4)	8.3			
Octadecanoic acid, ME(Isomer #5)				
Octadecanoic acid, methyl ester	0.4			
Pyrene, 4,5-dihydro-	1.5	0.8	0.6	0.7
Pyrene	4.6	3.0	2.1	2.3
cis-11,14 Eicosadienoic acid, methyl ester				

Table II-B-10: AF-B100 WAF concentrations (μg/L or ppb) with blank subtracted

Compound name	WAF_01[1]	WAF_02	WAF_03	WAF_04
Benzene	0.5	0.2	0.2	0.3
Butane, 2,2,3,3-tetramethyl-	3.2			
Toluene	0.6	0.1	0.0	0.1
Acetic acid, butyl ester	40.2	56.4	69.1	3.1
Ethylbenzene	0.4			
m-Xylene	0.8			
p-Xylene	0.3			
o-Xylene	0.5			
Oxime-, methoxy-phenyl-_	2.1	0.7	0.8	0.6
Benzene, (1-methylethyl)-				
Hexanoic acid, methyl ester	50.5	21.7	25.7	1.6
Benzene, propyl-	0.2			
Benzene, 1-ethyl-2-methyl-	0.3			
Benzene, 1-ethyl-4-methyl-	0.0			
Benzene, 1,2,3-trimethyl-	0.2	0.0		
Benzene, 1,3,5-trimethyl-	0.4			
Benzene, 1,2,3-trimethyl-	1.0			
Benzene, (1-methylpropyl)-		0.0		
Benzene, 1-methyl-2-(1-methylethyl)-				
Benzene, 1,2,4-trimethyl-	0.5			
Benzene, 1-methyl-2-(1-methylethyl)-	0.1			
Indane	0.3			
Benzene, 1,3-diethyl-	0.1			
Benzene, 1-methyl-3-propyl-	0.4			
Benzene, 1,2,3,4-tetramethyl-	2.5	0.3	0.0	0.0
Benzene, 1-methyl-4-(1-methylethyl)-	0.5			
Benzene, 1-methyl-4-propyl-	0.3			
Benzene, 2-ethyl-1,4-dimethyl-	0.3			
Benzene, 1-ethyl-2,4-dimethyl-	0.3			
Benzene, 4-ethenyl-1,2-dimethyl-				
Benzene, 1-ethyl-2,4-dimethyl-	0.3			
Benzene, 1-ethenyl-4-ethyl-	0.3	0.0		
Benzene, 1-ethyl-2,4-dimethyl-				
Benzene, 1-methyl-4-(1-methylpropyl)-	0.8	0.1		
Benzene, 1-ethyl-2,3-dimethyl-		0.1		
Benzene, 1-methyl-4-(1-methylpropyl)-				
Benzene, 1,2,3,4-tetramethyl-	0.3	0.1		
Benzene, 1,2,3,4-tetramethyl-	0.6	0.3		
Benzene, 1-methyl-4-(1-methylpropyl)-	0.1			
Benzene, (2-methyl-1-butenyl)-				
Indan, 1-methyl-	0.5	0.1		
1H-Indene, 2,3-dihydro-4-methyl-	1.1	0.6		
Benzene, 1,2,4,5-tetramethyl-	0.5	0.3		
Benzene, 1-methyl-4-(1-methylpropyl)-	0.5			
Benzene, 1-methyl-4-(1-methylpropyl)-	0.4			
Naphthalene, 1,2,3,4-tetrahydro-	1.2	0.7		

Compound name	WAF_01[1]	WAF_02	WAF_03	WAF_04
Benzene, 2-ethyl-1,3-dimethyl-	0.3	0.1		
1H-Indene,2,3-dihydro-2,2-dimethyl-	7.1	10.4	9.5	9.0
1H-Indene,2,3-dihydro-2,2-dimethyl-	1.0	0.5		0.1
Naphthalene	0.3	0.3	0.1	0.1
1H-Indene, 2,3-dihydro-1,6-dimethyl-	0.2	0.4		
Benzene, 1,3-dimethyl-5-(1-methylethyl)	0.2			0.1
Benzene, 1,3-dimethyl-5-(1-methylethyl)				
Naphthalene, 1,2,3,4-tetrahydro-2-methy	0.6	0.2		
Naphthalene, 1,2,3,4-tetrahydro-1-methy	0.5	0.4		
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy	0.6	0.3		
Benzocycloheptene				
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.6	0.3		
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.8	0.5		
Naphthalene, 1,2,3,4-tetrahydro-6-methy	1.4	0.7		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.0			
Phenol, 2-(2-methyl-2-propenyl)-				
1H-Indene, 2,3-dihydro-4,7-dimethyl-	0.1	0.2		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim				
Naphthalene, 1,2,3,4-tetrahydro-5-methy	1.1	0.7		
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-	0.1	0.1		
Naphthalene, 1-methyl-	0.3	0.1		
Decanoic acid, methyl ester	197.9	0.3	0.2	
Naphthalene, 1-methyl-		0.1		
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	2.0	0.5		
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	0.8	0.2		
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro	0.1	0.3	0.7	
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim				
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim		0.2		
(1,4-Dimethylpent-2-enyl)benzene				
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim		0.1		
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	0.3	0.2		
Ethanol, 2-(2-butoxyethoxy)-, acetate	28.2	25.2	28.3	4.2
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	0.8	0.2		
Methyl 4-oxododecanoate	17.4	10.8	10.7	1.8
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro				
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-				
Biphenyl	0.4			
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	0.9	0.2		
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	0.1			
Diphenylmethane	0.1	0.0		
Naphthalene, 1,4-dimethyl-				
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim		0.0		
Naphthalene, 1,7-dimethyl-		0.0		
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim		0.0		
Naphthalene, 2,6-dimethyl-				
Nonanoic acid, 9-oxo-, methyl ester	20.1	21.7	26.6	
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	0.1			
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	0.4	0.1		
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t				

Compound name	WAF_01[1]	WAF_02	WAF_03	WAF_04
Naphthalene, 1,2-dimethyl-				
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t	0.1			
1,4-Dimethyl-2-cyclopentylbenzene				
1,1'-Biphenyl, 4-methyl-	0.5			
1,1'-Biphenyl, 4-methyl-	0.0			
Dodecanoic acid, methyl ester	980.4	1.3	0.9	
Naphthalene, 1,4,6-trimethyl-				
Naphthalene, 1,6,7-trimethyl-				
Naphthalene, 1,6,7-trimethyl-				
Nonanedioic acid, dimethyl ester	53.3	34.9	34.3	
Naphthalene, 2,3,6-trimethyl-				
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	17.7	4.9	5.1	17.9
4,4'-Dimethylbiphenyl				
Naphthalene, 1-(2-propenyl)-				
Methyl myristoleate	736.7	0.2	2.1	
Methyl tetradecanoate	10145.0	2.6	1.0	
4,4'-Dimethylbiphenyl	0.4			
Pentadecanoic acid, methyl ester	865.0			
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr	12.2	13.7	12.9	
9-Hexadecenoic acid, methyl ester, (Z)-	974.7			
Methyl palmitoleate	14445.3			
Hexadecanoic acid, methyl ester	89326.2	16.6	6.4	8.8
cis-10-Heptadecenoic acid, methyl ester	2223.3			
Heptadecanoic acid, methyl ester	2567.7			0.2
9,12-Octadecadienoic acid (Z,Z)-, methy	55098.9	9.4	1.8	
Octadecenoic acid, ME (Isomers #3-4)		21.7	4.8	2.9
Octadecanoic acid, ME(Isomer #5)		8.9	4.4	1.4
Octadecanoic acid, methyl ester		6.1	1.6	2.3
Pyrene, 4,5-dihydro-	2.3			
Pyrene				
cis-11,14 Eicosadienoic acid, methyl ester	3058.2			

[1] The AF-B20 WAF_01 had visible oil droplets on the surface of the WAF before extraction indicating that the water had been contaminated during mixing so the excessively high levels of FAME in this sample are not valid.

Table II-B-11: AF-B20 WAF concentrations (µg/L or ppb) with blank subtracted

Compound name	WAF_01[1]	WAF_01[2]	WAF_02	WAF_03	WAF_04
Benzene	0.7	9.0	0.2	0.2	0.3
Butane, 2,2,3,3-tetramethyl-		54.0			
Toluene	59.9	341.3	28.2	23.4	40.5
Acetic acid, butyl ester	68.1	55.6	20.6	26.1	8.3
Ethylbenzene	81.0	223.0	36.7	35.3	49.3
m-Xylene	154.4	375.1	65.6	63.8	85.6
p-Xylene	60.0	151.6	26.8	25.9	35.0
o-Xylene	155.5	339.3	75.1	69.7	81.4
Oxime-, methoxy-phenyl-_	1.9	1.0	0.9	0.8	0.7
Benzene, (1-methylethyl)-	10.6	23.7	3.9	4.2	5.9
Hexanoic acid, methyl ester	28.8	14.4	3.7	4.6	2.0
Benzene, propyl-	37.8	80.2	13.9	15.3	19.8
Benzene, 1-ethyl-2-methyl-	145.2	273.3	56.9	61.5	70.5
Benzene, 1-ethyl-4-methyl-	72.1	136.8	27.9	30.0	35.2
Benzene, 1,2,3-trimethyl-	51.7	90.7	20.5	21.3	23.6
Benzene, 1,3,5-trimethyl-	119.8	208.3	51.9	52.6	55.5
Benzene, 1,2,3-trimethyl-	284.8	467.4	122.5	124.2	125.6
Benzene, (1-methylpropyl)-	8.0	21.2	1.2	3.8	1.8
Benzene, 1-methyl-2-(1-methylethyl)-	12.1	20.5	3.9	4.6	5.3
Benzene, 1,2,4-trimethyl-	130.6	198.8	61.8	59.1	55.6
Benzene, 1-methyl-2-(1-methylethyl)-	8.0	13.7	2.5	3.0	3.5
Indane	92.0	137.5	46.9	43.6	39.2
Benzene, 1,3-diethyl-	21.3	34.1	7.2	8.3	9.1
Benzene, 1-methyl-3-propyl-	37.1	60.8	11.4	13.5	15.4
Benzene, 1,2,3,4-tetramethyl-	37.5	59.4	11.8	13.5	15.3
Benzene, 1-methyl-4-(1-methylethyl)-	52.9	81.1	18.1	20.4	21.4
Benzene, 1-methyl-4-propyl-	46.2	73.5	16.3	17.9	19.1
Benzene, 2-ethyl-1,4-dimethyl-	48.8	71.8	18.7	19.8	19.7
Benzene, 1-ethyl-2,4-dimethyl-	51.6	75.9	19.6	20.8	20.4
Benzene, 4-ethenyl-1,2-dimethyl-	20.6	30.0	9.2	9.0	8.5
Benzene, 1-ethyl-2,4-dimethyl-	61.3	87.4	23.1	24.8	24.1
Benzene, 1-ethenyl-4-ethyl-	52.5	76.9	23.7	23.3	21.5
Benzene, 1-ethyl-2,4-dimethyl-	9.0	12.7	3.9	4.0	3.7
Benzene, 1-methyl-4-(1-methylpropyl)-	22.2	33.0	5.5	6.9	8.4
Benzene, 1-ethyl-2,3-dimethyl-	37.9	53.1	16.5	16.5	15.1
Benzene, 1-methyl-4-(1-methylpropyl)-	6.6	9.5	1.5	1.9	2.4
Benzene, 1,2,3,4-tetramethyl-	33.4	44.8	14.2	14.2	12.6
Benzene, 1,2,3,4-tetramethyl-	58.2	77.5	25.3	25.1	22.1
Benzene, 1-methyl-4-(1-methylpropyl)-	6.9	9.2	1.7	2.1	2.4
Benzene, (2-methyl-1-butenyl)-	5.5	8.0	2.0	2.1	4.4
Indan, 1-methyl-	108.7	142.6	51.6	49.0	42.1
1H-Indene, 2,3-dihydro-4-methyl-	170.5	223.8	84.4	77.6	66.3
Benzene, 1,2,4,5-tetramethyl-	74.7	96.4	34.6	32.9	28.3
Benzene, 1-methyl-4-(1-methylpropyl)-	24.8	32.4	7.5	8.5	8.6
Benzene, 1-methyl-4-(1-methylpropyl)-	15.5	19.2	4.8	5.4	5.5
Naphthalene, 1,2,3,4-tetrahydro-	194.4	254.3	99.8	90.9	76.5

Compound name	WAF_01[1]	WAF_01[2]	WAF_02	WAF_03	WAF_04
Benzene, 2-ethyl-1,3-dimethyl-	7.7	9.6	2.3	2.6	2.6
1H-Indene,2,3-dihydro-2,2-dimethyl-	4.3	8.0	5.8	4.9	2.6
1H-Indene,2,3-dihydro-2,2-dimethyl-	62.6	81.8	26.3	26.3	23.0
Naphthalene	50.9	66.7	28.0	24.1	19.8
1H-Indene, 2,3-dihydro-1,6-dimethyl-	42.0	54.1	18.5	18.0	15.5
Benzene, 1,3-dimethyl-5-(1-methylethyl)	9.4	12.2	3.7	3.8	3.3
Benzene, 1,3-dimethyl-5-(1-methylethyl)	5.3	6.8	2.3	2.3	2.0
Naphthalene, 1,2,3,4-tetrahydro-2-methy	43.7	57.3	19.5	18.6	15.9
Naphthalene, 1,2,3,4-tetrahydro-1-methy	41.5	52.4	19.6	18.4	15.4
Bicyclo[4.2.1]nona-2,4,7-triene, 7-ethy	38.1	46.0	17.0	16.4	13.9
Benzocycloheptene	8.9	1.7	0.7	0.6	0.5
1H-Indene, 2,3-dihydro-4,7-dimethyl-	47.9	57.0	21.3	20.4	17.0
1H-Indene, 2,3-dihydro-4,7-dimethyl-	50.5	61.4	24.3	22.7	18.0
Naphthalene, 1,2,3,4-tetrahydro-6-methy	113.6	137.5	54.1	50.5	40.5
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	9.8	10.6	2.8	3.2	3.0
Phenol, 2-(2-methyl-2-propenyl)-	11.2	13.2	5.1	4.8	3.9
1H-Indene, 2,3-dihydro-4,7-dimethyl-	29.0	34.5	13.9	13.0	10.3
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	5.7	7.1	2.4	2.4	2.0
Naphthalene, 1,2,3,4-tetrahydro-5-methy	96.5	117.2	49.1	44.3	34.8
1H-Indene, 2,3-dihydro-1,4,7-trimethyl-	13.9	18.5	6.1	5.9	4.9
Naphthalene, 1-methyl-	34.0	44.5	18.5	15.8	12.1
Decanoic acid, methyl ester	9.9	0.7	0.2	0.1	0.1
Naphthalene, 1-methyl-	32.8	41.7	18.4	15.4	11.8
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	53.7	65.1	22.2	22.2	18.0
1H-Indene, 2,3-dihydro-1,1,5-trimethyl-	22.1	27.6	9.9	9.6	7.8
Naphthalene, 2-ethyl-1,2,3,4-tetrahydro	5.3	5.9	2.4	2.3	1.6
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	5.9	6.6	2.7	2.6	2.1
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	19.6	15.8	8.8	11.4	7.1
(1,4-Dimethylpent-2-enyl)benzene	10.2	10.8	5.3	5.2	4.2
Naphthalene, 1,2,3,4-tetrahydro-1,4-dim	13.9	16.8	6.4	5.8	4.9
Naphthalene, 1,2,3,4-tetrahydro-1,5-dim	15.4	19.7	7.6	7.2	5.5
Ethanol, 2-(2-butoxyethoxy)-, acetate	20.8	31.2	12.9	17.6	7.9
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	18.8	22.3	8.5	8.5	6.6
Methyl 4-oxododecanoate	9.7	10.7	3.5	4.3	2.0
Naphthalene, 5-ethyl-1,2,3,4-tetrahydro	8.6	10.3	4.9	4.4	3.4
Acenaphthylene, 1,2,2a,3,4,5-hexahydro-	3.8	4.7	1.9	1.8	1.4
Biphenyl	64.9	83.1	36.0	30.4	22.9
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	32.3	40.2	15.5	14.5	11.0
Naphthalene, 1,2,3,4-tetrahydro-5,7-dim	15.0	18.0	7.0	6.6	5.0
Diphenylmethane	27.5	35.7	14.4	12.6	9.2
Naphthalene, 1,4-dimethyl-	8.8	11.2	4.4	3.9	2.8
Naphthalene, 1,2,3,4-tetrahydro-6,7-dim	15.6	19.1	7.6	7.0	5.4
Naphthalene, 1,7-dimethyl-	21.6	28.3	11.7	9.9	7.2
Naphthalene, 1,2,3,4-tetrahydro-5,6-dim	18.4	21.7	9.0	8.4	6.4
Naphthalene, 2,6-dimethyl-	14.7	19.3	7.8	6.7	4.9
Nonanoic acid, 9-oxo-, methyl ester	22.2	22.9	5.8	6.7	
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	5.6	6.5	2.4	2.5	1.9
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	27.0	32.5	11.6	11.7	9.0
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-t	3.6	3.7	1.3	1.3	1.0

Compound name	WAF_01[1]	WAF_01[2]	WAF_02	WAF_03	WAF_04
Naphthalene, 1,2-dimethyl-	4.6	4.0	1.8	1.5	1.1
Naphthalene, 1,2,3,4-tetrahydro-1,4,6-t	8.4	9.7	3.7	3.6	2.8
1,4-Dimethyl-2-cyclopentylbenzene	8.0	9.5	3.7	3.5	2.8
1,1'-Biphenyl, 4-methyl-	37.8	50.0	20.7	17.1	12.4
1,1'-Biphenyl, 4-methyl-	22.1	28.5	11.8	9.8	7.2
Dodecanoic acid, methyl ester	81.8	1.4	0.2	0.3	
Naphthalene, 1,4,6-trimethyl-	4.0	5.4	2.4	1.9	1.4
Naphthalene, 1,6,7-trimethyl-	7.5	9.5	3.9	3.3	2.5
Naphthalene, 1,6,7-trimethyl-	7.5	9.4	3.8	3.2	2.4
Nonanedioic acid, dimethyl ester	42.8	27.4	5.0	7.3	1.4
Naphthalene, 2,3,6-trimethyl-	6.8	9.1	3.8	3.2	2.3
1,4-Benzenediol, 2-(1,1-dimethylethyl)-	15.3	12.0	5.0	5.4	7.2
4,4'-Dimethylbiphenyl	5.2	6.6	2.7	2.3	1.7
Naphthalene, 1-(2-propenyl)-	10.4	14.4	6.4	5.1	3.7
Methyl myristoleate	60.1[3]				
Methyl tetradecanoate	484.2				
4,4'-Dimethylbiphenyl	4.1	4.5	2.4	1.9	1.4
Pentadecanoic acid, methyl ester	27.9				
Naphthalene, 1,2,3,4,4a,5,8,8a-octahydr	10.5	12.1	3.9	5.4	
9-Hexadecenoic acid, methyl ester, (Z)-	468.3	0.3			
Methyl palmitoleate	556.3				
Hexadecanoic acid, methyl ester	5981.3	0.7	5.5	2.6	2.9
cis-10-Heptadecenoic acid, methyl ester	95.3	4.2			
Heptadecanoic acid, methyl ester	121.9	0.6			
9,12-Octadecadienoic acid (Z,Z)-, methy	3094.6		1.1		
Octadecenoic acid, ME (Isomers #3-4)	9907.0		5.2		
Octadecanoic acid, ME(Isomer #5)	6118.5		0.2		
Octadecanoic acid, methyl ester	3699.3	0.6	2.8	0.8	1.2
Pyrene, 4,5-dihydro-	1.5	1.9	1.1	0.8	0.5
Pyrene	4.3	7.4	3.5	2.6	1.8
cis-11,14 Eicosadienoic acid, methyl ester	200.8				

[1] The first column of WAF_01 results is the average of two measurements made of the same mixture subsequent to the initial range finding experiment. These values had excessive levels of FAME compared to the original measurements during the range finding experiment. [2] The results from the range finding experiment are reported here. [3] the values in the box are likely from contamination of the WAF with fresh B-100 fuel.

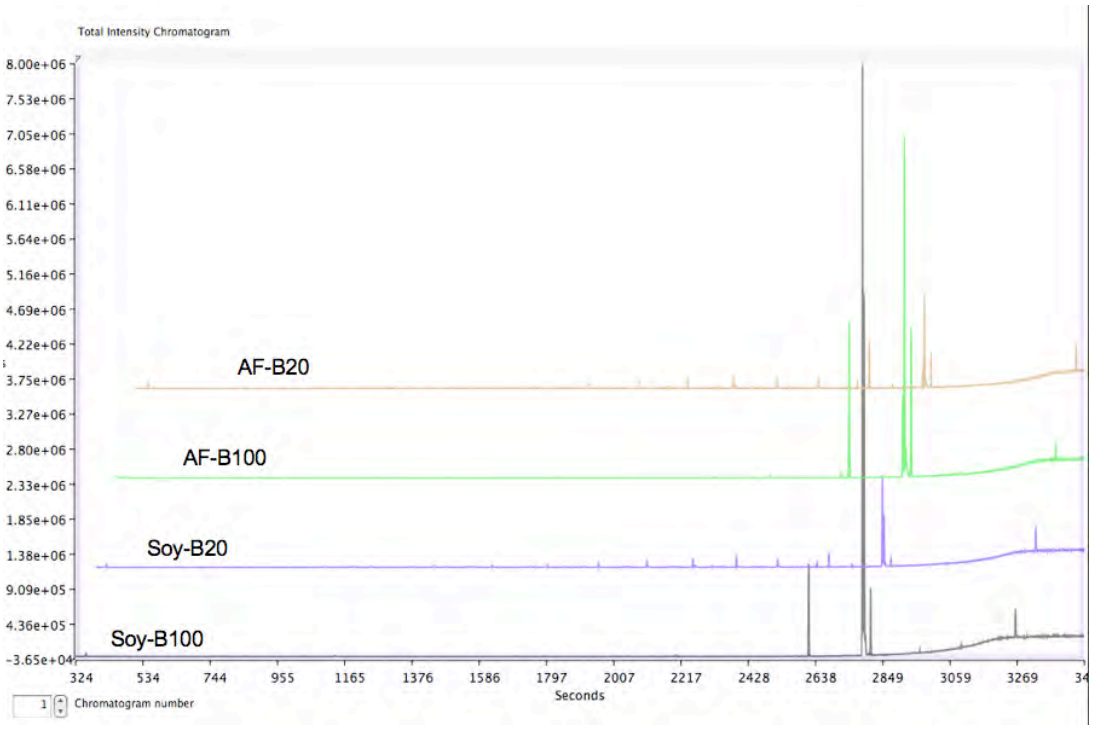

Figure II-B-1. Overlay total ion chromatogram of 1:1000 (v/v) dilution of each neat fuel in MeOH injected (2 µL) with 5:1 split.

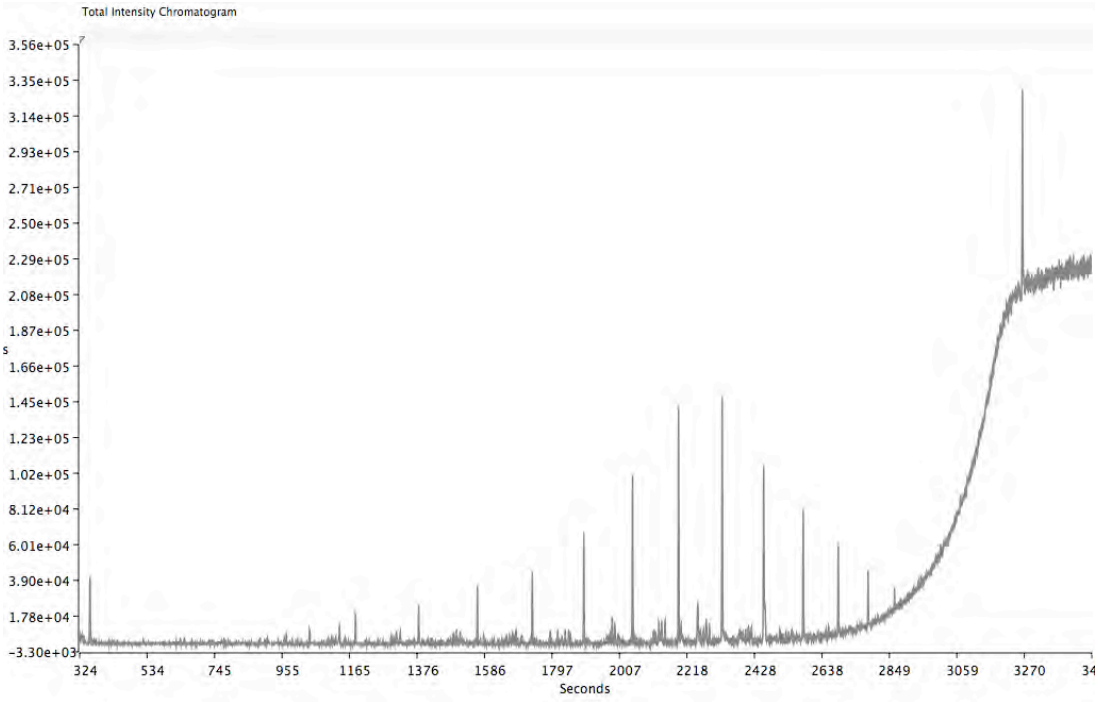

Figure II-B-2. CARB Diesel total ion chromatogram of 1:1000 (v/v) dilution of neat fuel in MeOH injected (2 µL) with 5:1 split.

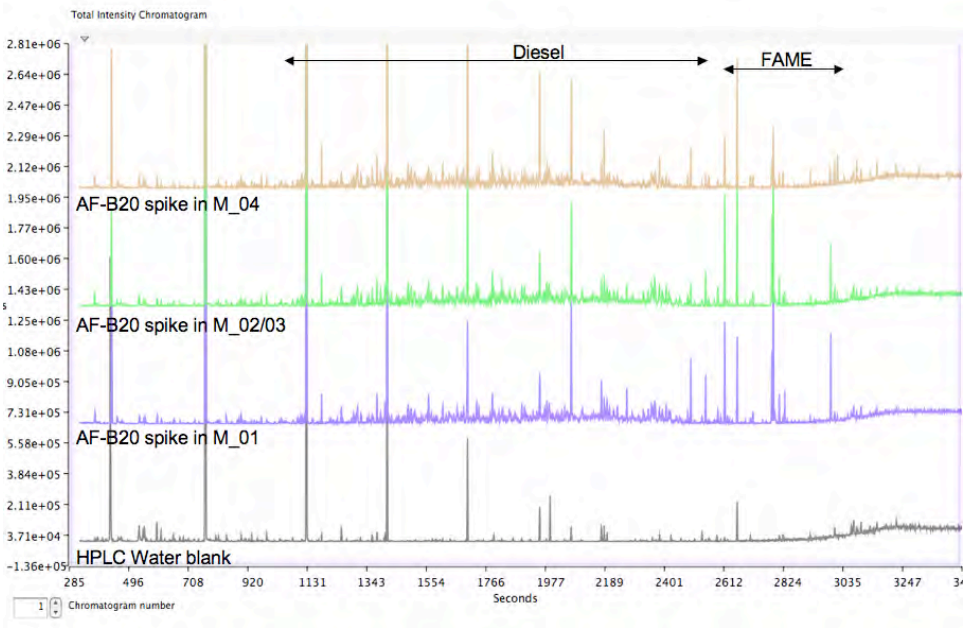

Figure II-B-3. Overlay of 1:1000 (v/v) dilution of neat AF-B20 fuel in MeOH spiked (4 µL) in each of the test waters (40 mL). The HPLC water blank is 40 mL of the water used as makeup volume in the WAF analysis. The large evenly spaced peaks are siloxanes from the stir-bar coating and are not quantified in the method.

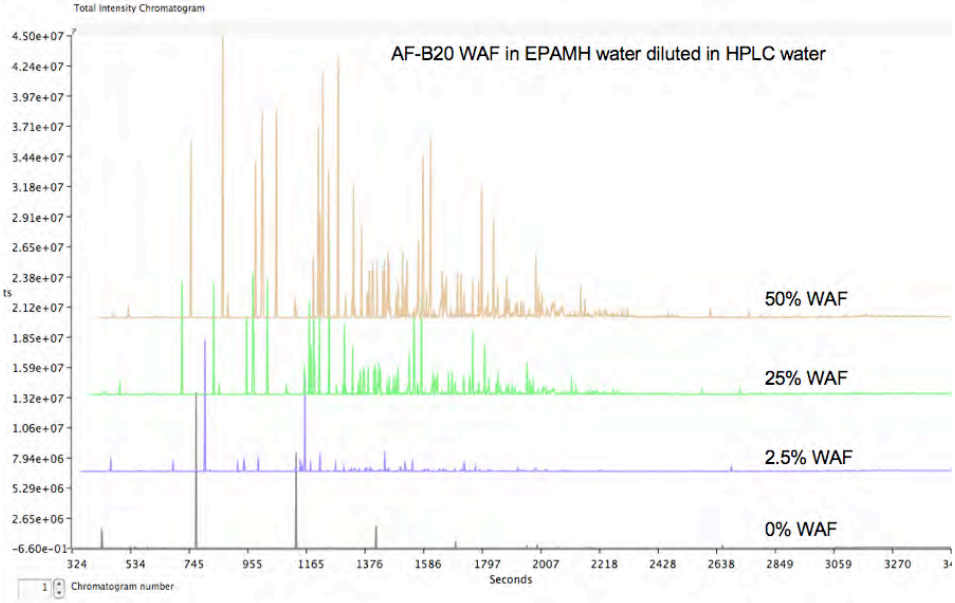

Figure II-B-4. Range finding experiment with increasing fractions of AF-B20 WAF_01 in HPLC water. The optimal dilution for the SBSE analysis was identified as 25% WAF in HPLC water.

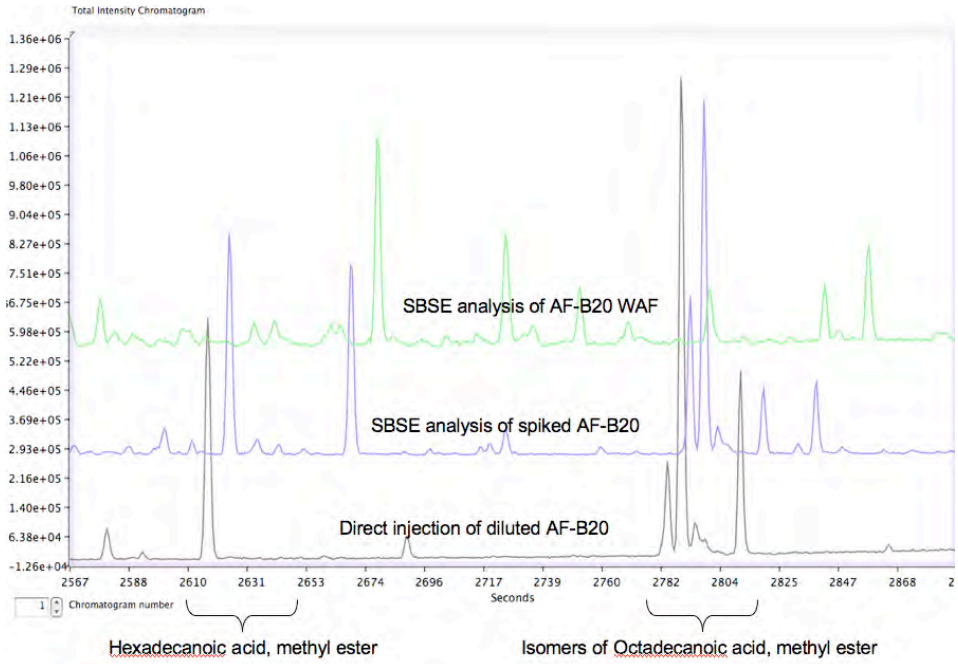

Figure II-B-5. Zoomed overlay in the region of the chromatogram where FAME elutes showing the presence of hexadecanoic acid methyl ester and the isomers of octadecanoic acid methyl ester in the raw fuel and in the spiked water but significantly reduced or absent in the WAF. The peak eluting at about 2660 seconds in the SBSE chromatograms is a siloxane from the stir bar and not part of the WAF or fuel.

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8. Appendix II-C: 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, 2009; 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.

METHODS AND MATERIALS

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 manufacture'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 noticeable effects on infiltration induced by the additive itself. The resulting suite of experiments is given in Table II-C-1.

Table II-C-1. 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.

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 digital photography of the dual infiltrating plumes, a sandbox design was developed using wood to build a three-sided frame, 16 inches by 11 inches. The frame was used to separate two glass walls of same dimension (Figure II-C-1). 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 II-C-1: 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 II-C-1) 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.

RESULTS AND DISCUSSION

Figure II-C-2 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 II-C-3, C-4, C-5, and C-6, 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 II-C-3. The images themselves show the different qualitative behavior seen for Animal Fat B-100 (e.g., Figure II-C-2).

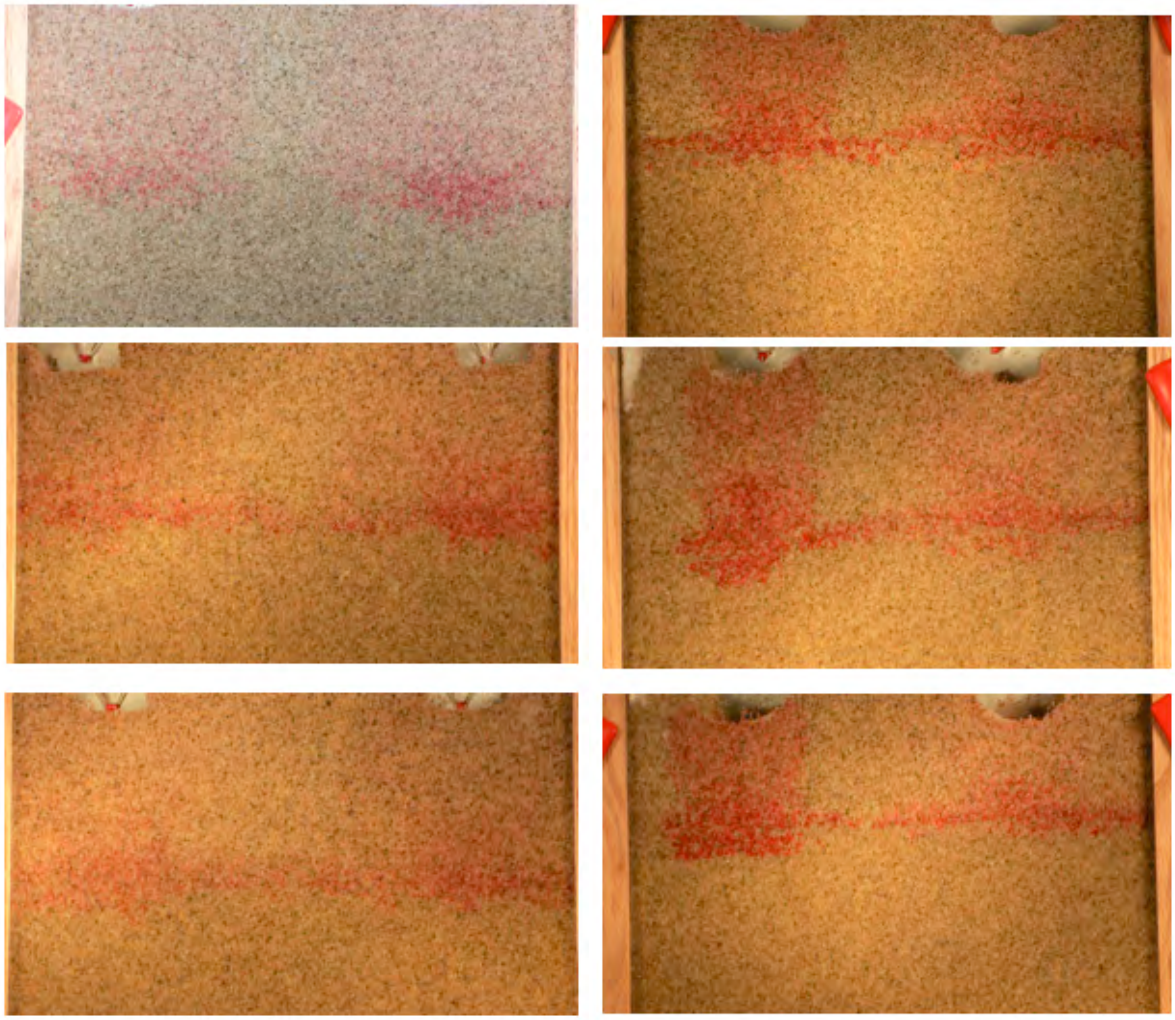

Figure II-C-2. Example final digital images. Left panel: the triplicate images for Soy B-20 at 2 hours; the left hand plume is Soy B-20 and the right-hand plume is ULSD. Right pane: those for the Animal Fat B-100 at 2 hours; the left hand plume is Animal Fat B-100 and the right-hand plume is ULSD. Note 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.

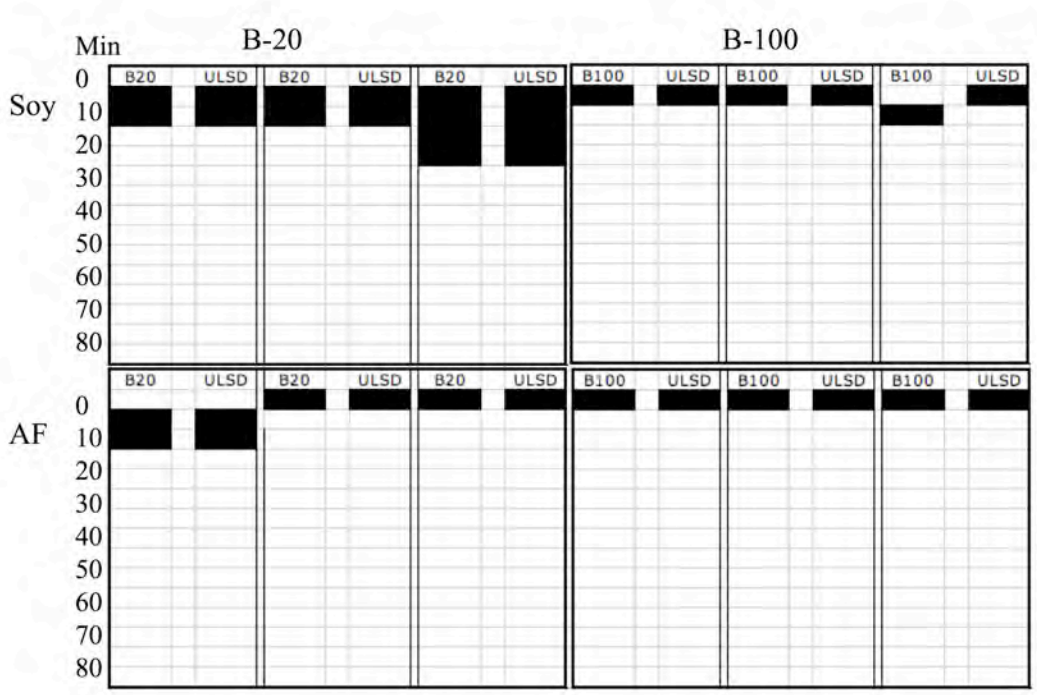

Figure II-C-3: Characteristic times to formation of the U-shaped plume for each of the four blends (Soy B-20, Soy B-100, Animal Fat (AF) B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

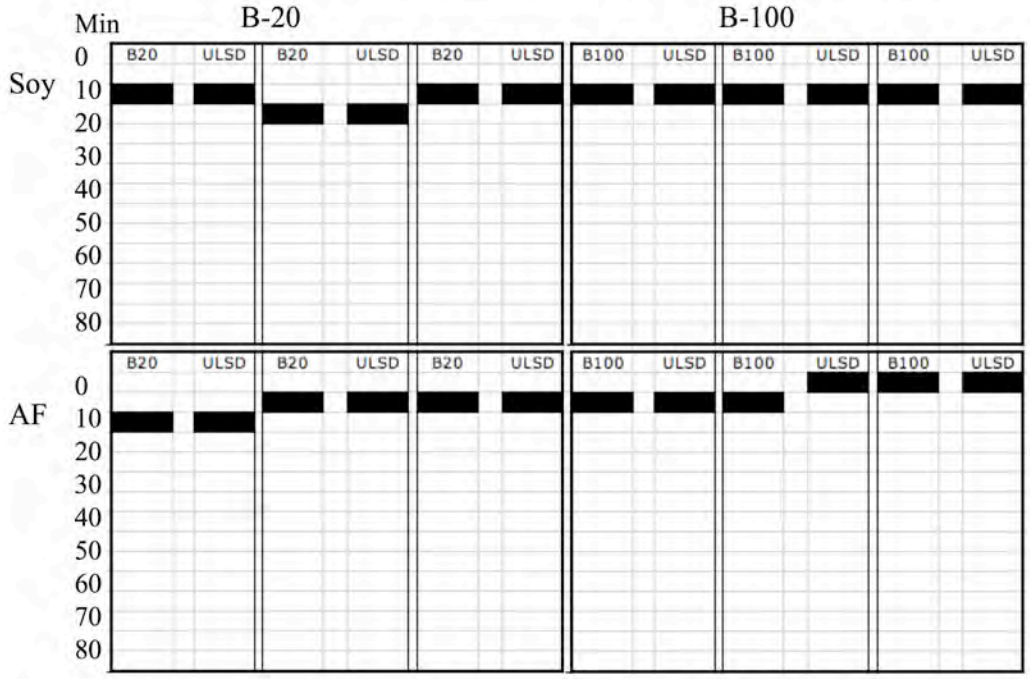

Figure II-C-4: Characteristic times to plume plume separation from the sand surface for each of the four blends (Soy B-20, Soy B-100, Animal Fat (AF) B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

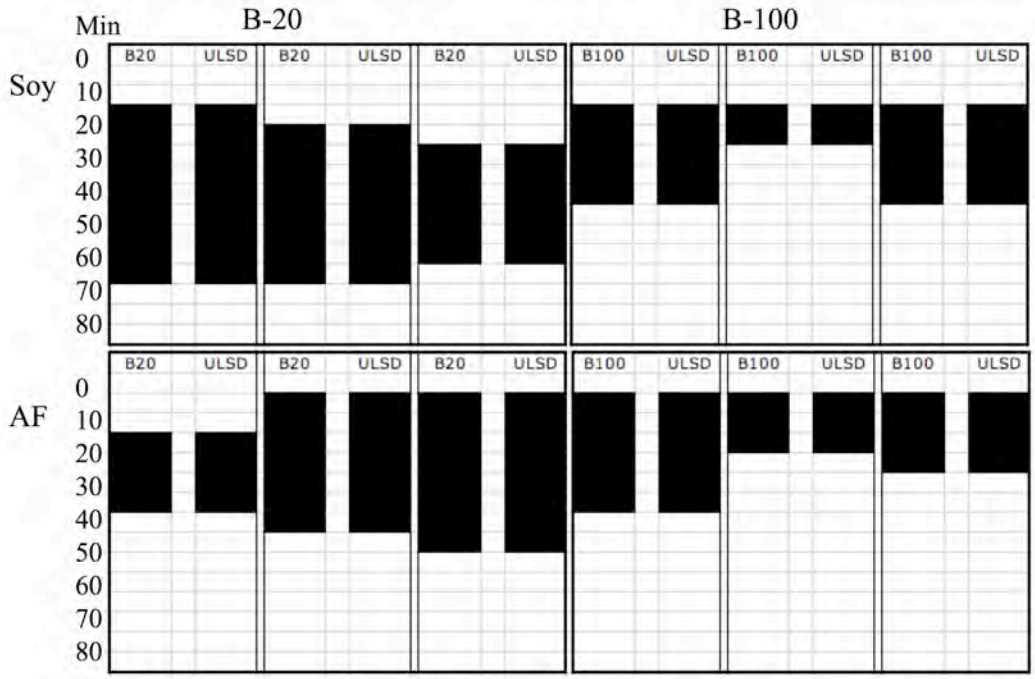

Figure II-C-5: Characteristic times for commencement of lens spreading on the water table for each fuel (Soy B-20, Soy B-100, B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

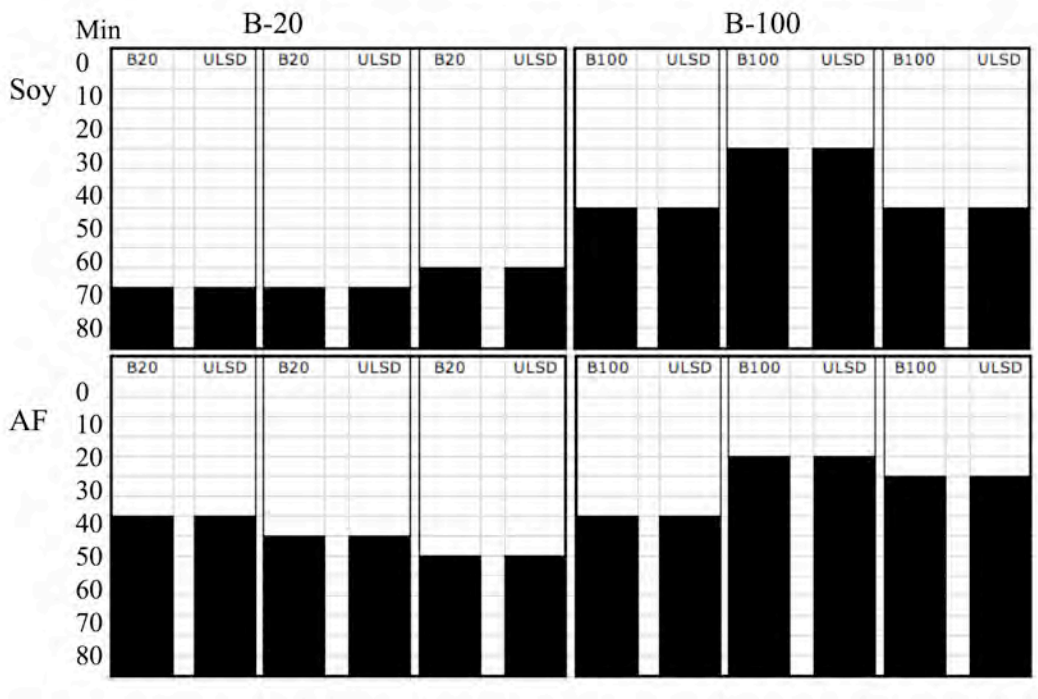

Figure II-C-6: Characteristic times for lens formation on the water table for each of the four blends (Soy B-20, Soy B-100, AF B-20, AF B-100) relative to ULSD in side-by-side comparison. The three columns per fuel blend show the results for each of the three replicates.

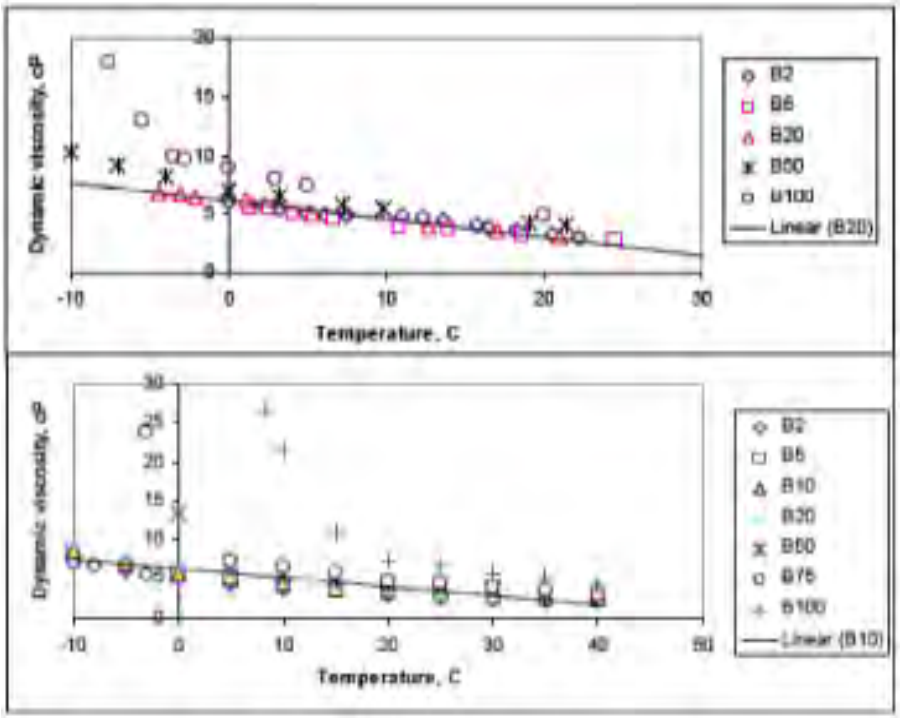

Figure II-C-7. Biodiesel fuel blend (Soy, top; Animal fat, bottom) viscosities for a range of temperatures. Note relatively large increase in Animal Fat B-100 viscosity for temperatures below 20 degrees C. From Yang et al., 2008.

DISCUSSION

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. An important distinguishing characteristic 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).

CONCLUSIONS

- The antioxidant additive did not affect the infiltration of animal fat B-100
- Soy biodiesel blends at both 20 and 100 percent, 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.

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9. Appendix II-D: 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 recommended, 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.

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.

METHODS AND MATERIALS

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 is listed in Table II-D-1 below.

The microcosms were prepared using a 250 mL flask that consists of 190 ml mineral medium, 2g soil (Yolo, silty-loam) as bacterial inoculum and addition of 5µL 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 II-D-1: 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 AA
	Animal fat biodiesel 100% + bioextend	AF B-100
	Soy biodiesel 100% + bioextend	Soy B-100

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 5uL, 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.

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 II-D-1) 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 II-D-1 shows the arrangement of each experiment and code used for each fuel type. The duration of each experiment was 28-30 days.

Figure II-D-1: 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.

Fuel Carbon Content

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

RESULTS AND DISCUSSION

Initial Carbon Content of Fuel Blends

Initial carbon contents for the fuels tested are shown in Table II-D-2. 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 5mL. The volumetric mass densities (data not

shown) range from 0.86 to 1.02g/mL, and the resulting initial carbon contents (last column of Table II-D-2) range from 3.78 to 4.15 for the biofuel blends compared to 4.54 for the ULSD.

Table II-D-2: Carbon content of the 12 biodiesel blends and one 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

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 D1.

$C + O_2 \rightarrow CO_2$ Equation D1

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 II-D-3). 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.

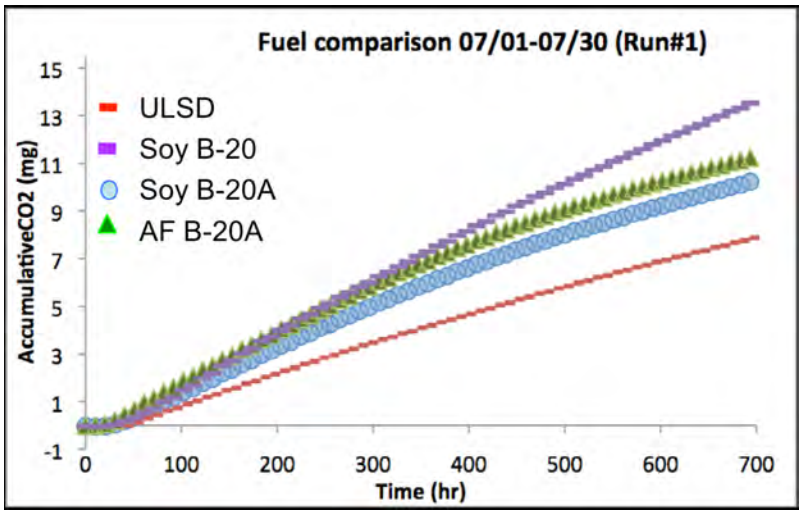
Table II-D-3 – Percent degradation of different fuel types

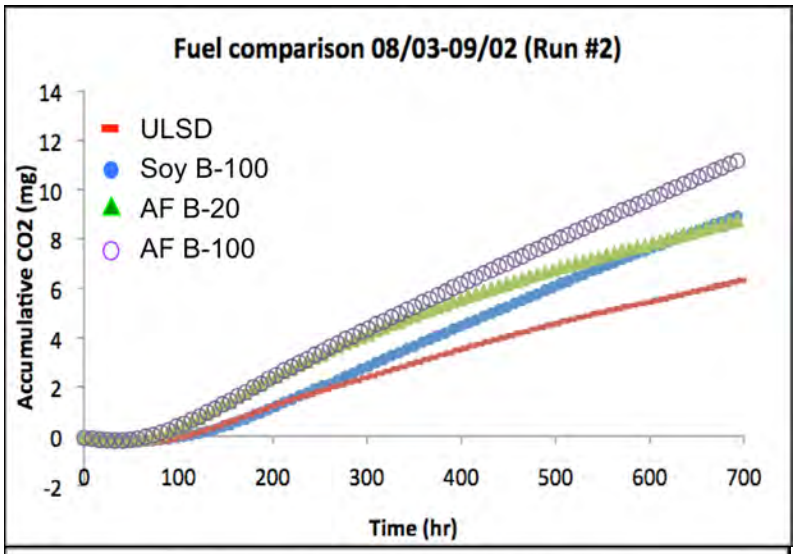
Experiment	Fuel Type	Accumulated CO2 (mg)	Equivalent oxidized carbon (mg)	Percent degradation
#1	ULSD	7.87	2.15	47.40
	Soy B-20 A	10.23	2.80	71.48
	AF B-20 A	11.24	3.07	78.43
	Soy B-20	13.53	3.70	92.40
#2	ULSD	6.37	1.74	38.36
	Soy B-100	9.04	2.47	62.70
	AF B-20	8.83	2.41	58.18
	AF B-100	11.31	3.09	75.99
#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

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 II-D-2 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.

Figure II-D-3 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 vs. Soy biodiesel blends observed at the end of the ~29-day experiments shown in Figure II-D-2.





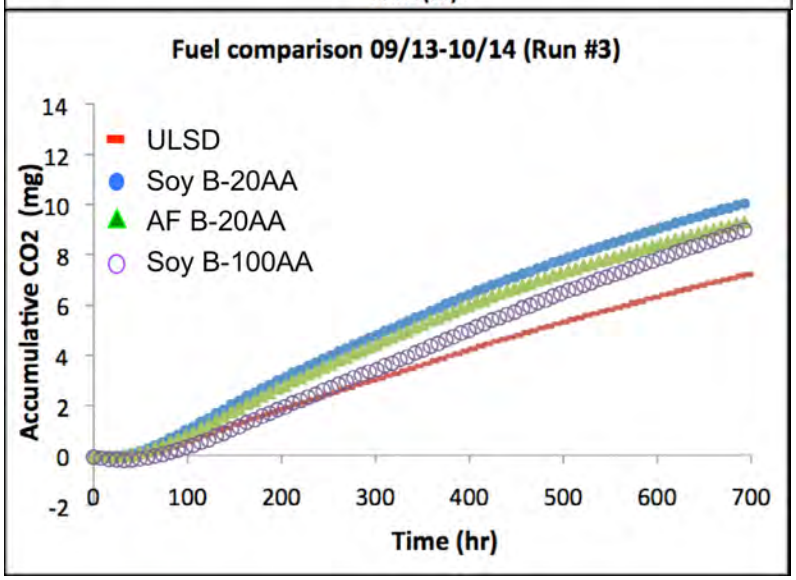


Figure II-D-2. Respirometry data on CO2 production in experimental suites #1 (top), #2 (middle) and #3 (bottom).

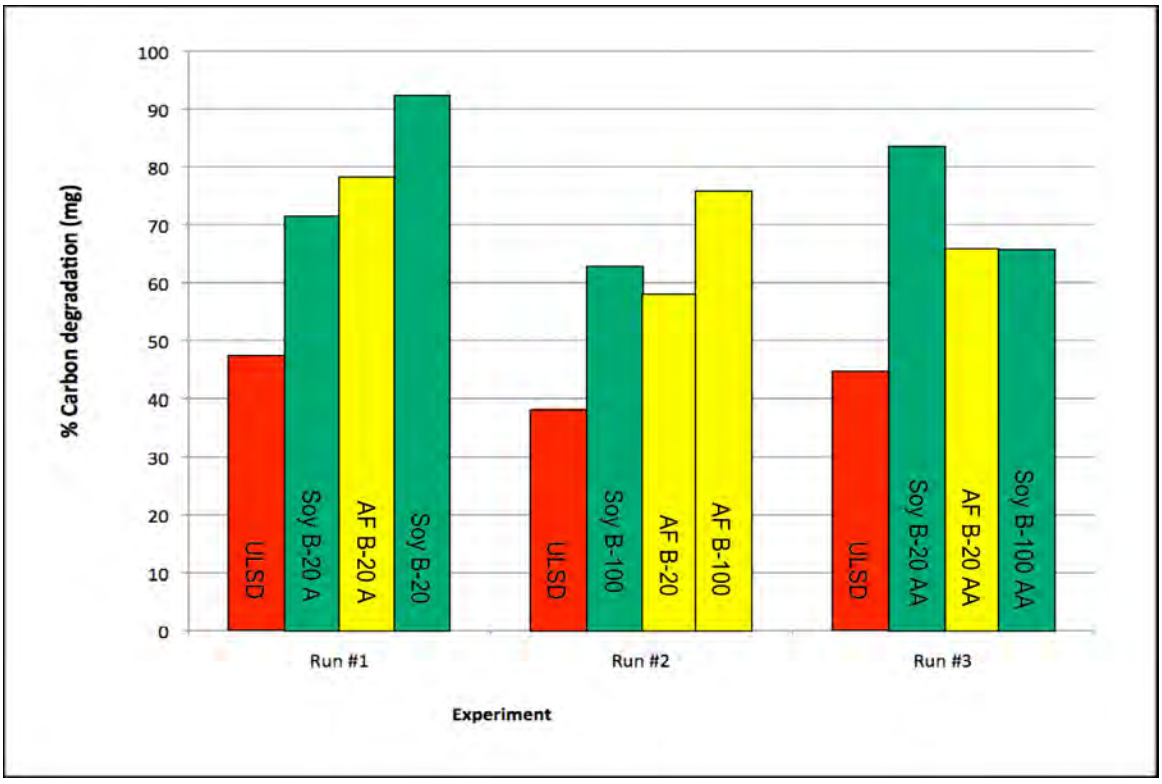


Figure II-D-3: Comparison of percent of carbon biodegradation with different fuel types in microcosm respirometry at the end of the experiments.

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.

CONCLUSIONS

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

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[3] http://ecb.jrc.it/Documents/TECHNICAL_GUIDANCE_DOCUMENT/EDITION_2/tgdpart2_2ed.pdf.

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