

## **Attachment 5**

### **Reviews of Biodiesel Assessment Reports and Supporting Documents (7)**

- a) Edward J. Bouwer, Ph.D. - John Hopkins University
- b) Tracey Holloway, Ph.D. - University of Wisconsin - Madison
- c) An Li, Ph.D. - University of Illinois - Chicago
- d) Stephen Nesnow, Ph.D. - Stephen Nesnow Consulting
- e) Lisa A. Rodenburg, Ph.D. - Rutgers University
- f) Paul White, Ph.D. - University of Ottawa
- g) Xiusheng (Harrison) Yang, Ph.D. - University of Connecticut



January 7, 2014

Gerald W. Bowes, Ph.D.  
Manager, California Environmental Protection Agency  
Scientific Peer Review Program  
Office of Research, Planning, and Performance

Dear Dr. Bowes:

I have reviewed the **Staff Report: Multimedia Evaluation of Biodiesel** including 10 appendices. My expertise is microbial engineering that is applied to biodegradation of organic contaminants, transport and fate of bacteria in soil and aquifers, biofilm reactors, and contaminated sediments. I am providing external scientific peer review comments below mainly for the two sections on Water Evaluation and Soil and Hazardous Waste Evaluation.

**Water Evaluation.** Biodiesel is largely a mixture of fatty acid methyl esters (FAME). The FAME compounds tend to biodegrade at a faster rate than the compounds in CARB diesel. A general tendency is that liquid products from biomass are highly biodegradable under the proper conditions. For example, most liquid petroleum hydrocarbons (e.g., gasoline, diesel, jet fuel, oils, and FAME) can be biodegraded under aerobic conditions by many different species of bacteria. Several of these species of bacteria capable of petroleum hydrocarbon biodegradation are commonly found in rivers, lakes, and oceans and in the subsurface. Consequently, these liquid products tend not to persist for long periods when they are released to the environment. Furthermore, the vadose zone infiltration experiments showed that the vertical and horizontal extent of migration for biodiesel and CARB diesel were similar. The increased biodegradability of biodiesel in comparison to CARB diesel and similar transport properties means there is not an expected increase in risk from the use of biodiesel in comparison to CARB diesel when they come in contact with surface waters or groundwaters. I agree with the conclusion that there are likely to be minimal additional risks to the waters of California from the use of biodiesel.

The one factor that “clouds” the above conclusion is that additives are likely to be introduced in almost all biodiesel blends. These additives address issues of oxidation, corrosion, foaming, cold temperature flow properties, biodegradation during storage, and water separation. As long as the expectation holds that biodiesel will employ additives similar to those used currently in CARB diesel, then it follows that the health and environmental impacts of the two mixtures will be similar. If different additives are employed that might make the biodiesel mixture either more toxic or less biodegradable, then additional studies will need to be conducted to demonstrate the environmental health and safety of the biodiesel mixture planned for use.

**Soil and Hazardous Waste Evaluation.** Essentially, the same analysis provided for the Water Evaluation above applies for this topic. The enhanced biodegradability of biodiesel with FAME compounds in comparison to CARB diesel indicates that there will be less persistence of

biodiesel in the subsurface following releases or spills. Consequently, there is not likely to be an increased risk to the environment with the use of biodiesel. The limited knowledge regarding the additives that will be used for biodiesel does add uncertainty to this conclusion. If such additives are different from the ones used for CARB diesel, then there is potential for the biodiesel mixture to behave differently in the environment, such as increased toxicity or reduced biodegradability. If different additives are used for biodiesel, then additional studies are recommended to properly document the new transport and fate properties.

In addition to the above comments for the major conclusions offered by the Staff Report, I provide following comments on specific sections of the report:

1. The Opening Glossary should contain CARB. The opening section does not define CARB diesel (page 4). CARB diesel is defined later in the report. If a reader starts with the opening section as I did, it will be confusing to not have a definition of CARB diesel up front. In Appendix G, the term “conventional petroleum diesel” or simply “petroleum diesel” is used. I suspect that CARB diesel and conventional petroleum diesel are terms for the same product. The broader community is likely to be more familiar with the term conventional petroleum diesel or petroleum diesel in comparison to CARB diesel.
2. Add CARB to the list of acronyms on page 8 of Appendix A. ARB is listed, but not CARB.
3. In Appendix F on page 2 of the 3-page memo from Donn Diebert, the opening sentence of the last paragraph is unclear. The sentence states that biodiesel appears to react differently in the environment than does CARB diesel. It is not clear if the three characteristics listed before the last paragraph are the main characteristics that are different. The opening sentence should provide more detail on the differences between biodiesel and CARB diesel by either referring to the characteristics listed or adding new characteristics that are most important for the differences. The knowledge about reaction differences is important for assessing the fate in the environment.
4. The bullet point at the top of page xi in Appendix G should mention that there are many bacterial groups indigenous to aquatic systems and soils that are capable of biodegrading biodiesel and petroleum hydrocarbons. This contributes to the high potential for biodegradation in these media.
5. On page 7 in the Appendix G report, there is a discussion that material compatibility is an important consideration. The second paragraph indicates some of the materials that are poorly compatible with biodiesel. It would be helpful to also provide examples of materials that are compatible with biodiesel. This will help educate the reader on materials to avoid along with those that are good to use. The same modifications are recommended to a similar discussion of materials compatibility that appears on page 25 in Appendix G. There is more discussion about materials compatibility in a section labeled “6. Tier III Appendices” within the

Appendix G tab on page I-26. More advice on compatible materials will be helpful. The impression given by this discussion is that most materials can be incompatible. It would be good to conclude with some suggested materials that can be used with high confidence.

6. As acknowledged thoroughly in the report, the presence of additives in the biodiesel is a source of uncertainty for the chemical and physical properties of the biodiesel. There is a statement in the last paragraph on page 24 of Appendix G that “it is reasonable to assume that most of the additives used in biodiesel are currently used in CARB ULSD”. It would be helpful to provide some documentation that this assumption is true. The database might be limited, but are the current stocks of biodiesel using similar additives as CARB ULSD? Any evidence to support this statement will be helpful to support a conclusion that biodiesel is just as acceptable as CARB diesel.
7. In a section labeled “6. Tier III Appendices” within the Appendix G tab, there is a discussion of subsurface fate and transport properties on page I-5. The third sentence makes the statement that the composition of biodiesel differs significantly from that of petroleum diesel. Consequently, the behavior of the two liquids is likely to be different in the environment. It would be helpful if this discussion can include more specifics about the composition differences so it is easier to understand if the biodiesel might be more or less problematic. For example, we learn from the biodegradation studies that biodiesel is more biodegradable than petroleum biodiesel, so this is a positive attribute.
8. In a section labeled “6. Tier III Appendices” within the Appendix G tab, there is a discussion of biodegradation of biodiesel components on page I-55. Near the bottom of page I-55, the observed percentages for degradation are reported with 4 significant figures (e.g., 85.54% degradation). A batch biodegradation test cannot achieve this degree of precision, so it is recommended that the results be rounded to 2 or at most 3 significant figures. More detail needs to be provided for the results shown in Table 6.2 on page I-56 that are used to support the mechanism of co-metabolism. What is the ratio of diesel and biodiesel in the mixture? Was the total mass of diesel and biodiesel in the mixture the same as the mass of diesel alone? Without reporting the initial masses or concentrations, it is not clear from the reported data if more mass of diesel biodegraded in the presence of the biodiesel. Finally, there is a statement in the middle of page I-56 that microorganisms metabolize biodiesel and diesel at roughly the same rates. This statement is inconsistent with the reported data on page I-55 that after 28 days, the biodiesel exhibited 85 to 88% removal in comparison to 26% removal for petroleum diesel. The biodegradation rate for biodiesel is markedly faster than for petroleum diesel. The results shown in Table 6.3 also support this conclusion that biodegradation rates are faster for biodiesel.
9. Section 6.4.3 on page I-57 is called Biodegradation Under Aerobic and Anaerobic Conditions. It is better to explicitly mention the electron acceptor involved rather

than to call the conditions “anaerobic”. For example, the situation with nitrate as the electron acceptor can be called nitrate respiration or denitrification. It is difficult to generalize from denitrification to all anaerobic conditions. The data on biodegradation for a wide range of anaerobic conditions are certainly sparse.

10. The Table II-D-3 on page II-84 within the Appendix G tab seems to arbitrarily show all of the data with two decimal points. This means that some of the percent degradation numbers are shown with 5 significant figures (e.g., 125.42). This level of precision is not possible for batch biodegradation testing, so the numbers should be reported with 2 or at most 3 significant figures. Furthermore, the last three tests have percent degradation values greater than 100%. The sources for this error should be discussed in the text.
11. Typos: Appendix G, page x: line 2 from the bottom: “compare” should be “compared”. Same error on page 11, line 4 in the second full paragraph. On page 25 of Appendix G, “alliviate” should be “alleviate” in the second to last line of the first full paragraph. On page 27 of Appendix G, “volitalize” should be “volatilize”. On page I-73 of Appendix G, “month” should be “months” in line 7 from the bottom. On page II-3 of Appendix G, “biodegradable” should be “biodegradable” in line 13 from the top. On page II-3 of Appendix G, “test” should be “tests” in line 2 from the bottom. On page II-17 of Appendix G, “without algae inoculate” should be “without an algae inoculum” in line 6 in the middle paragraph. On page II-20 of Appendix G, the caption for Figure II-A-1 is incomplete (i.e., it looks like some text is missing or is cutoff). On page II-32 of Appendix G, there is a statement about an error with the reference cited in the last line of text. The same error occurs on the next page II-33 in line 18 from the top. On page II-87 of Appendix G, “biodegradability” should be “biodegradability” in the second bullet in the middle of the page.

Sincerely,



Edward J. Bouwer, Ph.D.  
Abel Wolman Professor of Environmental Engineering  
Department Chair

## *Review of*

### "Staff Report: Multimedia Evaluation of Biodiesel" Prepared by the Multimedia Working Group

*Tracey Holloway, Ph.D.  
University of Wisconsin--Madison*

The California Air Resources Board (ARB) is proposing the development of new regulation for biodiesel. Biodiesel is considered a potentially desirable fuel alternative, given the lower carbon intensity relative to petroleum diesel fuel and possible other benefits. In this report, all conclusions about biodiesel are given relative to diesel fuel meeting ARB specifications, referred to in the report as "CARB diesel."

This review follows the topical areas of the MMWG report:

#### 1. Biodiesel

Overall, the conclusions of the staff report are supported by the California Biodiesel Multimedia Evaluation (Final Tier I, II, and III reports) from researchers at UC Davis and UC Berkeley. In particular, the major conclusion that biodiesel use "does not pose a significant adverse impact on public health or the environment relative to diesel fuel" is in line with the findings of the Multimedia Evaluation.

The impacts of biodiesel relative to CARB diesel depend strongly on the percentage blend of petroleum diesel with biodiesel. However, the treatment of these categories and terminology is inconsistent through the report. For example:

- p. 4 introduces four categories of blending: B10, B20, B50, and B100 (where B10 = a 10% by volume blending of biodiesel with CARB diesel; B20, 20% blending, and so on).
- p. 8 report emissions for B5 blends, but not B10;
- p. 11 discusses B5 in the context of underground storage tanks (UST);
- Appendix A p. 4 defines only B5 and B20, as follows "(6) 'B5' means a biodiesel blend containing no more than five percent biodiesel by volume" and "(7) 'B20' means a biodiesel blend containing more than five and up to 20 percent biodiesel by volume." In this definition, both B10 and B20 would fall into the B20 category.
- Appendix A p. 5 defines "(8) 'CARB Diesel fuel' means ... which may be comingled with up to five (5) volume percent biodiesel..."Combining these definitions, B5 and CARB Diesel both have between 0 and 5 percent biodiesel by volume mixed with petroleum diesel meeting ARB standards.

The proposed regulation order and report would be improved by clearly defining the terms, especially clarifying whether B5 means a 5% blend of biodiesel, or a range from 0-5%

biodiesel, or some other range. Similarly, whether B20 means a 20% blend of biodiesel, or a range from 6-20% of biodiesel, or some other range.

#### a. Air Emissions Evaluation

The conclusion of "the use of biodiesel does not pose a significant adverse impact on public health or the environment from potential air quality impacts" is supported by the Multimedia Evaluation and discussion in the MMWG staff report. This conclusion is based on an analysis of criteria pollutant emissions (including ozone precursor emissions), toxic air emissions, and greenhouse gas emissions. Conclusions are drawn primarily from emission tests conducted at UC Riverside and at ARB test facilities. All types of emissions decrease except NO<sub>x</sub>, and even then only in heavy-duty vehicles that do not meet newer emissions standards.

Overall, the findings of the air emissions evaluation are well supported. However, the discussion of results could be improved in a few respects. These are noted below.

Section 1. (p. 7) is labeled "Criteria Pollutants." This section should begin with a discussion of what pollutants fall into this category, and which are evaluated here for biodiesel. Currently, this information is provided on p. 8, paragraph 2. However, this overview would be more helpful at the beginning of the section.

As written, Section 1 includes PM, nitrogen oxides (NO<sub>x</sub> = NO + NO<sub>2</sub>), total hydrocarbons (THC), carbon monoxide (CO) and carbon dioxide (CO<sub>2</sub>). However, THC and CO<sub>2</sub> are not criteria pollutants and do not belong in this section. SO<sub>2</sub> is a criteria pollutant that is not discussed here, but which may be reduced (per EPA<sup>1</sup>) by substituting biodiesel for petroleum diesel. NO<sub>x</sub> includes both the criteria pollutant NO<sub>2</sub> as well as NO, which is not a criteria pollutant. It would be helpful to know the size distribution of the PM emissions, for consistency with the criteria emissions categories of PM<sub>2.5</sub> and PM<sub>10</sub>. Section 1 should report on all criteria pollutant emissions (or precursor emissions) in some way, and omit discussion of emissions that are not criteria pollutants.

Details are provided on the test vehicles used for emission tests (p. 7-8). It would be helpful to know how these were selected, and whether they are typical of the California vehicle fleet.

As noted, discussion of CO<sub>2</sub> emissions should be removed from Section 1, because CO<sub>2</sub> is not a criteria pollutant. It would fit more clearly in Section 4 (p. 10) on Greenhouse Gas Emissions. In addition, this section should be edited to clarify that the measured increase in CO<sub>2</sub> emissions does not suggest that biodiesel leads to a net increase in carbon emissions. It may be useful to note that a) end-of-pipe CO<sub>2</sub> emissions are only one component in determining a fuel's lifecycle carbon emissions (including uptake by feedstocks); b) an increase in CO<sub>2</sub> reflects more complete combustion, and is an expected result of decreased THC and CO emissions; c) the vast majority of THC and CO convert to CO<sub>2</sub> in the atmosphere, so the total CO<sub>2</sub> produced by the biodiesel combustion process is determined by direct CO<sub>2</sub> emissions, as well as THC and CO. As written, the discussion of CO<sub>2</sub> emissions could be misleading and a source of potential confusion.

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<sup>1</sup> <http://nepis.epa.gov/Adobe/PDF/P1009IYE.pdf>

Section 2 (p. 9) discusses "Toxic Air Contaminants." The discussion notes that the reduction in PM emissions would be expected to decrease toxic risk from diesel PM. This is a reasonable conclusion. In addition, some discussion should be included on the PM speciation from biodiesel versus petroleum diesel.

Section 3 (p. 9) discusses "Ozone Precursors." Because ozone is a criteria pollutant, this section would seem to be a better fit with Section 1 and/or follow directly afterward. For the benefit of readers unfamiliar with ozone chemistry, some brief comment should be added explaining that THC and NO<sub>x</sub> emissions determine ozone concentrations.

As written, Section 3 only discusses one ozone precursor: NO<sub>x</sub>. At a minimum, it should include both THC and NO<sub>x</sub>. Because THC is not a criteria pollutant, the discussion of THC from Section 1 would fit better here. Furthermore, the expected ozone impacts of THC reductions and NO<sub>x</sub> increases deserve some discussion. It may be beyond the scope of this report to comment on the expected ozone response to these competing precursor sensitivities. However, some qualitative comment would be helpful to frame the importance of the THC and NO<sub>x</sub> response to biodiesel.

Section 4 (p. 10) reports on Greenhouse Gas Emissions. This section would benefit from a number of changes. First, clarifying which greenhouse gas emissions have been evaluated - it appears only CO<sub>2</sub>. As noted above, the CO<sub>2</sub> discussion from Section 1 should be moved to Section 4. The discussion notes an increase in fuel consumption due to the lower energy density of biodiesel. However, this analysis is of limited value, given that the fuel consumption impacts are given quantitatively, whereas the energy density changes are given only qualitatively. It would be helpful to include a more appropriate metric to compare the net CO<sub>2</sub> emissions from vehicle operation with CARB diesel versus biodiesel.

The difference between end-of-pipe emissions and life-cycle emissions should be more clearly defined in section 4. Overall, the paragraph (p. 10) discussing lifecycle emissions is unclear. It would benefit from more detail on what steps in the lifecycle were considered. In addition, it would be helpful to note that the 95% reduction in GHG emissions would arise from waste-oil feedstock use, whereas the 15% reduction in GHG emissions would arise from soybean production in the Midwestern U.S.

## b. Water Evaluation

Overall, the MMWG conclusion that "there are minimal additional risks to beneficial uses of California waters posed by biodiesel" is well supported. However, the summary presentation of study findings could be clarified on a few points.

1. Water impacts (p. 11). There are two main impacts discussed in this section: aquatic toxicity, where there are results, and agricultural impacts, where there are no results from the current multimedia review. It would be helpful to break these two topics into separate paragraphs. More detail should be provided on the toxicity findings from the multimedia evaluation. Similarly, Section 3 would benefit from more detail clarifying issues related to biodegradability. Sections 2 and 4 seem to have an appropriate level of detail for the topic.

### c. Public Health Evaluation

Overall, the public health evaluation seemed to be redundant with the air emissions evaluation, and lacking any specific discussion of health impacts. The public health conclusions are supported, in that Section 1 ("Combustion Emissions") summarizes the same changes in emissions presented in the Air Evaluation (p. 7-9). However, the report would be strengthened with a clearer discussion of health impacts.

At a minimum, the public health evaluation should address the conclusions on both air and water impacts in terms of health outcomes. For example, discussing the health outcomes of the PM reductions - both in terms of acute effects and toxicity - on exposed populations.

Section 2 (p. 13), entitled "Impact on Atmospheric Carbon Dioxide" does not have clear health linkages discussed. Overall, this explanation is unclear. Topically the material fits better in Section A4 where greenhouse emissions and lifecycle impacts are discussed.

### d. Soil and Hazardous Waste Evaluation

Hazardous waste is outside the expertise of this reviewer. However, the discussion overall was clearly presented and seemed consistent with findings from the Multimedia Evaluation. It would seem appropriate, however, to define the term "vadose zone infiltration."

## Review Comments

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Submitted to Dr. Gerald W. Bowes ([Gerald.Bowes@waterboards.ca.gov](mailto:Gerald.Bowes@waterboards.ca.gov)) on January 7, 2014

**Document Reviewed:** Staff Report: Multimedia Evaluation of Biodiesel by the Multimedia Working Group (MMWG), California Environmental Protection Agency (CEPA), November 2013

**Topic/Area Reviewed:** Surface and Ground Water Quality

The document reviewed here is a Staff Report prepared by MMWG of CEPA for the California Environmental Policy Council (CEPC), which will determine whether the proposed regulation on commercialization of new alternative diesel fuels poses significant adverse impact on public health or the environment. This is part of the process towards legally accepting and commercializing alternative diesel fuels in California.

The assignment to this reviewer is to help determine whether the scientific portions, particularly in the water quality section, of the MMWG Staff Report are based upon sound scientific knowledge, methods, and practice. The sections regarding water quality impacts were written based on the report from the State Water Resources Control Board (SWRCB) (Appendix D). The scientific knowledge is provided primarily in the Final Tiers I, II, and III Reports (Appendix G).

I have read the main Staff Report and its Appendices A, D, and G. I consider the tiered multimedia evaluation well designed, and the Tiers Reports (Appendix G) were well written. Tier I provided an excellent review of the key knowledge gaps through literature search, and presented a very good work plan. In Tier II, laboratory experiments were conducted in aquatic toxicity, transport in porous media, and aerobic biodegradation. CARB also conducted engine and chassis emission tests showing reductions for most air pollutants, demonstrating the major advantage of alternative diesel over petroleum based CARB diesel. All experimental results are highly valuable and the findings can be far-reaching, although some may be considered preliminary or screening in nature. However, constraints in time and budget prevented the experiments on materials compatibility and aqueous solubility; both of which are highly important to water quality impact evaluation. Tier III is a summary of all the work with qualitative risk assessment in some sections. A quantitative risk assessment and a full life cycle analysis may be difficult at this stage due to the lack of needed data. The Proposed Regulation Order (Appendix A) specifies the stages for commercializing new alternative diesel fuels; its implementation would further ensure that the impacts on the ecological environment and public health progressively change in a positive direction.

Provided below are my Overall comments, Comments on water quality impact assessment, and Document specific minor comments.

### Overall Comments

1. Within the scopes of my review and my expertise, I do not found major flaws in the scientific knowledge, methods, and practice presented in the main Staff Report and its Appendices A, D and G.

2. Large scale use of pure biodiesel (B100), as well as diesel blends with >20% B100, is considered premature at present, given the current knowledge gaps and uncertainties in several key areas.
3. In the main Staff Report, section I-C, I suggest summarizing the limitations of this multimedia evaluation immediately following the major MMWG conclusion on page 6. Some limitations are well described in the Tiers Reports, but are absent in the Staff Report. The limitations are different from the conditions in the Recommendations (page 17).
4. In contrast to the general positive impact on air quality due to reduced direct air emissions, the effects of switching to biodiesel on natural waters could be adverse and extensive. Most of the priority issues identified in Tier I Conclusions are related to water quality, including additives impacts, subsurface fate and transport, biodegradation, production and storage release. Unfortunately, these issues were not sufficiently investigated during Tier II experimental test stage. This leads to high uncertainty in making conclusions on the impact of using biodiesel on water quality. I consider this is the major weakness of this multimedia evaluation. Other regulations (such as the laws and regulations on underground storage tank and the hazardous waste, as mentioned in main Staff Report, section II-B) will help prevent water pollution; but they are not relevant to scientific assessment of biodiesel impact on surface and ground water quality.
5. Additives impacts remain a top concern. Additives, particularly those needed for biodiesel, are neither defined nor emphasized in the Proposed Regulation Order (Appendix A). Tier III assessment suggests no substantive change in additive impact in the case of B20, based on the expectation that most currently used additives would continue to be used (Tier III Report, page viii, 1<sup>st</sup> paragraph). Does this mean that no new additives will be used in new fuels covered by the proposed regulation? Given the needs of adding additives to biodiesel to control oxidation, corrosion, degradation, NO<sub>x</sub> formation and others as well as cetane value enhancement, there seems a disconnection between the findings of the Tiers conclusions and the proposed regulation.
6. The assessment of the supply and demand is not within the scope of this multimedia assessment. According to Hill et al. (2006), even dedicating all U.S. corn and soybean production to biofuels would meet only 12% of the gasoline and 6% of diesel demands in the country. Even with B20 or lower blends, whether all the available resources would meet the demand is unclear.
7. Biodiesel and renewable diesel were assessed separately. The advantages of each over the other were not quantitatively or qualitatively compared. According to UOP (2005), renewable diesel has a lower environmental impact than biodiesel and requires less capital investment to produce. This is in agreement with what I learned from reading the documents provided. However, I failed to find answers to the questions whether biodiesel is indeed needed and why biodiesel is being proposed as the first alternative diesel fuel in California, given the apparent advantages of the renewable diesel.
8. Tier-I Report, page I-20, is the only section about algae as a feedstock, and the discussion is highly positive. It is not clear what type of algae is relevant to biodiesel production. Given that California has long ocean shorelines, are there brackish water resources suitable for algae production? Are there any foreseen adverse impacts, besides the limitations associated with a narrow range of growing and harvesting conditions?
9. In the near future, the major feedstock could be soybeans grown in the US Midwest, where most adverse impact will occur. Although a complete evaluation of the impact outside California is beyond this work, a summary of available information on the impacts of the upstream processes (feedstock production, extraction, blending, etc.) on the environment and human health could have been included.
10. No occupational exposure and risk of any sort are included in this multimedia evaluation.

## Comments on Surface and Ground Water Quality Assessment

1. Please see my overall comments 4 and 5 above.
2. In the main Staff Report, the conclusion on water quality impact (page 16, part B) needs to be more specific. The current version is not consistent with summary section II-B, which indicates an increase in toxicity in part 1 and decreased biodegradation in part 3. It is not clear how the results summarized in section II-B lead to a conclusion of “minimal additional risks” in the Conclusions. In addition, the last several words should be changed from “public health or the environment” to “the quality of surface water and groundwater in California”.
3. The incompatibility of biodiesel with underground storage tank (UST) as well as other infrastructure equipment calls for work plans needed in the cases of leaks into groundwater. Merely requiring affirmative statements of compatibility from biodiesel manufacturers and lead detection (main Staff Report, page 11, part 2) does not seem sufficient to ensure no adverse impact on groundwater.
4. Main Staff Report, page 11, part 4 indicates no significant areas of concerns when comparing biodiesel and CARB diesel with regard to waste discharges from manufacturing. This may not be correct because the manufacturing processes and chemical compositions are completely different between biodiesel and petroleum based CARB diesel, as detailed in the Tier I Report. The transportation and distribution may also differ between the two. Many chemicals are reviewed in Tier I Report, including acid and base as catalysts, various additives, etc. Not mentioned in Tier I Report and elsewhere is the possible incidental environmental release of glycerin, which is the major by-product of biodiesel production, and it is known to disrupt the microbial cleaning processes used in wastewater treatment (GAO, 2009) and has caused discharge problems (NYT, 2008).
5. NOx mitigating agent di-tert-butyl peroxide (DTBP) is the only additive included in Proposed Regulation Order (Appendix A, page 26). It is not clear whether this chemical has been used among the currently in-use additives added to CARB Diesel, or it is a new additive for new diesel fuels. Information on the basic physicochemical properties, environmental behavior, and the potential impacts of DTBP are not found in this multimedia evaluation.
6. Potentially positive impacts on water quality, if appropriate, could be mentioned somewhere in these documents. For example, plant feedstock production may help prevent soil erosion, remediate contaminated sites, build wetland and prairie, etc.

## Document Specific Minor Comments

### Main Report (19 pages)

Table of Contents: I suggest changing II title from “Summary” to “Section Summaries” or “Summaries of Reports from Participating State Agencies”, in order to avoid confusion with the summary of this Main Report.

Page 1, section A: There are three bulleted lines for air, water and wastes, respectively. It is not clear why public health is not included here. Risk assessment on the public health focuses on human, in contrast to those on environmental media. The same can be said for the bulleted lines in Page 2, section 2.

Page 5, section C: I suggest including one brief sentence on line 4 indicating that CARB diesel is conventional petroleum based ultra-low sulfur diesel, along with a brief time line. One or more references

should be helpful, directing readers to information on CARB diesel development and adoption, quantity of use in the state, its environmental and human health impacts, etc. This is especially helpful to stakeholders and interested parties who reside outside California and are unfamiliar with the phrase "CARB diesel".

Page 10, first paragraph, ending phrase: The words "and fuels" are confusing to me.

#### Appendix A – Proposed Regulation Order (36 page)

Page 4, (a), (1): If ADF means any non-CARB diesel fuel that does not consist solely of hydrocarbons, a question arises whether "renewable diesel" as defined in the 3-tier multimedia evaluation is an ADF. The renewable diesel, to my understanding, consists of predominantly hydrocarbons.

Page 5, (8): The definition for "CARB Diesel fuel" in this proposed regulation appears different from that for "CARB Diesel" used in the 3-tier multimedia evaluation. The former includes 5%v of FAME, while the latter is a pure ultra-low-sulfur diesel (ULSD) derived from petroleum.

Page 22, top lines: The definition of NBV is repeated.

Page 22, Table A.2. "Limit" column: The sign " $\geq$ " for both total aromatics and polycyclic aromatic hydrocarbons could be " $\leq$ ".

Page 30, Table A.9, column "fuel Specifications", row 4 for PAHs w%: The 10% maximum seems incorrect for PAHs in a reference fuel. Please check.

#### Appendix D – SWRCB Submittal (5 pages)

Relevant to this review is Attachment #1 (2 pages).

Most part of Attachment #1 is the same as presented in the main Staff Report. Thus, same comments as explained above are applicable.

#### Appendix G – Final Tier III Report (31 pages)

Page vi, 9<sup>th</sup> line from bottom: There is an extra "that".

Page x, line 12: There is an extra "from".

Page x, line 21: Should the word "transport" be "transportation"?

Page 17, line 10 from bottom: Is "~10 cm" correct? Given in Tier II Report, page II-11, line 3 is "~20 cm".

Page 17, line 9 from bottom: "Bioextent" should be "Bioextent-30".

Page 18: At the end of section 3.2, it is helpful to add the environmental and remediation implications of the lens geometry from AF B100, as it is different from others.

Pages 21-29: Section 4 Conclusions has substantial overlap with the Executive Summary on pages iv – xi, therefore reads redundant.

#### Appendix G, Appendix – Tier I Report (94 pages)

I found this Report is of high quality. It is comprehensive and has sufficient details and depth. It is easy to read and has little redundancy.

A summary of the history and the current status of alternative diesels in California would be very helpful but is not found.

Page I-33, line 4 from bottom: The word "centane" should be "cetane", and a period is needed at the end.

Page I-39, first paragraph in 4.4.3, line 3: The word "that" might be "of".

Page I-39, line 3 from bottom: The word “course” could be “coarse”.

#### Appendix G, Appendix – Tier II Report (87 pages)

Page Pages II-29 to II-68 – Chemical Analysis: This work aimed at discovering the compounds responsible for the increased toxicity. It was a difficult task, and the methods using stir bar sorptive extraction (SBSE) and GCMS appear appropriate. From the multimedia evaluation perspective, the results from this work are considered by this reviewer as screening in nature. Future work is needed, and it would be more efficient to focus on the additives, based on Figure 1 (page II-9) and the toxicity test (page II-15 to II-28) which suggested strong impact of the additives on toxicity.

Page II-9, bottom two lines: I suggest rewriting the names of the antioxidant additives. They appear as 4 separate ones, but were just two. For acetic acid butyl ester, please delete the comma after acid, or simply change to butyl acetate. For 1,4-Benzenediol, 2-(1,1-dimethylethyl), the name tert-butylhydroquinone (TBHQ) is less confusing.

Page II-10, first two paragraphs: The results from GCMS for the additives are highly variable, resulting in high uncertainty or failure in identifying the source of increased toxicity. I doubt the SBSE extraction efficiencies for these two compounds, especially acetic acid butyl ester which has a log Kow of only 1.8 (EPI, 2013).

Page II-77, line 5: Figure B7 should be Figure II-C-7.

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Peer Review of Staff Report: Multimedia Evaluation of Biodiesel (Biodiesel Staff Report)

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January 6, 2014

## 1. Preface

The purpose of this document is to review The Staff Report: Multimedia Evaluation of Biodiesel to determine whether the scientific portions of the MMWG staff report is based upon “sound scientific knowledge, methods, and practices.” The Staff Report: Multimedia Evaluation of Biodiesel is based on three previous documents California Biodiesel Multimedia Evaluation Final Tier I, II and III Reports that contain data and analyses from government reports, literature documents, and from reports of studies commissioned by the CARB.

## 2. General comments

Emissions from diesel fueled engines are a complex mixture consisting of both gaseous and particulate components. The gaseous phase contains ozone, sulfur oxides and the criteria pollutants, carbon monoxide, particulate matter, nitrogen dioxide and ozone. Many organic compounds are also present, such as acetaldehyde, acrolein, benzene, 1,3-butadiene, ethylbenzene, formaldehyde, polycyclic aromatic hydrocarbons and nitro-polycyclic aromatic hydrocarbons. Particulate matter, benzene, 1,3-butadiene, formaldehyde and benzo[a]pyrene are carcinogenic in experimental animals and are classified as human carcinogens and acetaldehyde, ethylbenzene and a number of other polycyclic aromatic hydrocarbons and nitro-polycyclic aromatic hydrocarbons have been classified as probably or possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC, 2013). The particulate phase also contains trace metals such as lead, manganese arsenic and chromium and metals from the catalyst after treatment system, vanadium, copper and iron. Arsenic and arsenic inorganic compounds and chromium VI are classified as human carcinogens by IARC while lead and inorganic lead compounds as classified as probably or possibly carcinogenic to human, respectively by IARC. Moreover, diesel engine exhaust, diesel exhaust particles, diesel-exhaust condensates, and organic solvent extracts of diesel-engine exhaust were genotoxic. Increases in bulky DNA adducts were detected the lung tissues of rodents exposed to whole diesel exhaust and in workers exposed to diesel exhaust. In addition to lung cancer, diesel exhaust exposure in humans has been linked to lung inflammation, cardiovascular disease and cardiopulmonary disease (Madden et al., 2011).

The biological and toxicological information available for biodiesel emissions are very limited compared to the rich compendium available for diesel emissions and many of the biological and toxicological measures available for conventional diesel are not available for biodiesel. Therefore, surrogate measures need to be employed to make meaningful comparisons between the emission types. These measures include chemical and physical analyses of the biodiesel emissions and to a small extent some toxicological data on the biodiesel emissions.

The Staff Report bases the comparisons (chemical, physical and toxicological) of the biodiesel fuel emissions to those properties of CARB diesel emissions. The crux of the document’s conclusion is that the selected parameters (chemical, physical and toxicological) examined were lower (with some exceptions) in emissions from engines fueled with biodiesel compared to CARB diesel with the exception of oxides of nitrogen and acrolein that had higher levels. Thus, the public health risk would not be greater than that already established for CARB diesel. The underlying premise is that lower levels of

specific emissions will equate to lower human health risk or adverse health effects. This premise is generally consistent with the quantitative results from many studies in animals and in human populations of each of the specific constituent within diesel exhaust emissions as well as studies in animals and human populations exposed to whole diesel exhaust emissions. Much of the data on emissions from the combustion of biodiesel is from quantitative chemical analysis and that is used to equate to lower toxic or adverse effects in exposed humans. The agents selected for comparison are from the group of EPA criteria pollutants and from selected VOCs commonly found in diesel exhaust and in ambient air. Each exhibits its own toxicity profile. There are few studies in whole animals exposed to complete exhaust emissions and a number of toxicological evaluations of the particulate matter and of organic extracts of particulates. There are no studies that I know of in humans exposed to complete exhaust emissions from biodiesel. Genotoxicity evaluations for the most part are based on organic extracts of particulates using bacterial tests for mutagenic activity; some evaluations were conducted with the vapor phase fraction. Some genotoxicity data in mammalian cells in culture are also available.

**The MMWG concludes that the use of biodiesel fuel in California, as specified in the biodiesel multimedia evaluation, does not pose a significant adverse impact on public health or the environment relative to diesel fuel meeting Air Resources Board (ARB) motor vehicle diesel fuel specifications (CARB diesel).**

Based on the results of the biodiesel multimedia evaluation and the information provided in the University of California (UC) final report, "California Biodiesel Multimedia Evaluation Final Tier III Report" (Ginn, T.R. *et al.*, May 2013), the MMWG makes the overall conclusion that biodiesel specifically evaluated within the scope of the biodiesel multimedia evaluation will not cause a significant adverse impact on public health or the environment relative to CARB diesel. The MMWG based their conclusion on each individual agency's assessment of the biodiesel multimedia evaluation. (Biodiesel Staff Report, Chapter 3)

**Public Health Evaluation. Office of Environmental Health Hazard Assessment (OEHHA) staff concludes that the substitution of biodiesel for CARB diesel reduces the rate of addition of carbon dioxide to the atmosphere and reduces the amount of particulate matter (PM), benzene, ethyl benzene, and polycyclic aromatic hydrocarbons (PAHs) released into the atmosphere, but may increase emissions of oxides of nitrogen (NO<sub>x</sub>) and acrolein for certain blends.** OEHHA staff evaluated potential human health impacts from the use of biodiesel and made conclusions based on their analysis of potential impacts on atmospheric carbon dioxide and combustion emissions results. (Biodiesel Staff Report, Chapter 2 and 3).

### 3. Peer review of the scientific issues

The basic premise of the conclusion: "that biodiesel specifically evaluated within the scope of the biodiesel multimedia evaluation will not cause a significant adverse impact on public health or the environment relative to CARB diesel" is based in large part on the measurements of the levels of key toxic components of emissions from biodiesel and CARB diesel and to a lesser degree on some toxicological measurements of these emissions.

Some of the issues of concern include: Are the metrics used to compare the levels and toxicity of individual or groups of pollutants of biodiesel to CARB diesel appropriate, relevant, specific, sensitive and accurate?; Are the CARB biodiesel results consistent with those reported by others in the literature?; Are all of most toxic components known to be present in diesel exhaust being measured in the CARB biodiesel studies?; Are the proportions of PAH and/or N-PAH the same?; Are the selected indicators of adverse human health accurate and comprehensive?; Are there additional markers that could be included?

Carbon dioxide, a major greenhouse gas is generated through the combustion of both diesel and biodiesel fuels. However, the plant feedstock used in the production of biomass fixes carbon dioxide from the atmosphere through photosynthesis thus recycling the carbon pool. This process does not occur using petroleum derived diesel fuel. There seems to be little difference in the levels of carbon dioxide exhaust emissions between biodiesel and diesel. However, the carbon dioxide released from biodiesel combustion is offset by the carbon dioxide incorporated in the plant feed stock. A National Renewable Energy Laboratory/U.S. Department of Energy life cycle study showed that the production and use of biodiesel fuel using urban buses, resulted in a 78.5% reduction in carbon dioxide emissions compared to carbon dioxide emissions from conventional diesel fuel emissions (NREL, 1998). This is within the range cited in the Report.

The Public Health Evaluation conclusion that the use of biodiesel compared to CARB diesel reduces the amount of particulate matter and polycyclic aromatic hydrocarbons released into the atmosphere, but may increase emissions of oxides of nitrogen ( $\text{NO}_x$ ) is supported by studies described in the Staff Report based on the California Biodiesel Multimedia Evaluation Final Tier III Report, a number of government-conducted studies as well as studies reported in the open literature. The average emission impacts in terms of particulate matter levels of biodiesel compared to conventional diesel for heavy-duty highway engines were reduced by increasing levels of biodiesel, while oxides of nitrogen levels increased slightly (EPA, 2002). The literature on the levels of combustion emissions from biodiesel fueled engines compared to those from diesel fueled engines in the range of B5 to B100 was recently reviewed (Bünger et al., 2012). They reported that in most studies biodiesel emissions had lower levels of particulate matter compared to conventional diesel emissions and that the levels of many polycyclic aromatic hydrocarbons levels and some nitro polycyclic aromatic hydrocarbons were also lower in biodiesel emissions. They also reported that the levels of nitrogen oxides were increased in biodiesel emissions. Thus, the Public Health Evaluation conclusion that combustion emissions from biodiesel fueled engines compared to those from CARB diesel fueled engines leads to lower emissions of particulate matter and polycyclic aromatic hydrocarbons is consistent with the published literature as is the increase in nitrogen oxides. However, as pointed out in the report although there is a reduction in particulate matter emissions in biodiesel blends, “there is some uncertainty that that a drop in total PM mass may not necessarily equate with an overall reduction in the number of UFP emitted from combustion. This is an issue of national interest and more testing would be required to fully address it”. In fact, it has been recently reported that higher numbers of ultrafine particles (UFPs, < 100 nm) were emitted from a diesel engine combusting pure waste cooking oil biodiesel compared to ULSD supporting this concern (Betha and Balasubramanian, 2013).

The Public Health Evaluation conclusion that benzene levels are lower in emissions from biodiesel fueled vehicles compared to those vehicles using CARB diesel from the Durbin et al (2011) report is consistent with those measurements found in Magara-Gomez et al. (2012) and the NREL (1998) report.

Ethylbenzene has the potential to cause hepatic, CNS and renal damage. The Public Health Evaluation conclusion that ethylbenzene levels are lower in emissions from biodiesel fueled vehicles compared to those vehicles using CARB diesel from Durbin et al (2011) is supported by the results of a number of studies including that of Magara-Gomez et al. (2012) who found decreases in ethylbenzene emission rates from biodiesel blends compared to ULSD.

There are several different conclusions regarding the levels of acrolein in the Staff Report and in the literature. In part C. Office of Environmental Health Hazard Assessment Evaluation, P 13 it states "In tests using a Caterpillar C-15 engine, the amount of acrolein was increased in emissions from combustion of B100 and B50 from both plant and animal sources when compared to the amount of acrolein in emissions from CARB diesel combustion." Under C. Conclusions on Public Health Impact, P 16, L5 it states "Limited emissions testing resulted in a non-statistical increase in acrolein for a higher B50 biodiesel blend level (i.e., confidence interval less than 95%). Furthermore, the statistical analysis for acrolein emissions results was compared to only one data point for the control sample". The first conclusion is likely based on the data from Cahill and Okamoto (2012) using a 2000 Caterpillar C-15 engine and a UDDS drive cycle. Cahill and Okamoto (2012) used N values of 2-3. The second conclusion is likely based on Figure 10-18 (P 174) in the Durbin et al (2011) report who 2000 Caterpillar C-15 engine and a UDDS drive and used N values of 1 and 2. The Bünger et al. (2012) analysis of papers comparing acrolein levels in B5, B10, B20 and 100% biodiesel emissions concludes shows general increased levels of acrolein in biodiesel emissions but the results are highly variable. My recommendation is to make one clear and consistent concluding statement regarding the levels of acrolein compared to CARB diesel a statement that encompasses the all of the available data.

The role of oxy-PAHs needs to be more fully described in the Staff Report. Durbin et al. (2011) states "The emission trends for Oxy-PAH emissions showed different trends for different compounds, with some compounds showing generally higher emissions in soy and animal-based biodiesels compared to CARB diesel, whereas others decreased in animal biodiesel and renewable diesel. For all toxic species, emission levels were significantly reduced in the DPF-equipped vehicle, and there were few fuel related trends." Oxy PAH levels were also increased in studies using methyl ester blends of vegetative and animal based oils compared to EN590 using a diesel passenger car (Karavalakis et al., 2009, 2011). One issue that has not been fully discussed is the apparent increase in the levels of 1,2-naphthoquinone as described in Durbin et al. (2011). This might be due to increased oxygen content of the ester-based biofuels. Inspection of the mean and standard deviation results of CARB animal and A100 levels in Figure 10-47 (Durbin et al., 2011) indicate that CARB animal and A100 levels of 1,2-naphthoquinone appears to be statistically significantly different. 1,2-Naphthoquinone is cytotoxic (Flowers-Geary et al., 1993) and genotoxic (Saeed et al., 2008) and 1,2-naphthoquinone and its analog 1,4-naphthoquinone each induce reactive oxygen species (ROS) (Thornalley et al., 1984 ). 1,4-Naphthoquinone, a component of particulates collected from road tunnel emissions is also cytotoxic and induced ROS and DNA damage in human lung epithelial cells, as did the road tunnel particles themselves (Shang et al., 2013). It is well

known that several ROS forms induce cytotoxicity and genotoxicity and the formation of ROS can lead to adverse health outcomes.

The toxicities of exhausts in rats from a biodiesel fueled engine were reported in by studies described in the California Biodiesel Multimedia Evaluation Final Tier III Report. “CARB diesel, biodiesel, and renewable diesel all induced inflammatory markers, such as COX-2 and IL-8 in human macrophages and the mucin related MUC5AC markers in Clara type cells, with the inflammatory markers higher in the 2000 Caterpillar C-15 engine vehicle than the 2007 MBE4000 engine vehicle. For the comet assay, at the limited dose levels tested, there was little increase of chromosomal damage (gross DNA damage) from the various fuels tested” (Durbin et al., 2011). The mutagenic activities of combustion emissions (as organic particulate extracts and some vapor phase fractions) from biodiesel fueled engines compared to those from conventional diesel fueled engines were reported in the California Biodiesel Multimedia Evaluation Final Tier III Report based in part on Durbin et al. (2011) and were also reviewed by Bünger et al. (2012). The available data indicate, with some exceptions, a general lowering of mutagenic activity based mainly on the data from bacterial assays which is consistent the lower levels of polycyclic aromatic hydrocarbons and nitro polycyclic aromatic hydrocarbons in the biodiesel emission extracts. It noted that in a recent study, organic extracts of particles from emissions of engines using rapeseed methyl ester and EN 590 fuels both produced DNA adduct levels to comparable extents in an acellular assay using calf thymus DNA both in the presence and absence of an exogenous metabolic activation system, suggesting equal genotoxic activities of the two extracts (Topinka et al., 2012). No increases in micronuclei in bone marrow or sister chromatid exchanges in peripheral blood lymphocytes were found in rats exposed by subchronic inhalation to emissions from a diesel engine burning soybean based biodiesel fuel (Finch, 2002).

There is an increasing body of new literature on inflammation, lung tissue damage, oxidative stress and oxidative damage where biodiesel emissions (particulate matter or complete emissions) have been shown to be more toxic than those from conventional diesel emissions. The results of many of these studies are summarized or quoted here. “Concentrations of inflammatory mediators (Interleukin-6, IL-6; Interferon-gamma-induced Protein 10, IP-10; Granulocyte stimulating factor, G-CSF) in the medium of B20-treated cells and in bronchioalveolar lavage fluid of mice exposed to B20 were ~20–30% higher than control or B0 PM, suggesting that addition of biodiesel to diesel fuels will reduce PM emissions but not necessarily adverse health outcomes (Fukagawa et al., 2013)”. Human bronchial BEAS-2B cells were exposed to particulate matter collected from diesel passenger vehicles with and without a diesel particulate filter using a rapeseed biodiesel (B50) blend or to diesel fuels. The particulate matter from the B50 blend induced increased cytotoxicity and IL-6 release in the cells compared to the diesel fuel per distance driven. These differences were observed irrespective of the use of a diesel particulate filter (Gerlofs-Nijland et al. 2013). Rat alveolar macrophages exposed to exhaust particles from heavy duty diesel engine combusting B20 biofuel resulted in an increased production of PGE<sub>2</sub> relative to particles from diesel fuel combustion (Bhavaraju et al., 2013). Mice were exposed by pharyngeal aspiration to diesel particulate matter collected from a diesel engine using biodiesel (NEXSOL BD-100) and ULSD. Biomarkers of tissue damage and inflammation were significantly elevated in the lungs of mice exposed to the biodiesel particulates. Inflammatory cytokines/chemokines/growth factors were up-regulated to

a greater extent and oxidatively modified proteins and 4-hydroxynonenal levels were increased by biodiesel particulates compared to diesel particulates (Yanamala et al., 2013). Mice were exposed by inhalation to combustion emissions of soy biodiesel (B100) and diesel. “B100 combustion emissions produced a significant accumulation of oxidatively modified proteins (carbonyls), an increase in 4-hydroxynonenal (4-HNE), a reduction of protein thiols, a depletion of antioxidant glutathione (GSH), a dose-related rise in the levels of biomarkers of tissue damage (lactate dehydrogenase, LDH) in lungs, and inflammation (myeloperoxidase, MPO) in both lungs and liver. Significant differences in the levels of inflammatory cytokines interleukin (IL)-6, IL-10, IL-12p70, monocyte chemoattractant protein (MCP)-1, interferon (IFN)  $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$  were detected in lungs and liver upon B100 and D100 complete emission exposures. Overall, the tissue damage, oxidative stress, inflammation, and cytokine response were more pronounced in mice exposed to biodiesel complete emissions” (Shvedova et al., 2013).

Overall, the Public Health Evaluation is generally supported by the data in the Staff Report with exceptions noted above.

### **3. MMWG’s Recommendations to the California Environmental Policy Council**

**The MMWG recommends that the California Environmental Policy Council (CEPC) find that the use of biodiesel and renewable diesel, as specified in the respective multimedia evaluations, does not pose a significant adverse impact on public health or the environment.** Based on the MMWG’s conclusions in Chapter 3 of the Biodiesel Staff Report and the Renewable Diesel Staff Report, the MMWG proposes recommendations to the CEPC. (Biodiesel Staff Report and Renewable Diesel Staff Report, Chapter 4).

The MMWG conclusions “that the use of biodiesel fuel in California, as specified in the biodiesel multimedia evaluation, does not pose a significant adverse impact on public health or the environment relative to diesel fuel meeting Air Resources Board (ARB) motor vehicle diesel fuel specifications (CARB diesel)” is supported by many of the analytical chemical and biological measures of toxic components of emissions from CARB vs. biodiesel fueled engines as found in the Staff Report suggesting a lessened impact on public health, however, the recent toxicological data give me some concern that not enough studies have been conducted to unequivocally conclude that substitution of biodiesel for CARB diesel will not adversely affect public health and that the ARB should proceed with caution.

### **4. The Big Picture**

In the holistic view, based on multiple lines of evidence from studies found in government reports and in the peer-reviewed literature it seems clear that the levels of a number of key constituent of emissions from the combustion of biodiesel fueled engines are lower than those measured in the emissions from conventional diesel fueled engines. These are carbon monoxide, total hydrocarbons, particulate matter and polycyclic aromatic hydrocarbons. Moreover the overall carbon dioxide levels released to the atmosphere are reduced due to recycling of the carbon dioxide by the vegetative feedstock. The levels of a number of VOCs (e.g. benzene, ethylbenzene) are also decreased. However increases in the emissions of nitrogen oxides and acrolein have been reported and there is an increase in the proportion of ultrafines in the particulate matter emissions. These results are in concert with a recent Health Canada and Environment Canada modeling study where the authors concluded: “Although modeling

and data limitations exist, the results of this study suggested that the use of biodiesel fuel blends compared to ULSD was expected to result in very minimal changes in air quality and health benefits/costs across Canada, and these were likely to diminish over time” (Rouleau et al., 2013). However, the levels of many of the constituents cited above have not been determined for the many different combinations of engine types (heavy and light duty) technology (old, new, catalyst type, test cycle and load), feed stock sources (plant and animal based) and mixture blends, therefore, some caution needs to be exercised in accepting these conclusions without further data on the most prevalent combinations. Decisions on the impact of the toxicity of emissions from the multitude of combinations should be revisited after more data is available and the recent toxicological data given weight in the current decision process.

In my opinion, the conclusions and scientific portions of the multimedia evaluation were based upon sound scientific knowledge, methods, and practices. The Report should be updated to incorporate the new chemical, physical and toxicological data now available.

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**External scientific peer review of the Multimedia Working Group's assessment of the biodiesel multimedia evaluation**

As reviewers we are specifically asked to evaluate the following statements:

**A. Air emissions evaluation. Air resources board staff concludes that the use of biodiesel does not pose a significant adverse impact on public health or the environment from potential air quality impacts.**

I find that this conclusion of the report is based on sound scientific knowledge, methods, and practices. While there were clearly gaps in this knowledge, these have been largely filled by the tier II investigations. These investigations showed that there are some tradeoffs associated with the air emissions from biodiesel. In particular, biodiesel appears to generate more NO<sub>x</sub> than regular diesel, however, these higher NO<sub>x</sub> emissions are offset by lower emissions of VOCs, such that the overall generation of ozone is about the same as for regular diesel. It is clear that biodiesel has significantly lower emissions of particulate matter, many hazardous air pollutants, and most VOCs. However, it should be noted that because there are so many possible sources of biodiesel, the emissions factors for all of these pollutants are likely to be highly variable. Thus although the reductions in emissions are statistically significant for the specific biodiesels investigated, they may not be significant for all biodiesels that may be sold in California. Nevertheless, it appears that there are no worrisome increases in emissions associated with biodiesel. The increase in production of NO<sub>x</sub> is small and not worrisome.

**B. Water evaluation. State water resources control board staff concludes that there are minimal additional risks to the beneficial uses of California waters posed by biodiesel than posed by CARB diesel alone.**

I find that this conclusion of the report is based on sound scientific knowledge, methods, and practices. Biodiesel has been demonstrated to be more biodegradable than regular diesel. In addition, the higher biodegradability of biodiesel augments the biodegradability of the regular diesel with which it is blended. The one area of concern for biodiesel is its tendency to act as a cosolvent and increase the solubility of other contaminants. This may be of concern in groundwater. However, it is probably of much

less concern than the cosolvent properties of ethanol with which gasoline is often blended.

- C. **Public health evaluation. Office of environmental health hazard assessment staff concludes that the substitution of biodiesel for CARB diesel reduces the rate of emission of carbon dioxide to the atmosphere and reduces the amount of particulate matter, benzene, ethylbenzene, and polycyclic aromatic hydrocarbons released into the atmosphere, but may increase emissions of oxides of nitrogen and acrolein for certain blends.**

I find that this conclusion of the report is based on sound scientific knowledge, methods, and practices. As noted above the increase NO<sub>x</sub> emissions are offset by the decrease VOC emissions and may lead to less overall ozone production. However, it should be noted that these tests were performed on a limited number of biodiesel blends. While it certainly appears that the overall trend for biodiesel is to produce less of many of these hazardous pollutants, additional types of biodiesel should probably be investigated.

- D. **Soil and hazardous waste evaluation. Department of toxic substances control staff concludes that biodiesel aerobically degrades more readily than CARB diesel, has potentially higher aquatic toxicity for a small subset of tested species and generally has no significant difference in vadose zone infiltration rate.**

I find that these conclusions of the report are based on sound scientific knowledge, methods, and practices. The higher aquatic toxicity of biodiesel may be related to its cosolvent properties, which increase the solubility and bioavailability of other toxic material within the diesel. However this property of the biodiesel also causes it to disperse more readily and because it is more biodegradable this means that spills of biodiesel may very well be less of a concern than spills of regular diesel.

In addition, as a reviewer I have been asked to evaluate the following statement:

**The MMWG recommends that the California environmental policy council find that the use of biodiesel and renewable diesel, as specified in the respective multimedia evaluations, does not pose a significant adverse impact on public health or the environment.**

I find that this conclusion is based on sound scientific knowledge, methods, and practices. The concerns related to biodiesel are more significant than those related to renewable diesel, because the chemical composition of biodiesel is demonstrably different from that of regular diesel. However the tier II assessment has filled and many of our knowledge gaps, and demonstrated that the air and water impacts of biodiesel are not likely to be any worse than those of regular diesel. The few negative impacts of biodiesel (increased NO<sub>x</sub> emissions) are more than offset by the positive qualities of biodiesel, which include

decreases in emissions of most hazardous air pollutants and decreased carbon dioxide emissions. In addition, biodiesel has been shown to be more readily biodegradable than regular diesel. Thus biodiesel may be regarded as a safer alternative to regular diesel. As opposed to renewable diesel, the use of biodiesel may require development of new types of additives, changes in materials used to store and transport biodiesel, and the building of new facilities to produce biodiesel. In those respects, the environmental impacts of biodiesel have more uncertainty associated with them.

As a reviewer, and also been asked whether there are additional scientific issues that are not described in the report. Several of the issues requiring further study are already mentioned in the report, including the effects of ultra fine particles, the possible cosolvent effects of biodiesel (which could mobilize contaminants that are, for example, sorbed to soils outside of leaking underground storage tanks), and the environmental impacts of additives. It is possible that new additives may have to be developed for use with biodiesel and these should be carefully vetted before being approved. The cosolvent effects could be positive in some cases, such as leading to greater dispersal of oil spills, for example. Perhaps it should be investigated whether biodiesel can be used as a dispersant for oil spills.

Other issues that should be investigated include synergistic effects of additives on the other properties of biodiesel. If the amount of additives to be used is a significant percentage of the amount of biodiesel, they will require their own life cycle and environmental impact assessment. Another issue raised by the report is the compatibility of various pipeline and tank materials with biodiesel. This is mentioned for example on page 25.

In comments below, I also note that sensitive populations should be taken into account when evaluating health effects of biodiesel. In particular people, especially children, who suffer from asthma or allergies may be at higher risk of health effects from biodiesel due to allergic response. Because biodiesel is derived from oils which themselves can cause allergic reactions, such as palm oil and soy oil, biodiesel has the potential to be an allergen. This is less of a concern with renewable diesel, because the chemical structure of renewable diesel has been shown to be so similar to that of regular diesel. However, it should be noted that regular diesel itself has been shown to cause allergic response.

#### **Additional comments.**

#### **If 6. Tier III appendices**

#### **California biodiesel multimedia evaluation tier one report**

Page I-3. This section focuses on some of the vehicle operability issues associated with the use of biodiesel blends. The impacts to a vehicle's fuel system can result in reduced reliability and increased maintenance costs. The next generation of environmental impact assessment for biodiesel should consider whether retrofitting of existing equipment or production of new vehicle equipment is going to require changes to engine design that could have environmental impacts. For example if the use of biodiesel would require, say, catalytic converters or other equipment that might contain platinum or other heavy metals, the production of those heavy metals has significant environmental impacts and should be considered in the assessment of biodiesel. (I am not suggesting that biodiesel will require catalytic converters, I'm only using them as an example of a technology that was designed to protect the environment but used a chemical—platinum—that has significant environmental impacts.) Page I-22 discusses the fact that most modern engines without modifications can run on biodiesel, however, there are impacts on the engine's durability and reliability. The next round of environmental assessment should consider whether more frequent vehicle replacement is going to be required. If so, the impact of all these new vehicles should be considered.

Page I-6. The report notes that the biodiesel used in many of the studies described in this report was at least six months old, which is the maximum recommended storage time for biodiesel. It might also be pointed out that this may represent a worst case scenario. Emissions of particulates, NOx, etc. are likely to be worse with this relatively old fuel.

Page I-13 describes the possible need to build new facilities for the processing or production of biodiesel. If such facilities are to be built, this will have a huge impact on a life cycle assessment of biodiesel. The next round of environmental impact assessment for biodiesel should consider these impacts and should try to estimate whether these facilities are going to be built, how many are going to be built, and what the environmental impacts of those facilities will be.

Page I-26 notes that acceptable materials for storage and transport of biodiesel include aluminum, steel, and fluorinated polyethylene or polypropylene. In particular, the fluorinated compounds are a big environmental problem and should be avoided at all costs. If increased use of biodiesel is going to require the use of these kinds of fluorinated compounds this could be a serious problem.

Page I-55. Typo about halfway down the page. "Fatty acids are oxidize at the \_ carbon"

Page I-59 refers to specific sensitive populations at risk of exposure, yet I did not see anything in the report about this. Although asthma is mentioned as a possible problem

with biodiesel, this requires more discussion. Another important issue to investigate with regard to biodiesel is allergy. Many people are allergic to the raw oils such as palm oil or soy oil. Is there any reason to believe that the combustion products of biodiesels derived from these sources may cause an allergic response? People with known allergic responses are definitely a sensitive population that should be considered. Such allergies could be respiratory or dermal. There is some literature showing that regular diesel fuel is allergenic.

**7. Appendix II-B: chemical analysis of the water accommodated fractions of Bio fuels using stir bar sorptive extraction**

Page II-32 missing reference at bottom of page

Page II-33 another missing reference

Page II-83 Amount of diesel added to each microcosm is given as 5 mL, when it should be 5 $\mu$ L.

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## **External Peer Review of “Multimedia Evaluation of Biodiesel”**

### ***Re-statement of Objectives –***

External peer reviewers were instructed to evaluate the scientific portions of the Multimedia Working Group (MMWG) report and related documents to ensure that they are based on “sound scientific knowledge, methods and practices”.

This review is primarily focussed on the *Public Health Evaluation* by the Office of Environmental Health Hazard Assessment (OEHHA), as well as additional components of the evaluation that relate to the toxicological hazards of biodiesel and biodiesel emissions (e.g., results of aquatic toxicity tests). The review encompasses the MMWG Staff Report “Multimedia Evaluation of Biodiesel”, as well as the Tier I, Tier II and Tier III reports, and related documents (e.g., *Impact Assessment of Biodiesel on Exhaust Emissions from Compression Ignition Engines*).

### ***Recap of MMWG Conclusions to be addressed by Peer Reviewers (Biodiesel) –***

- (1) ARB staff concludes that the use of biodiesel does not pose a significant adverse impact on public health or the environment from potential air quality impacts.
- (2) SWRCB staff concludes that there are minimal additional risks to beneficial uses of California waters posed by biodiesel than that posed by CARB diesel alone.
- (3) OEHHA staff concludes that the substitution of biodiesel for CARB diesel reduces the rate of addition of carbon dioxide to the atmosphere and reduces the amount of particulate matter (PM), benzene, ethyl benzene, and polycyclic aromatic hydrocarbons (PAHs) released into the atmosphere, but may increase emissions of oxides of nitrogen (NOx) and acrolein for certain blends.
- (4) DTSC staff concludes that biodiesel aerobically degrades more readily than CARB diesel, has potentially higher aquatic toxicity for a small subset of tested species, and generally has no significant difference in vadose zone infiltration rate.

### ***Evaluation of MMWG Conclusions –***

Noteworthy shortcomings regarding the quality of the MMWG Evaluation of Biodiesel (i.e., the staff report), and the associated Tier I, II and III reports, **precluded effective scholarly evaluation of the aforementioned conclusions**. More specifically, in this reviewer’s opinion, the MMWG evaluation of the available scientific information regarding the relative toxicological activity of biodiesel emissions is incomplete and superficial. **Consequently, it was necessary for this reviewer to collect, review and evaluate all publicly-available scientific information pertaining to the relative toxicological activity of biodiesel and petroleum diesel emissions**. The resulting review is provided in *Part II* of this document, and a detailed summary of the publicly-available scientific information is presented in a series of appended tables (i.e., Appendix D). *Part III* of this document comprises concluding remarks based on a thorough analysis of all publicly-available scientific information. *Part I* contains the more detailed peer review of the MMWG staff report and related documents.

Following a complete review of the available scientific information (i.e., Parts II and III of this document), including the information presented in the MMWG documents; this reviewer was able to render a professional, scholarly opinion regarding the MMWG conclusions. Noting that in some instances the available information may be incomplete and “less than ideal”, **this reviewer nonetheless supports the ARB and OEHHA conclusions listed above** (i.e., 1 and 3). Although some of the published scientific information available to date shows enhanced toxicological activity for biodiesel emissions, relative to petroleum diesel, **the weight of evidence supports the ARB and OEHHA conclusions**. With respect to the SWRCB and DTSC conclusions, this reviewer’s analysis of the presented information did not reveal any problems or inconsistencies. However, it should be noted that this reviewer is not qualified to critically evaluate statements related to aerobic degradation or

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vadose zone infiltration.

**Despite publicly-available scientific studies that have documented enhanced toxicological hazards for biodiesel emissions, the weight of evidence, in this reviewer's opinion, permits support of the MMWG's recommendations to the California Environmental Policy Council (i.e., "that the use of biodiesel, as specified in the multimedia evaluations, does not pose a significant adverse impact on public health and the environment"). However, as noted below, the WG must acknowledge studies that have documented enhanced toxicological hazards for biodiesel emissions; particularly those that noted effects generated under experimental conditions that have been linked to adverse effects in humans (e.g., inflammation).**

This reviewer certainly acknowledges that comprehensive statements regarding the relative toxicological hazards of biodiesel and conventional diesel emissions are hampered by variations in exhaust composition attributable to engine design, fuel formulations and blending rate, biodiesel source, aftertreatment, and test cycle; and moreover, that the available scientific data may indeed be "less than ideal". **Nonetheless, this evaluation of the WG conclusion can reasonably be regarded as an informed appraisal based on available information and professional judgement.**

Despite the aforementioned support of the MMWG conclusions, the MMWG is *strongly* encouraged to revise the reports such that they constitute a comprehensive, well balanced, scholarly evaluation of the available scientific information. The California H&SC states that multimedia evaluations "must be based on the best available scientific data"; and moreover, that the multimedia evaluation process must include a summary of the information available to date (i.e., "literature review") with identification of noteworthy knowledge gaps (Tier I). Although the Tier I report does identify some important knowledge gaps, it does not provide an acceptable scholarly summary of relevant toxicological information on biodiesel emissions available to date. **It is critical for the WG to effectively summarise all publicly-available evidence in order to effectively demonstrate that the risk of adverse health effects attributable to biodiesel emissions, or emissions of biodiesel-petroleum diesel blends, are similar or lower in comparison with conventional diesel emissions. Concurrently, it is also critical for the WG to acknowledge that a limited number of studies have documented enhanced toxicological hazards for biodiesel emissions; moreover, hazards related to pathophysiologic changes associated with an increased likelihood of human morbidity and mortality (e.g., pulmonary inflammation, oxidative stress, pulmonary tissue damage, cardiovascular irregularities).**

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***Peer Review of MMWG Evaluation of Biodiesel–***

***Part I – Peer Review of the MMWG Evaluation Staff Report and Related Documents***

It is this reviewer's understanding that the Tier I report, which constitutes the first step in "evaluating the cumulative health and ecological impacts from releases to air, surface water, groundwater and soil at all stages of the life cycle of biodiesel blends", should be based on the latest information available to date. In this regard, the Tier I review, and analysis of available information related to the toxicological hazards of biodiesel combustion emissions relative to conventional petroleum diesel combustion emissions, is incomplete and superficial, and, dare I say, naïve with respect to its analysis and interpretation. Moreover, in some instances the presentation of available information lacks the details required for the reader to understand and appreciate the relevant scientific publications (e.g., lack of units for air toxics emission rates). For example, the Tier I report states that the use of biodiesel is expected to contribute to large reductions in hydrocarbon (HC), particulate matter (PM) and carbon monoxide (CO) releases; however, the units upon which this statement is based are not provided (e.g., p. I-35). Although, as the report indicates, numerous studies have indeed documented noteworthy declines in key air toxics such as HC, CO and PM, for biodiesel and biodiesel blends relative to conventional diesel, the discussion in the Tier I report is superficial. For example, although the USEPA (2002) report on biodiesel exhaust emissions notes that B20 blends can be expected to contribute to average declines in PM, CO and HC emissions of 10.1%, 11.0% and 21.1%, respectively, relative to conventional diesel, it also notes that declines in emissions recorded in individual studies are dependent on engine design, fuel formulation and properties, and engine test cycle. Moreover, it is clear from the EPA report that declines in the aforementioned criteria air pollutants can be highly variable. For example, changes in HC emissions for B20 blends, relative to conventional diesel, can range from almost -100% to almost +100%. Variations in relative changes in PM and CO emission rates are somewhat lower (e.g., negligible to -60% for PM). Although this reviewer does acknowledge that there is strong evidence to support the assertion that biodiesel emissions rates for criteria air pollutants such as CO, PM and HCs are in fact reduced, the Tier I review and analysis of the available information is superficial. Similar statements can be made regarding other criteria air pollutants such as NO<sub>x</sub>, as well as air toxics such PAHs and aldehydes. Increased NO<sub>x</sub> has been clearly linked to an enhanced risk of human morbidity and mortality and the potential impacts on human health are only superficially acknowledged. It may be true that advanced emission control will offset the hazards associated with the increased NO<sub>x</sub> emissions rates; however, it is reasonable to expect the authors to present a more complete, quantitative evaluation.

From this reviewer's point of view, the most serious deficiencies in the MMWG evaluation of biodiesel relate to the incomplete review of publicly-available scientific information on the toxicological properties of biodiesel emissions relative to conventional diesel emissions. More specifically, in this reviewer's opinion, the review on pages I-42 and I-43 and I-59 to I-64 of the Tier I report is remarkably incomplete and superficial, and this superficial analysis is carried over into the Tier III report. Moreover, with respect to the organization of the Tier I report, it is unclear why information on the toxicological properties of emissions are introduced in Section 4 (Use of Biodiesel), and then discussed in more detail in Section 7 (Biodiesel Toxicity). Nevertheless, the combined information presented in both sections only reviews and discusses 7 of the roughly 45 scientific publications that have compared the toxicological properties of biodiesel and conventional diesel emissions. Moreover, the review is confusing for reader in the sense that it does not clearly distinguish between studies that examined fuels and fuel blends, and studies that examined combustion emissions. Furthermore, the discussion mixes up studies that conducted toxicological assessments based on measurements of air toxics emission rates with studies that assessed toxicological properties using *in vivo* and *in vitro* toxicity assessment tools. Finally, the presented

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review of the pertinent scientific literature ignores critical information regarding the metric(s) used to express the magnitude of toxicological potency (i.e., potency unit). The units employed for quantitative evaluation of the results have a critical bearing on the relevance of the results for the assessment of human health risk. For example, the cited Turrio-Baldassarri et al (2004) study, which examined the mutagenic activity of organic extracts of PM from conventional diesel and B20 RME, compares mutagenic activity expressed per  $\mu\text{g}$  of extractable organic matter (EOM) and per unit of engine work (kWhr). The former unit is useful for studies that are interested in identifying the putative toxicants in combustion emissions, the latter, which requires information on EOM emission rates (e.g.,  $\mu\text{g}$  per kWhr), is more useful for assessing the likelihood of post-emission adverse human effects. The Bunger et al study cited by the MMWG (i.e., 2000a), which examined the *cytotoxicity* of organic extracts of PM from conventional diesel and RME blends, notes that the potency of RME emissions, expressed per L of exhaust, is more pronounced. Interestingly, the MMWG review of the Bunger et al (2000) publications (i.e., 2000a and 2000b) only discusses *mutagenic* activity, and notes the reduction in potency associated with RME and SME emissions. In addition, the review, which is presented on page I-61, fails to acknowledge the units used for the potency comparison. Importantly, the Bunger et al (2000b) publication includes a comparative analysis of potency expressed per unit mass and per hour of engine operation. The latter unit is far more relevant for assessment of human risk. The noteworthy declines in the potency of biodiesel emissions described in the publication appear to be related to reduced potency of the PM, *as well as* the reductions in PM and PAH emission rates (e.g., g or  $\mu\text{g}$  per engine hour).

The superficial nature of the MMWG's review of the available information regarding the toxicological properties of biodiesel emissions relative to conventional diesel ***precludes an effective peer review of the WG's conclusions.*** Moreover, the external peer review process provides the latitude to include any scientific information that is deemed to be pertinent to the review of the MMWG documents. Consequently, **it was necessary for this reviewer to collect and review all pertinent publicly-available scientific information.** This evaluation of available information is contained in *Part II* of this document; a review and analysis of the available literature pertaining to the relative toxicological properties of biodiesel and conventional diesel emissions.

It is certainly important to acknowledge that the Tier II analyses of biodiesel and renewable diesel emissions (i.e., the Durbin et al, 2011 report) constitutes a comprehensive comparative analysis of biodiesel and conventional diesel emissions. Nevertheless, it is also important to note that there are serious shortcomings in the Durbin et al report regarding the description of the methods employed for the toxicological analyses. Moreover, the concluding remarks and executive summary do not even comment on the relative ability of the emissions to induce inflammatory and oxidative stress responses. More specifically, the results presented indicate that extracts of biodiesel DEP from the 2000 model year engine without aftertreatment generally show a reduced ability to induce inflammatory signalling (COX-2, IL-8) or oxidative stress (HO-1), relative to conventional diesel, with noteworthy declines associated with NExBTL HVO blends. These results are never discussed in any meaningful way. Nevertheless, it is important to acknowledge the documented declines (i.e., in Durbin et al, 2011) in criteria air pollutants (e.g., PM, CO and HC) associated with biodiesel emissions; and moreover, the frequent observations of increasing reductions in emission rates for increases in blending rate. Similarly, it is important to acknowledge documented declines in biodiesel exhaust emission rates for air toxics such as PAHs, BTEX, and some carbonyl compounds, and the noteworthy increases in toxic aldehydes such as acrolein. Some researchers have suggested that compounds such as acrolein, which is a noteworthy irritant, may be responsible for documented increases in the toxicological activity of biodiesel emissions (Bunger et al, 2000; Bunger et al, 1998). Indeed, the increased risk of mucous membrane irritation in road maintenance workers exposed to

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RME emissions, relative to conventional diesel, may be due to increased emissions rates of reactive aldehydes (Bunger et al, 2012<sup>1</sup>).

Although this peer review focussed primarily on toxicological hazards (i.e., health hazards) of biodiesel emissions, the information pertaining to the relative toxicological activity of biodiesel (i.e., the fuel) and conventional diesel were also reviewed. The Tier I report provides an effective overview of available information on the relative aquatic toxicity of biodiesels and conventional diesel; and moreover, identifies knowledge gaps that are effectively addressed in Tier II using well-established EPA methods. The conclusions of the MMWG, which state that the biodiesel blends “exhibit somewhat increased toxicity to subsets of tested species compared to ULSD” are supported by the data. Nevertheless, it is also important to note that the Tier I review of the literature is incomplete. For example, important studies by Leme et al 2012<sup>2</sup>) used water and soil spill simulations to show that soy-derived B100 yielded samples with an increased capacity to induce genetic damage in bacteria, mammalian cells and plants (i.e., *Allium cepa* root tips).

#### ***Miscellaneous Editorial Comments –***

Although this review did not include detailed examination of spelling, grammar, or stylistic issues, a few of the more obvious problems are highlighted below.

Biodiesel Tier I, page I-4: The authors are reminded that it is critical to provide units when referring to changes in emission rates.

Biodiesel Tier I, page I-35: The authors often use statements such as “large reductions”, but fail to qualify. How will the reader know what “large means”. Is it 10% or 95%?

Biodiesel Tier I, page I-43: The authors refer to TEFs but fail to note what endpoint is being discussed. Presumably it’s carcinogenic activity. Although some agencies use the term TEF to refer to carcinogenic activity relative to BaP, the authors are reminded that many readers will be more familiar with the terminology used the EPA’s Integrated Risk Information System (IRIS) employs the acronym RPF (Relative Potency factor).

Biodiesel Tier I, page I-61: “...thoroughly tested by the EPA and is “safe”. Where does this statement come from? The EPA is extremely reluctant to use adjectives such as “safe”. The agency is far more likely to use statements such as “negligible increase in risk above background”.

Biodiesel Tier I, section 7: Please pay attention to units!

Biodiesel Tier I, Section 7 (carried through to Tier III): Many vague statements need to be clarified. For example, “premature death” from what type(s) of effects? “More investigations in biological systems” – what systems? What endpoints? What route(s) of exposure?

Biodiesel Tier II, page II-32: Reference problem at bottom of page.

Throughout the Biodiesel Tier I, II and III reports: The quality of reproduced graphics (e.g., page II-77) is marginal. In some cases it is very difficult to make out the axes labels.

Durbin et al (2011), pages 222 and 224: “Marcophage” should be macrophage.

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<sup>1</sup> Original source – Hasford, B. et al. 1997. Respiratory symptoms and lung function after exposure to exhaust from rapeseed oil in comparison to regular diesel. *Proceedings of the 9<sup>th</sup> International Conference on Occupational Respiratory Diseases: Advances in Prevention of Occupational Respiratory Diseases*. Elsevier, Kyoto, Japan, pp. 131-135.

<sup>2</sup> Leme et al. 2012. *J. Hazard Mat* **199-200**:343-349; Leme et al. 2012. *Chemosphere* **86**:512-520.

***Part II – Review of Published Information on the Relative Toxicological Properties of Biodiesel and Petroleum Diesel Combustion Emissions***

**1. INTRODUCTION – Toxicological Assessments of Vehicular Emissions for Comparisons of Petroleum-based Diesel, Biodiesel, Renewable Diesel, and Biodiesel or Renewable Diesel Blends**

It is important for the MMWG to acknowledge that studies investigating the toxicological activity of diesel emissions can be conducted on diluted exhaust, exhaust particulate matter, filtered exhaust (i.e. gaseous portion), extracts of particulate matter, or concentrates of semi-volatile organics (i.e. SVOCs) adsorbed on a solid matrix (e.g. XAD resin). The nature of the toxicological assessment, and the units employed to express the observed responses, will influence the interpretation of the results in a human health context.

The most sophisticated *in vivo* studies involve inhalation exposures whereby experimental animals are exposed to diluted vehicular emissions. Doses delivered via inhalation exposure are generally expressed as mg PM per m<sup>3</sup> in the exposure chamber, with additional information provided regarding the duration and frequency of the exposure. Other *in vivo* studies generally involve delivery of exhaust particulate suspensions or particulate extracts to the pulmonary system via intratracheal or intrapharyngeal instillation, or delivery of particulates or particulate extracts via oral gavage, dietary intake with food, topical treatment, or intraperitoneal (IP) injection. Intratracheal, intraperitoneal or dietary doses are generally expressed as total mg PM delivered/consumed or mg per kg body weight.

The majority of *in vitro* assessments of effects associated with vehicular emissions involve exposures of cells suspended in liquid medium, cells attached to solid culture surfaces (e.g. polystyrene), or cells imbedded in agar. More recently, it has become possible to hold cultured cells, including primary human cells or 3-dimensional tissue constructs, on semi permeable membranes and expose the cells at an air-liquid interface (<sup>1-3</sup>). However, such systems (e.g. VitroCell® or Cultex®) have rarely been employed to examine the toxicity of vehicular emissions (e.g., <sup>4</sup>). Thus, most *in vitro* assessments involve exposures to collected PM, organic extracts of PM, or concentrates of SVOCs. PM collection can present a substantial technical challenge, with most studies collecting PM on glass fibre filters. In some instances bulk, size-fractionated PM is collected using devices such as cascade impactors. In either case, preparation of PM extracts generally involves extraction of the “soluble organic fraction (SOF) using solvents such as dichloromethane (DCM), acetone, hexane, ethanol, methanol, or solvent mixtures. Extracts are generally exchanged with a bioassay-compatible solvent such as dimethyl sulfoxide (DMSO) before testing. SVOCs are generally collected by passing filtered exhaust over a solid adsorbent matrix (e.g. XAD resin) followed by solvent elution and concentration.

Concentration units employed for *in vitro* assessments of toxicological effects induced by vehicular emissions vary depending on the nature of the test article and the experimental system. Exposure concentrations for PM suspensions are generally expressed as mass of PM (µg or mg) per assay unit (e.g. agar plate or mL of culture medium). Exposure concentrations for organic extracts of PM are often expressed as µg of EOM, or µL of extract, per assay unit. Measures of EOM per unit mass of PM can be used to convert these concentration values into equivalent mg of PM per assay unit. In addition, measures of engine work, engine run time, fuel consumption, or distance travelled, can be employed to convert exposure concentrations to equivalent amounts of engine work in kWhr or hph, equivalent volume of fuel consumed, equivalent hour of engine operation, or equivalent distance travelled. Concentrations of SVOCs collected by adsorption on solid resins (e.g. XAD) are generally expressed as µg EOM per assay unit. With respect to the potential for adverse human effects, the potency of the sampled material (e.g., PM extracts or PM suspensions in effect per unit PM mass or EOM mass) must be considered in conjunction with the expected magnitude of the exposure.

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Emission rates per unit engine work (e.g., g PM per brake hph) can be used to express results per unit of engine work, which can more readily be interpreted in a human health context.

## **2. Summary of Studies Comparing the Toxicological Properties of Diesel Engine Emissions for Biodiesel- or Renewable Diesel-fuelled Engines to Petroleum Diesel-fuelled Engines**

Few studies have employed *in vivo* animal exposures to compare diesel emissions associated with engines or vehicles operated using petroleum-based fuels with emissions associated with biodiesels (i.e., FAMES), other renewable fuels (e.g., HVO), or fuel blends. Only two studies examined effects elicited by diluted exhaust, and both studies noted evidence that biodiesel emissions can be associated with increased severity of toxicological responses. For example, Brito et al (2010) noted that SEE emissions are associated with increases in cardiovascular irregularities in Balb/c mice <sup>(5)</sup>. Steiner et al (2013) employed an air-liquid interface system to demonstrate that diluted RME emissions (B100) induced increased cytotoxicity and oxidative stress in an *ex vivo* 3D human airway model, relative to petroleum diesel. The authors noted some decrease in inflammatory stress for biodiesel <sup>(6)</sup>. In addition, Yanamala et al (2013) showed that pharyngeal aspiration of PM from corn-derived FAME induced increased pulmonary damage, oxidative stress and inflammation in C57BL/6 mice, relative to petroleum diesel PM <sup>(7)</sup>. Importantly, the doses examined in the Yanamala et al study equate to human occupational exposures of 156.25 working days at an allowable MSHA concentration limit of 160 µg total carbon per m<sup>3</sup>. With respect to carcinogenic hazard, a single study examined the emission rate of carcinogenic PAHs, expressed as total BaP equivalents, and concluded that the carcinogenic hazards of biodiesel emissions (source unspecified) are likely to be lower than petroleum-based diesel for PM-associated PAHs in primary and secondary aerosols <sup>(8)</sup>.

Several *in vitro* studies have employed cultured animal cells to assess the toxicological activity of diesel PM suspensions. With respect to proinflammatory signalling, several studies have noted similar or reduced activity for biodiesel emissions, relative to petroleum diesel (expressed per unit mass of PM or kWhr). For example, Bhavaraju et al (2013), Hemmingsen et al (2011), and Ihalainen et al (2009) showed that exposures of several types of cells (e.g., rat alveolar macrophages, human pulmonary and endothelial cells) to biodiesel PM, including PM associated with RME, AFME and HVO, can contribute to modest declines in inflammatory signalling, relative to petroleum diesel PM <sup>(9-11)</sup>. Similarly, with respect to cytotoxicity and/or cell death (i.e., apoptosis), several studies have shown similar or reduced activity for biodiesel emissions. Studies by Betha et al (2012), Bhavaraju et al (2013) and Ackland et al (2007) noted that cytotoxic responses to biodiesel-derived PM (e.g., waste cooking oil FAME) in human and rodent cells are similar or lower relative to petroleum diesel PM <sup>(9, 12, 13)</sup>. Nevertheless, it should be noted that the Betha et al (2012) study noted greater cytotoxicity for the biodiesel PM (i.e., waste cooking oil FAME) for samples collected from high load tests. The same study also noted increased oxidative stress (GSH/GSSG ratio) associated with biodiesel PM collected under high load conditions. With respect to genotoxic activity, a single study noted similar or reduced responses for biodiesel PM (i.e., RME, AFME), relative to petroleum diesel <sup>(10)</sup>. Finally, a single study noted that exposure of fresh rat alveolar macrophages to biodiesel PM resulted in an increase in macrophage activation (i.e., PGE<sub>2</sub> release), relative to petroleum diesel <sup>(9)</sup>.

Several *in vitro* studies have employed cultured animal cells to assess the toxicological activity of organic extracts of diesel PM (i.e., SOF of collected PM). With respect to proinflammatory responses, several studies present fairly strong evidence that extracts of biodiesel PM (i.e., diesel exhaust particulates or DEP) have an enhanced ability to induce inflammatory signalling, relative to petroleum diesel. However, there is also evidence that extracts of RME DEP have a reduced ability to elicit inflammatory signalling. More specifically, two noteworthy studies showed that biodiesel DEP extracts have an enhanced ability to induce inflammatory signalling (i.e., IL-6 and IL-8 release) in

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human BEAS-2B bronchial epithelial cells (<sup>14, 15</sup>). Importantly, the Gerlofs-Nijland et al study also examined PM emission rates and noted that reductions associated with fuel changes (i.e., from petroleum diesel to biodiesel) and/or aftertreatment (e.g., DPF) may not be sufficient to offset the increased hazard associated with biodiesel DEP SOF. Nevertheless, two studies by Jalava et al noted that the SOF from RME DEP had a reduced ability to induce inflammatory signalling (i.e., *Tnf- $\alpha$* , *Mip-2* release) in murine macrophages (<sup>16, 17</sup>). The same studies noted an increase in inflammatory signalling for HVO, relative to petroleum diesel. Extensive analyses by Durbin et al (2011) of DEPs and SVOCs from two heavy-duty engines revealed that extracts of biodiesel DEP from the 2000 model year engine without aftertreatment generally show a reduced ability to induce inflammatory signalling (*COX-2*, *IL-8*) or oxidative stress (*HO-1*), with noteworthy declines associated with NExBTL HVO blends (<sup>18</sup>). Although analyses of extracts of DEP emitted by the 2007 model year engine (SME and AFME blends only) with advanced aftertreatment (i.e., DOC/DPF combination) showed higher responses for extracts of biodiesel DEP, all responses were several orders of magnitude below those obtained for the aforementioned 2000 model year engine.

With respect to cytotoxicity and/or cell death, several studies have noted an increased response for SOF samples from biodiesel DEP and/or SVOC samples compared with petroleum diesel. For example, studies by Bungler et al (2000), Gerlofs-Nijland et al (2013) and Lui et al (2008) noted increased cytotoxicity in mouse fibroblasts, human BEAS-2B cells and luminescent bacteria, respectively, with the Bungler et al (DEP extract) and Lui et al (SVOC) results based on responses expressed per L of exhaust (<sup>14, 19, 20</sup>). Again, the Gerlofs-Nijland et al study noted that DEP reductions associated with fuel changes and/or aftertreatment may not be sufficient to offset the increased hazard of biodiesel DEP SOF. In contrast, in their analyses of mouse fibroblasts, murine macrophages and human BEAS-2B cells, studies by Bungler et al (1998), Jalava et al (2010), Jalava et al (2012) and Swanson et al (2009), respectively, documented little or no difference in the cytotoxic activity of SOF from biodiesel DEP, relative to petroleum diesel (<sup>15-17, 21</sup>). In addition, the study by Kooter et al (2011), of mouse macrophages exposed to DEP extracts, noted a decline in the cytotoxicity of biodiesel DEP extracts (<sup>22</sup>). The same study also noted no difference in oxidative stress (*Ho-1* expression) signalling between biodiesel DEP extracts and petroleum diesel DEP extracts. With respect to genotoxic activity, the aforementioned Jalava et al studies noted some reductions in the ability of SOF from biodiesel DEP to induce DNA strand breaks in murine macrophages, with the most pronounced decline, relative to petroleum diesel, associated with RME (<sup>16, 17</sup>).

A single study investigated the ability of extracts from biodiesel and conventional diesel DEP to damage naked DNA *in vitro*. More specifically, Topinka et al (2012) examined extracts of biodiesel (RME and RSO) DEP and conventional diesel DEP, and noted no appreciable fuel-related differences in ability to induce DNA adducts (i.e., per mg equivalent PM) (<sup>23</sup>).

### **3. Summary of Studies Comparing the Mutagenic Activity of Organic Extracts of Diesel Particulates from Biodiesel- or Renewable Diesel-fuelled Engines to Extracts of Diesel Particulates from Petroleum Diesel-fuelled Engines**

A careful review of the literature revealed 27 studies that employed the Salmonella mutagenicity assay to compare the mutagenic activities of SOFs from biodiesel DEP and petroleum diesel DEP. Of these, 17 studies provide evidence that the SOF of biodiesel DEP is less potent relative to petroleum diesel. In contrast, 9 studies provide evidence of increased mutagenic activity for the SOF of biodiesel DEP. However, interpretation of the results requires scrutiny of the potency units employed to compare biodiesel and petroleum diesel derived samples. From a human hazard point of view, the most convincing studies compared mutagenic potency values expressed per unit distance (i.e. mile or km), per engine hour, per m<sup>3</sup> of exhaust, or per unit of engine work (i.e. kWhr or hph). Nine studies

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noted that the potency of biodiesel DEP extracts is significantly lower in comparison to samples derived from conventional petroleum diesel DEP. For example, studies by Krahl et al. (2003) and Bunger et al (2006) revealed significant reductions in mutagenic potency (per engine hour) for RME or SME relative to conventional diesel, with the former study noting that potency values for conventional diesel were 4- to 5-fold higher than biodiesel (<sup>24,25</sup>). Similar studies by Krahl et al (2005) and Westphal et al (2012) revealed significant reductions in mutagenic potency (per m<sup>3</sup> exhaust) for RME relative to conventional diesel, with the former study noting that extracts of conventional DEP yield samples with 2- to 8-fold higher potency than biodiesel (<sup>26,27</sup>). The Westphal et al (2012) study failed to reveal any fuel-related differences in the mutagenic activity of SVOC samples. Studies by Chase et al (2000), Bagley et al (1998), Kado and Kuzmicky (2003) and Rantanen et al (1993) revealed significant reductions in the mutagenic potency of DEP extracts, expressed per engine kWhr or hph, for biodiesel (i.e. SEE, SME, RME, CME, YGME, PLME, BTME) relative to conventional diesel (<sup>28-31</sup>). The Chase et al study also noted that SEE was associated with considerable reductions in the emission rates (per hph) of PM and PAHs. Moreover, the Rantanen et al (1993) study noted a correlation between mutagenic potency and PAH emission rates (both per kWhr). A study by Bunger et al (2000a) revealed significant reductions in the mutagenic potency, expressed per L of engine exhaust, of extracts from biodiesel DEP, compared with extracts from conventional diesel DEP (<sup>19</sup>). Interestingly, additional analyses showed higher PM emission rates for RME. Studies by Bunger et al (1998), Kado et al (1996), and Durbin et al (2011) revealed significant reductions in the mutagenic potency, expressed per engine mile or km, of extracts from biodiesel DEP compared with DEP from conventional diesel (<sup>18,21,32</sup>).

Several studies employed mutagenic potency values expressed per mg of DEP or per µg of EOM (extractable organic matter) to compare the mutagenic potency of extracts from biodiesel DEP and conventional diesel DEP. Studies by Bunger et al (2000b), Bunger et al (1998), Carraro et al (1997) and Kado et al (1996) noted that the mutagenic potency of extracts from biodiesel DEP is lower than extracts from conventional diesel DEP (<sup>21,32-34</sup>).

Several studies failed to reveal any differences between the mutagenic potency of extracts of biodiesel DEP compared with conventional diesel DEP. For example, in their examination of DEP from several light- and heavy-duty vehicles, studies by Krahl et al failed to detect any differences between the mutagenic potency (per L exhaust) of emissions associated with diesel fuel, RME, or diesel/GTL/RME blends (<sup>35,36</sup>). Similarly, in their study of emissions from a single cylinder research engine, Bunger et al (2000b) noted that the mutagenic potency (per engine hour) of DEP extracts are similar for conventional diesel, RME and SME (<sup>33</sup>). A study by Dorn and Zahoransky (2009) failed to detect mutagenic activity in extracts of DEP from conventional diesel or biodiesel (<sup>37</sup>). A study by Turrio-Baldassarri et al (2004) failed to detect any difference between the mutagenic potency, expressed per kWhr, of biodiesel (B20 RME) DEP extracts and extracts of DEP from conventional diesel (<sup>38</sup>).

In contrast to the aforementioned decreases in the mutagenic activity of extracts from biodiesel DEP, compared with DEP from conventional diesel, some studies have noted that the mutagenic potency of extracts from biodiesel DEP can be significantly greater than extracts from conventional diesel DEP. For example, studies by Bunger et al (2007) and Krahl et al (2007a, 2009b) noted increases in mutagenic potency (per L exhaust) for extracts of RME DEP in comparison with conventional diesel (<sup>39-41</sup>). Similarly, Kooter et al (2011) noted that the mutagenic potency (per µg PM) of extracts associated with biodiesel (source unspecified) is generally higher than conventional diesel (<sup>22</sup>). Of particular interest are studies that noted increased mutagenic potency for extracts of biodiesel DEP expressed per mg DEP or µg EOM (extractable organic matter), relative to extracts of DEP from

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conventional diesel, but a reversal in relative potency when values are expressed per unit of engine work. For example, Rantanen et al (1993) noted that extracts of DEP from RME emissions were more mutagenic (per  $\mu\text{g}$  EOM) than extracts of DEP from conventional diesel; however, when expressed per unit of engine work (kWhr), the RME samples proved to be less mutagenic relative to conventional diesel (<sup>31</sup>). Similarly, the study by Kado and Kuzmicky (2003) noted that the potency of extracts of some biodiesel DEP samples (per mg PM) were higher than extracts of DEP from conventional diesel; however, when expressed per unit of engine work (hph), the biodiesel potency values are lower relative to conventional diesel (<sup>30</sup>).

Several of the aforementioned studies revealed lower emission rates of PM and/or PAHs and other PACs (e.g., nitro-PAHs and oxy-PAHs) for biodiesels and biodiesel blends in comparison with conventional diesel. For example, studies by Krahl et al (2005, 2007b), Schroder et al (2012), Turrio-Baldassarri et al (2004), Westphal et al (2012, 2013), Kooter et al (2011) and Carraro et al (1997) noted that biodiesel is associated with lower emission rates of PM, PAHs, oxy-PAHs, or nitro-PAHs (<sup>22, 26, 27, 34, 35, 38, 42, 43</sup>).

***Part III - Concluding Remarks***

Although comprehensive statements regarding the relative toxicological hazards of biodiesel and conventional diesel emissions are hindered by variations in exhaust composition attributable to engine design, fuel formulations and blending rate, biodiesel source, aftertreatment, and test cycle, the evidence generated to date suggests that the risk of adverse effects attributable to biodiesel emissions, or emissions of biodiesel-petroleum diesel blends, are similar or lower than conventional ULSD emissions. Nevertheless, it is critical to note that several studies have clearly documented enhanced toxicological hazards for biodiesel emissions; moreover, hazards related to pathophysiological changes associated with an increased likelihood of human morbidity and mortality (e.g., pulmonary inflammation, oxidative stress, pulmonary tissue damage, cardiovascular irregularities). The most notable published studies include the *in vivo* murine studies of Brito et al (2010) and Yanamala et al (2013), and the air-liquid interface *ex vivo* study of Steiner et al (2013) that examined effects on 3D human airway epithelium constructs. In addition, several notable *in vitro* studies provide additional evidence suggesting the possibility of increased toxicological hazard for biodiesel emission. These include the *in vitro* DEP organic extract studies in human BEAS-2B bronchial epithelial cells by Swanson et al (2009) and Gerlofs-Nijland et al (2013), and the *in vitro* DEP extract studies in murine fibroblasts by Bunker et al (1998, 2000a) and Schroder et al (1999). Finally, several studies have shown that organic extracts from some biodiesel DEP have an enhanced ability, relative to extracts of conventional diesel DEP, to induce genetic damage and mutations that might be expected to increase the likelihood of cancer (<sup>39-41</sup>).

Several researchers have noted that the increased toxicological potency that has been observed for some biodiesel emissions may be associated with recorded increases in the emission rates of toxic aldehydes such as acrolein (<sup>19,21</sup>). Indeed, the increased risk (i.e., OR = 1.3 to 2.2) of mucous membrane irritation in road maintenance workers exposed to RME emissions, relative to conventional diesel, may be due to increased emissions rates of reactive aldehydes (<sup>45</sup>). Moreover, the increased toxicological activity of biodiesel DEP may be associated with an increase in its soluble organic fraction (i.e., µg EOM per mg PM) (<sup>7, 14, 20, 21, 25, 33</sup>).

**Although critical examination of the available information presented in the previous sections does indeed indicate, in this reviewer's opinion, that the risk of adverse health effects attributable to biodiesel emissions, or emissions of biodiesel-petroleum diesel blends, is similar or lower relative to conventional diesel fuel emissions, it is critical for the MMWG to acknowledge that there are some studies that have documented enhanced toxicological hazards for biodiesel emissions; moreover, hazards that are mechanistically related to pathophysiological changes associated with an increased likelihood of human morbidity and mortality (e.g., pulmonary inflammation, oxidative stress, pulmonary tissue damage, cardiovascular irregularities).** As indicated above, the most notable studies include the *in vivo* murine studies of Brito et al (2010) and Yanamala et al (2013), and the air-liquid interface *ex vivo* study of Steiner et al (2013), as well as several *in vitro* studies that provide additional evidence suggesting the possibility of increased toxicological hazard for biodiesel emissions. The latter includes studies by Swanson et al (2009) and Gerlofs-Nijland et al (2013) that examined the effects of DEP organic extracts on human BEAS-2B bronchial epithelial cells, and the *in vitro* DEP extract studies with murine fibroblasts conducted by Bunker et al (1998, 2000a) and Schroder et al (1999).

In this reviewer's opinion, the MMWG should also acknowledge studies which have shown that organic extracts from some biodiesel DEP have an enhanced ability, relative to extracts of conventional diesel DEP, to induce genetic damage and mutations that might be expected to increase the likelihood of cancer (e.g., Bunker et al, 2007; Krahl et al, 2007 and 2009).

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Finally, in this reviewer's opinion, the MMWG should also acknowledge that several researchers have noted the increased SOF of biodiesel DEP, compared to conventional diesel PM; and moreover, suggested that the differences may be responsible for the observed differences in toxicological activity (Yanamala et al, 2013; Liu et al, 2008, Bunger et al, 1998, Bunger et al, 2006, Gerlofs-Nijland et al, 2013; Bunger et al, 2000).

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**APPENDIX I: Summary of Published Information Regarding the Relative Toxicological Properties of Biodiesel and Petroleum Diesel Emissions.**

**Table 1. Summary of the published *in vivo* studies, or studies that estimated *in vivo* hazard using data on monitored toxics.**

Engine	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
Common rail direct injection 3.0L engine (Tata, Safari DICOR), photochemical reaction chamber for secondary aerosols, measurement of PM-bound PAHs.	DF, B20 (unspecified)	Conversion of PAHs to total BaP equivalents in ng/m <sup>3</sup> . Used TEFs from Nisbet and Lagoy (1992) for relative carcinogenicity.	Total BaP equivalents (i.e., total carcinogenic PAH emission rate).	Total BaP equivalents in secondary aerosols higher than primary. B20 lower than DF for both primary and secondary aerosols.	8
Branco BD-2500 diesel generator.	“Metropolitan diesel” with 3% biodiesel, SEE B50 and SEE B100.	Adult male Balb/c mice exposed to diluted exhaust (550 µg/ m <sup>3</sup> ) for 1 hr. 12 animals per exposure group.	Heart rate, heart rate variability and blood pressure, before exposure and 30, 60 mins after. Blood, BAL and bone marrow examination 24 hr after exposure.	No differences in inflammatory cell infiltration between DF and biodiesel blends. Some indication that cardiovascular irregularities increased with biodiesel relative to DF.	5
Isuzu C240 2.369L with DOC, 4 steady state conditions, high volume DEP sampling system.	ULSD and corn-derived FAME.	C57BL/6 mice exposed to DEP via pharyngeal aspiration, 0, 9 and 18 µg total C per mouse as aqueous suspension, sacrifice 1, 7 and 28 days after exposure.	Pulmonary inflammation (by BAL counts & cytokine levels), oxidative stress (by-products of lipid peroxidation), and morphological changes (by histopathological assessment).	Significant elevation in inflammatory markers for FAME relative to ULSD, evidence of increased tissue damage and oxidative stress for FAME relative to ULSD, significant elevation in inflammatory cytokines, chemokines, growth factors for FAME, histological examination showed impaired clearance and retention of FAME particulates.	7

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**Table 2. Summary of the published *in vitro* studies in cultured animal cells**

Engine	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
1979 1.6L Volkswagen Golf, ECE Euro 2 cycle, DEP collected on "filter papers".	DF, Biodiesel (unspecified) at B20, B40, B60, B80 B100.	A549 human alveolar adenocarcinoma cells exposed to 25 µg PM/mL for 5 days.	Induction of apoptosis (caspase III protein level, cytokeratin fragmentation)	Semi-quantitative analyses showed stronger induction of apoptosis by petroleum diesel, relative to biodiesel.	13
Yanmar single cylinder 296mL diesel generator, steady state at rated speed and 4 loads, DEP collected on Teflon® membranes and quartz filters.	ULSD, B100 and B50 (waste cooking oil).	A549 human alveolar adenocarcinoma cells directly exposed to PM on filters for 48 hr.	Cell viability and cytotoxicity, measured via production of fluorescent products, apoptosis as caspase III/VII, oxidative stress as GSH/GSSG ratio (Promega assays).	Cytotoxicity and oxidative stress higher for B100 relative to DF. Similar for apoptosis response. No significant difference between B100 and DF at lower engine loads, and largest difference at higher engine loads.	12
2002 Cummins 5.9L engine (EPA 2004 certified) with common rail fuel injection, EGR, DOC and DPF, steady state operation. DEP collected by "back-flush" of DPF.	DF and B20 (unspecified)	Freshly isolated rat alveolar macrophages exposed to 100-500 µg PM/mL for 24 hr.	Cytotoxicity (LDH release), inflammatory signalling ( <i>Cox-2</i> , <i>Mip-2</i> gene expression), and macrophage activation (PGE <sub>2</sub> release)	No difference in cytotoxicity between DF and B20. Some increased inflammatory signalling for DF. Some increased macrophage activation for B20.	9
Fendt tractor, 13-mode ESC, DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract.	DF and RME	L929 mouse fibroblasts exposed to solvent-exchanged extract (DMSO) in medium, 24 hr.	Cytotoxicity via Neutral Red uptake assay.	Reduction in cell viability more pronounced (at idling) for RME relative to DF (4-fold increase in potency expressed per L of exhaust). Difference not observed at rated power. RME yields higher particle emissions (g/hr).	19, 46
Volkswagen Vento 1.9L TDI with DOC, FTP-75, MVEG-A, and modified MVEG-A cycles. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract	DF and RME	L929 mouse fibroblasts exposed to solvent-exchanged extract (DMSO) in medium, 24 hr.	Cytotoxicity via Neutral Red uptake assay.	No significant difference between cytotoxic potency of RME and DF (based on relative concentration of extracts in culture medium). Slight increase in RME potency for FTP-75 only.	21

**Name:** Paul A. White, PhD

**Date:** January 14, 2014.

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Engine	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
Honda Accord (2.2L) 2.2i-CTDi (Euro4) with DOC and de-NOx, Peugeot (2.0L) 407 HDi with DOC and DPF, several composite driving cycles, DEP collected on Teflon®-coated GFFs, sonication MetOH extract.	DF, ULSD, RME	BEAS-2B bronchial epithelial cells exposed to DEP extracts suspended in culture medium, 24 hr, 0-200 µg equiv DEP per assay mL.	Cytotoxicity (necrosis, apoptosis) by flow cytometry, inflammatory stress via cytokine release (IL-6, IL-8).	On per mass basis, B50 significantly increased cytotoxicity and cytokine release. B50 and DPF both contribute to large reductions in PM emission rate. PM emission rate reduction for B50 may not be sufficient to compensate for increased potency on per mass basis.	14
Two light-duty diesel engines representing Euro2 and Euro4 standards. DEP collected on quartz filters.	ULSD, B20 RME, B20 AFME	A549 human alveolar adenocarcinoma cells, HUVEC cells, THP-1 cells exposed to 0.78–100µg PM/mL for 3 h.	DNA strand breaks in A549 cells by comet assay, and fpg-assisted comet assay, ICAM-1 and VCAM-1 expression in HUVEC cells, gene expression of <i>CCL-2</i> and <i>IL-8</i> in THP-1 cells.	All samples elicited concentration-related increases in DNA strand breaks and fpg-sensitive sites. RME B20 response lower than ULSD, AFME similar to diesel. With respect to <i>CCL-2</i> and <i>IL-8</i> expression, biodiesel responses similar or lower than DF. Levels of ICAM-1 and VACM-1 somewhat elevated for DF relative to biodiesel.	10
Kubota 1.123L D1105-T diesel engine (EPA Tier I), ISO C1 cycle, with or without DOC/POC, DEP collected using HVCI.	ULSD, HVO and RME	RAW264.7 mouse macrophage cells exposed to DEP suspension for 24 h	Production and release of proinflammatory cytokine TNF-α.	At 150 µg/mL decreased response for RME, relative to DF. HVO similar to DF. When based on per kW-hr exposures, reduced response for RME, especially with DOC/POC. Small reduction for HVO, relative to DF, without aftertreatment only. PM emission rates reduced for RME and HVO, relative to DF. Aftertreatment reduced PM emissions rates by 50-60%.	11
Kubota 1.123L D1105-T diesel engine (EPA Tier I), ISO C1 cycle, with or without DOC/POC, DEP collected using an HVCI with downstream polyurethane foam (PUF) and Teflon®-coated membrane, ultrasonic extraction with methanol.	ULSD, HVO and RME	RAW264.7 mouse macrophage cells exposed to 5–300µg/mL DEP extract and suspension of insoluble material for 24 h	DNA strand breaks by comet assay, proinflammatory cytokine production (Tnf-α, Mip-2), MTT reduction for cytotoxicity, apoptosis by flow cytometric analysis.	All samples yielded a significant concentration-related increase in cytotoxicity and DNA strand breaks. No difference in cytotoxicity across fuels types and aftertreatment. DOC/POC aftertreatment significantly reduced RME response only. ULSD and HVO elicited larger inflammatory response than RME. DOC/POC increased oxidative potential on a per mass basis; aftertreatment reduced PM emission rates by more than 50%.	16

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Engine	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
2005 Scania 6-cylinder 11.7L Euro 4 engine with EGR, Braunschweig (bus) cycle, with or without DOC/POC (for LSDF and HVO 100 only), DEP collected on Teflon® filter, ultrasonic extraction with methanol.	LSDF, RME (B100 and B30), HVO (B100 and B30)	RAW264.7 mouse macrophage cells exposed to 15–300µg/mL DEP extract and suspension of insoluble material for 24 h	MTT reduction for cytotoxicity, proinflammatory cytokine production (Tnf- $\alpha$ , Mip-2), apoptosis, cell cycle and membrane permeability by flow cytometry. DNA strand breaks by comet assay.	Little differences in cytotoxicity across the fuels and aftertreatment conditions examined. Higher inflammatory response for HVO samples; lowest for RME. Little differences in apoptosis across conditions examined; some indication of higher levels for HVO. DOC/POC greatly reduced PM emission rate and PAH content of PM.	17
Six cylinder 12L Euro III truck, no DOC, with or without DPF, 13-mode ESC, DEP collected on Teflon®-coated GFFs, ethanol/DCM (1:1) sonication extract	DF, B100, B5, B10, B20, PPO	RAW264.7 mouse macrophage cells exposed to DEP extract for 24 h	Cytotoxicity via LDH release, oxidative stress as <i>Ho-1</i> gene expression.	Biodiesel blends and PPO elicited less cytotoxicity relative to DF; B100 significantly more cytotoxic (unit unknown). No differences in <i>HO-1</i> expression. Biodiesel associated with reductions in PM (g/kWh), PAHs and oxy-PAHs (µg/kWh).	22
Four cylinder 2.976L diesel generator, steady state, DEP collected on GFFs, SVOCs collected on XAD-16, Soxhlet extraction with DCM.	DF and palm-derived FAME (B10, B30, B50, B75, B100)	BEAS-2B bronchial epithelial cells exposed to DEP extracts for 24 hr. <i>Vibrio fischeri</i> exposed to DEP extracts for 5- and 15 mins (Microtox assay)	Bacterial cytotoxicity as reduction in bioluminescence, mammalian cell cytotoxicity as reduction of MTT.	Microtox TUs show sharp reduction for biodiesel blends when expressed per µg SOF, but increase for biodiesel, with maximum at B50, for SVOCs when expressed per unit volume of exhaust. Appears to be result of increased SOF emission rate (g per kW-hr or g per L fuel) for biodiesel. Some indication of reduction in emission rate of insoluble material for biodiesel. Some indication that SVOCs of biodiesel emissions more cytotoxic than diesel.	20
1998 Opel Astra X20DTL (1.995L), continuous flow exposure system (air-liquid interface).	DF, RME (B20 and B100)	In vitro 3D human airway epithelial model, 2 or 6 hr exposures at low and high dilution.	Cytotoxicity as LDH release, oxidative stress as GSH, inflammatory response as TNF- $\alpha$ and IL-8, inflammation, necrosis, apoptosis and oxidative stress by gene expression ( <i>HO-1</i> , <i>TNF</i> , <i>IL-8</i> , <i>CASP7</i> , <i>FAS</i> )	Some indication of enhanced cytotoxicity and oxidative stress for B100, pro-inflammatory responses weak relative to air control, some indication of reduced inflammatory response for B20.	6

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Engine	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
1997 Caterpillar 3406E 14.6L engine, EPA heavy-duty transient cycle, DEP collected on Teflon®-coated GFFs, DCM extract.	DF, SME, SEE	BEAS-2B bronchial epithelial cells exposed to DMSO solutions of DEP extracts for 24 hr (equiv µg DEP per assay mL).	Cell viability via LDH release and MTT reduction, inflammatory stress via cytokine release (IL-8, IL-6).	No consistent changes in cytotoxicity, induction of cytokine release significant higher for biodiesel, relative to DF (for SOF expressed on a per mass DEP basis).	15
2000 Caterpillar C15 six cylinder 14.6L engine, 2007 MBE 4000 six cylinder 12.8L engine with EGR and DOC/DPF combination, chassis dynamometer UDDS and HHDDT, DEP collected on Teflon®-filters, PFE extraction with DCM followed by DCM/Tol, SVOCs on PUF/XAD cartridges, DCM extraction.	CARB DF, SME and AFME blends, renewable (NExBTL HVO)	Human U937 macrophages and NCI-H441 Clara cell line (exposure details not provided)	Expression of oxidative and inflammatory stress markers (CYP1A1, COX-2, IL-8, HO-1, MUC5AC). Details not provided. DNA damage by comet.	For C15, some evidence of declines in oxidative stress and inflammatory responses (per engine mile) for biodiesels relative to DF. Strong declines in oxidative stress for HVO (R100). For MBE 4000 some evidence for increase in oxidative stress and inflammatory signalling (SME and AFME only). No appreciable changes in DNA damage (all blends). Nevertheless, some indication of declines for HVO and SME relative to DF, reverse for AFME.	18

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**Table 3. Summary of published *in vitro* analyses of naked DNA exposed to diesel exhaust particulate extract**

Test Article	Fuels Examined	Exposure System	Endpoint(s) Examined	Results Obtained	Reference
2003 4.5L Cummins ISBe4 engine and 2007 Zetor Euro 3 engine, ESC, WHSC and NRSC driving cycles. DEP collected with a high-volume sampler, DCM extract.	DF, RME (B100) and RSO	Incubation of Calf thymus DNA with DEP extract for 24 h with and without rat liver S9.	Frequency of stable, bulky DNA adducts by <sup>32</sup> P-postlabelling.	Significant concentration-related increases in adduct frequency for all samples; higher responses with S9. Potency per mg PM similar for two engines, and similar across fuel types, diesel higher for WHSC. Similar potency trend per kWh.	<sup>23</sup>

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**Table 4. Summary of published results of Salmonella mutagenicity analyses of diesel exhaust particulate extracts**

Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEP and SVOCs from a 1983 Caterpillar 7L heavy-duty engine with DOC, custom 16-mode cycle representing light- and heavy-duty operation. DEP collected on Teflon®-coated GFFs, SVOCs on XAD, DCM Soxhlet extract of DEP and XAD	LSDF and SME	TA98, TA100, TA98NR and TA98/1,8DNP <sub>6</sub> , microsuspension preincubation version, Aroclor-induced rat liver S9	Mutagenic potency, per kWh, greater for LSDF compared to SME. Potency far greater for DEP extracts than SVOC samples, and DOC resulted in over 50% reduction in mutagenic activity associated with DEP and SVOC. SME emissions showed lower TPM, and reduced PAHs and 1NP relative to LSDF.	29
DEP and exhaust condensate from a Mercedes-Benz Euro 3 6.37L, 6-cylinder engine, 13-mode ESC, Teflon®-coated GFFs, DCM Soxhlet extract of DEP	DF, RSO, RME, GTL	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	All samples elicited significant positive responses. Potency (per L exhaust) higher without S9 for TA100 only. DEP extracts for RSO and heated RSO fuels yielded the highest potency samples (9.7- to 59 fold greater than DF for TA98 and 5.4- to 22.3-fold for TA100). DEP extracts for RME also significant higher than DF. Condensate samples for RSO and heated RSO also significantly elevated relative to DF (up to 13.5-fold).	39
DEP from a Fraymann single cylinder engine, 5 load modes (0–85%), with and without DOC. Teflon®-coated GFFs, DCM Soxhlet extract	DF, LSDF, RME, SME	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency (per hr engine operation) generally lower for RME and SME, compared to DF or LSDF. Under partial load DOC generally led to reduced mutagenicity. Under heavy-duty conditions (rated power), DOC frequently led to increases in mutagenic activity. Without DOC, PM emission rate (g per hr) significantly higher for biodiesel relative to diesel (especially LSDF). Authors note this is likely attributable to higher SOF (g per hr) for biodiesel.	25
DEP from a Fraymann single cylinder engine, 5 load modes (0–85%), without DOC. Teflon®-coated GFFs, DCM Soxhlet extract	DF, LSDF, RME, SME	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency, per mg DEP, frequently higher without S9, and DF potency far greater (up to 10-fold) than RME or SME. No response on TA100 for RME and SME. Potency per engine hr yielded similar results and indicates that DF potency is higher at increased load and speed. PAH emissions per engine hr greatest for DF and SME; generally lower for RME.	33

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Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEP from a Fendt tractor, 13-mode ESC, Teflon®-coated GFFs, DCM Soxhlet extract	DF and RME	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	All samples elicited significant positive responses. Both fuels yielded samples more potent (per L exhaust) without S9. At rated power, RME potency far lower than DF. At idling, little difference between with and without S9, and RME potency far lower than DF. DF 2- to 8-fold higher response on TA98 and 2- to 3-fold higher on TA100. RME yields higher particle emissions (g/hr).	19, 46
DEP from a Volkswagen Vento 1.9L TDI with DOC, FTP-75, MVEG-A, and modified MVEG-A cycles. Teflon®-coated GFFs, DCM Soxhlet extract	DF and RME	TA98, TA97a, TA102, TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Significant positive responses for DF and RME samples on TA98 and TA100, and potency (per mg DEP) generally higher without S9. Potency (per mg DEP) greater for DF compared to RME, particularly on TA98 (1.9- to 5.1-fold). Similar pattern for potency expressed per km. Potency generally higher for cycles that include a cold start (modified MVEG-A).	21
DEP from two light-duty (1.93L and 2.5L) engines with EGR, EUCD and FTP-75 cycles, with and without DPF, DOC or EGR modifications, DEP collected on Teflon®-coated GFFs, acetone sonication extract followed by separate acetone and benzene Soxhlet extractions	LSDF and biodiesel (unspecified)	TA98, TA100, standard plate-incorporation assay, unspecified S9	Mutagenic activity (per µg DEP) highest on TA98 without S9. DPF increased potency (per µg DEP or per km) for the 1.93L engine, and decreased potency for the 2.5L engine. DPF dramatically reduced PM emissions per km. Greater engine stress elicited greater mutagenic activity. Biodiesel potency (per µg DEP) lower than reference LSDF, and Biodiesel emissions lower in PAHs and nitro-PAHs. Evaluation of EGR showed reduced potency (per µg DEP or per km) with EGR.	34
DEP and SVOCs from a 4.6L, 6-cylinder Caterpillar engine, EPA heavy-duty transient test cycle. DEP collected on Teflon®-coated GFF, DCM Soxhlet extract, SVOCs on PUF plugs, supercritical CO <sub>2</sub> extraction	DF, RME, HySEE HySEE50 blend (HySEE-hydrogenated soy ethyl ester)	TA98 and TA100, microsuspension preincubation version, Aroclor-induced rat liver S9	Mutagenic potency of DEP extract (per hp-hr) higher without S9. HySEE potency lower than 50/50 blend with DF, which was lower than DF alone. SVOC samples from DF about 2-fold more mutagenic than HySEE. HySEE associated with considerable reductions in PM and PAH emission rates (per hp-hr).	28
DEP from 3 diesel engines, 1.686L, 4-cylinder light-duty, 10.8L, 6-cylinder heavy-duty with DPF and SCR, 10.52L, 6-cylinder, heavy-duty with DPF, DEP collected on GFF, DCM Soxhlet extract	DF and plant oils (peanut, rapeseed, soy, sunflower)	TA98, TA100, TA Mix, fluctuation assay (Xenometrics)	All samples in the range of the negative control with no evidence of differences in activity between the fuels.	37

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Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEP from a 1991 Detroit Diesel DDC Series 60, six cylinder 11.1L engine, heavy-duty transient cycle, DEP collected on Teflon®-coated GFFs, DCM sonication extract	DF, SME, CME, PLME, BTME, YGME (all B100)	TA98, microsuspension preincubation version, Aroclor-induced rat liver S9	For cold start only, DF and CME more potent without S9. For hot start only, DF, SME and CME appreciably greater without S9. All others more potent with S9. For cold start, with S9, potency (per µg PM equiv) of biodiesel samples all higher than DF. Without S9, all samples except SME more potent than DF. For hot start all biodiesel potency values greater than DF. Mutagenicity emission rates (rev per hph) higher for DF compared with any of the biodiesels. PM emission rate for DF almost 4-fold greater than biodiesel rates.	30
DEP from a Cummins 5.9L, heavy-duty engine, EPA heavy-duty cycle, with or without DOC, DEP collected on Teflon®-coated GFF, DCM sonication extract	DF, B20 REE, B50 REE, B100 REE	TA98, microsuspension preincubation version, Aroclor-induced rat liver S9	Mutagenic potency (per µg DEP) showed lowest mutagenicity for REE and highest for DF (with and without DOC). DOC contributed to increases in mutagenic potency per µg DEP. Similar potency pattern for potency expressed per mile. Higher potency with DOC. Some increase in 5- and 6-ring PAH emissions (µg per mile) for REE.	32
DEP and SVOCs from a Mercedes-Benz, 5.9L, 6-cylinder engine, 13-mode ESC, with and without DOC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract, SVOCs from condensates.	2 DFs, B100 RME, B20 RME	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency (unit not provided) uniformly higher without S9. Highest response for DF (reference fuel), with lowest for RME5 and RME. DOC further reduced activity of RME. No significant difference in potencies of SVOCs (per m <sup>3</sup> ), with complete elimination of activity by DOC.	47
DEP and SVOCs from a Mercedes-Benz, 6.37L, 6-cylinder engine, 13-mode ESC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract, and condensates from gas phase collected at 50 °C	DF, RME, GTL, RSO, modified RSO	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	All samples yielded a positive response, and all potency values (per litre exhaust gas) unchanged or reduced upon addition of S9. DEP extract for RSO yielded the highest potency values (9.7- to 17-fold higher than DF on TA98 and 5.4- to 6.4-fold higher than DF on TA100). Modified RSO potency 2.4- to 3.5-fold higher than RSO. RSO condensate samples also yielded the highest potency values (up to 3-fold DF). Modified RSO 3- to 5-fold higher than RSO. Few differences between DEP extracts for DF, RME and GTL, although RME significantly greater than DF on TA98 with S9 and TA100 without S9.	40, 41

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Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEP and SVOCs from 3 heavy-duty diesel engines, Mercedes-Benz, 6.37L, 6-cylinder engine, MAN, 6.87L, 6-cylinder engine, AVL single-cylinder, 1.47L engine, 13-mode ESC, ETC, and rated power. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract, SVOCs from condensates.	DF, GTL, B100 RME, B20 RME	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency (unit not provided) uniformly higher without S9. For Mercedes engine GTL lowest activity followed by DF. RME similar to DF, but RME20 significantly elevated. For AVL and MAN engines, RME20 significantly elevated relative to DF, but RME lower than DF. For SVOCs from the MAN engine, DF potency greater than RME blends. For the Mercedes and MAN engines, PM emission rates (g/kWh) for RME about half of DF.	48
DEP from a Mercedes-Benz 6.37L, 6-cylinder and an IVECO 5.9L, 6-cylinder diesel test engine with SCR, 13-mode ESC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract.	DF, RME, RSO, SMDS, B5 RME in SMDS, DF/RME/GTL blend.	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency values uniformly greater without S9. For the Mercedes engine, no significant difference in potency (per L exhaust gas) between DF, RME, SMDS and DF/RME/GTL blend. RO yielded significantly elevated potency (approximately 10-fold), also highest PM output in g/kWh. For the IVECO engine, SCR significantly reduced mutagenic potency, no difference between DF and RME, after 1000hrs SCR less effective. RME associated with reduced PM emissions (g/kWh).	35, 36
DEP from a Mercedes-Benz 6.37L, 6-cylinder engine, 13-mode ESC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract.	Two DFs, RME, GTL, 4 FAME mixtures from soy, palm and rapeseed	TA98, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency (per m <sup>3</sup> ) greater without S9 and highest for DF. RME potency less than half of DF potency. DEP emission rates lower (per kWh) for all FAMES.	26
DEP from a Mercedes-Benz 4.25L, 4-cylinder engine, 13-mode ESC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract.	DF, RME, LSDF, LSDF with high aromatic	TA98 and TA100, standard plate-incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency (per engine hr) lowest for RME. DF 4- to 5-fold higher than RME, LSDF 2- to 3-fold higher. No significant difference with and without S9. DEP emission rates (per kWh) highest for DF.	24
DEP from a 12L 6 cylinder Euro III truck, no DOC, with or without DFP, 13-mode ESC, DEP collected on Teflon®-coated GFFs, ethanol/DCM (1:1) sonication extract	DF, B100, B5, B10, B20, PPO (pure plant oil)	TA98 and YG1024, YG1029. Standard plate incorporation version, Aroclor-induced rat liver S9	No significant response in the presence of S9 for any sample. For TA98, significant response for B20 and PPO only. For YG1024, significant responses for B10, B100 and PPO only. Maximum responses on YG1024 for B100 and PPO (per µg PM). Biodiesel associated with reductions in PM (g/kWh), PAHs and oxy-PAHs (µg/kWh).	22

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Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEPs from four heavy-duty engines (8.5L, 6-cylinder, 7.4L, 6-cylinder and two 9.6L, cylinder), 13-mode ESC. DEP collected on Teflon®-coated GFF, DCM Soxhlet extract	DF, LSDF, 2 reformulated DFs, RME and RME30	TA98, TA98NR, YG1021, standard plate incorporation assay, Aroclor-induced rat liver S9	Mutagenic potency uniformly higher without S9. DF showed the highest mutagenic potency (per µg EOM), followed by LSDF reformulated DFs and RME. When expressed per kWh, RME potency lower than DF, but higher than other fuels (due to high EOM per unit mass). Good correlation between mutagenic potency per kWh and PAH emission per kWh. RME potency higher than predicted by PAH content.	31
DEP from Mercedes-Benz Euro III OM 906 6.37L six cylinder engine, ESC 13-mode test cycle, DEP collected on Teflon®-coated GFF, DCM Soxhlet extract.	DF, RME, LME, SME, PME, CME	TA98, TA100 with and without S9 (details not provided)	Responses higher without S9, and biodiesel responses (unit not provided) lower than DF. TA100 analyses of SME showed similar results relative to DF; B100 somewhat higher response. PM emission rates (g/kWh-hr) lower for all biodiesels, relative to DF. PAH emissions for biodiesels far lower, relative to DF (rate not provided).	42
DEP from an IVECO Euro 2 7.8L, 6-cylinder heavy-duty engine, 13-mode ESC. Teflon®-coated GFFs, toluene ASE extract, SVOCs on PUFs, hexane/acetone (1:1) ASE extract, fractionated on silica into 5 fraction with increasing polarity.	DF and B20 RME	TA98, TA100, TA98, TA98/1,8DNP <sub>6</sub> , YG1041 standard plate-incorporation assay, Aroclor-induced rat liver S9	All samples elicited significant positive responses. Potency per mg EOM showed little difference between DF and B20 on either strain. Expression of potency per kWh did not show any difference between DF and B20. Fractionation showed 80–83% of the mutagenicity in fractions containing nitro-PAHs, dinitro-PAHs and oxygenated PAHs. B20 emissions contained slightly lower levels of PAHs. Subsequent study showed greater potency (per kWh) on YG1041 without S9 relative to TA98.	38, 49
DEP and SVOCs from a heavy-duty, 6-cylinder 6.4L Mercedes-Benz OM 906 LA Euro 3-compliant engine, with and without DOC, ESC. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract, SVOC on chilled surface.	Low-sulphur DF, RME, B5 RME in diesel	TA98, TA100 standard plate incorporation assay, PB/5,6BF-induced rat liver S9	Mutagenic potency of DEP (per m <sup>3</sup> exhaust) modestly higher without S9. Without S9 potency highest for DF, and decreased for RME and 5% v/v RME. DOC contributed to modest reductions in potency without S9, and slight reductions with S9. DOC eliminated the mutagenic activity of SVOC.	27

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Test Article	Fuels Examined	Salmonella Strains <sup>a</sup> /Test Version	Results Obtained	Reference
DEP and SVOCs from a heavy-duty, 6-cylinder 6.4L Mercedes-Benz OM 906 LA Euro 3-compliant engine, ESC steady state cycle. DEP collected on Teflon®-coated GFFs, DCM Soxhlet extract, SVOC on chilled surface	DF, HVO, RME, JME	TA98, TA100 standard plate incorporation assay, with and without S9 (source not indicated)	Stronger responses for SVOC samples, relative to DEP extracts. SVOC samples and PM extracts for RME and JME elicited similar or greater responses on TA98 (unit not indicated), relative to DF. HVO responses much lower. RME and JME responses on TA100 substantial greater than DF. PM emission rates (g/kWhr) for RME and JME substantially lower than DF. HVO slightly lower. PAH emission rates (ng/test) substantially lower for biodiesels, relative to DF with HVO being the lowest.	43
2000 Caterpillar C15 six cylinder 14.6L engine, 2007 MBE 4000 six cylinder 12.8L engine with EGR and DOC/DPF combination, chassis dynamometer UDDS and HHDDT, DEP collected on Teflon®-filters, PFE extraction with DCM followed by DCM/Tol, SVOCs on PUF/XAD cartridges, DCM extraction.	CARB DF, SME and AFME blends, renewable (NExBTL HVO).	TA98, TA100, microsuspension preincubation version, rat liver S9	C15 engine DEP extracts, for both TA98 and TA100, general decline in potency (per engine mile) with increasing concentrations of biodiesel. For SVOCs, appreciable decline for HVO only. For MBE4000 samples, appreciable decline in potency for SME blends only.	18

<sup>a</sup>YG1021 – TA98 with plasmid pYG216, nitroreductase overproducing strain. YG1024 – TA98 with plasmid pYG219, O-acetyltransferase overproducing strain. YG1041 – TA98 with plasmid pYG233, nitroreductase and O-acetyl transferase overproducing strain. YG1026 – TA100 with plasmid pYG216, nitroreductase overproducing strain. YG1029 – TA100 with plasmid pYG219, O-acetyl transferase overproducing strain. YG1042 – TA100 with plasmid pYG233, nitroreductase and O-acetyl transferase overproducing strain.

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Review of  
Staff Report: Multimedia Evaluation of Biodiesel

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The staff report, prepared by the Multimedia Working Group (MMWG), provides an overall assessment of potential impacts on public health and the environment that may result from the production, use, and disposal of biodiesel, which is defined as a fuel composed on mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats and meets the specifications set forth by ASTM International Standard D6751. The report concludes that biodiesel will not cause a significant adverse impact on public health or the environment. The conclusion was made largely based on the results of the "California Biodiesel Multimedia Evaluation Final Tier III Report" from the researchers at University of California. As requested, this reviewer provides the following assessment and determination of whether each of the conclusions that constitute the basis of the staff report is based on sound scientific knowledge, methods, and practices, and if additional issues need to be addressed.

#### Overall Comments on the reports

The Staff Report is based on a cascade of studies conducted by University of California (UC) researchers. The PIs at UC are known scientists in the field. The evaluation procedure, as outlined in their final Tier III report, is sequential and logic. Literature cited in their reports is quite complete and up to date. Experiments were well designed and conducted. Data were carefully collected and analyzed. Therefore, it is fair to say that the UC final Tier III report and the Staff Report are based on sound scientific knowledge, methods, and practices. And consequently, the conclusions of the Staff Report are acceptable.

#### Comments on specific conclusion statements

1. Air Emissions Evaluation. Air Resources Board (ARB) staff concludes that the use of biodiesel does not pose a significant adverse impact on public health Or the environment from potential air quality impacts.

Based on engine and chassis emissions testing on multiple blends of biodiesel compared to the baseline California Air Resources Board (CARB) diesel fuel, the report concludes that for most of the criteria pollutants, toxic air contaminants, ozone precursors, and greenhouse gases, biodiesel blends could either reduce the emission into the atmosphere or impose only an insignificant adverse impact on air quality. This reviewer in general agrees with the findings of the evaluation studies that focused on the use of biodiesel. There might be a need to grow, storage, transport and process a large amount of biodiesel feedstock if portion of the biodiesel is produced with local resources. Further studies on the impact of these processes on air quality may be needed when large amount of biodiesel is used and produced in the state.

2. **Water Evaluation.** State Water Resources Control Board (SWRCB) staff concludes that there are minimal additional risks to beneficial uses of California waters by biodiesel than that posed by CARB diesel alone.

Water evaluation focused on aquatic toxicity and risks associated with fuel transport and storage (UST in particular). The report has concluded that biodiesel and biodiesel blends slightly increase the toxicity to subsets of screening species compared to CARB diesel, and that the adverse impact on public health and the environment is insignificant. Similar to the air emissions evaluation, the study does not include the effect of biodiesel production and distribution on water quality. Biodiesel is produced from biological feedstock, including plant and animal materials. Some are produced from community wastes (like “yellow grease”). It is not appropriate to assume that all biodiesel used in California will come from sources outside the state. If certain portion of the feedstock is from sources inside the state, or if certain portion of the production (conversion) process is done inside the state, an evaluation of the impact on water resources/quality by growing, transportation, storage, and conversion of large amount of biodiesel feedstock will be necessary.

3. **Public Health Evaluation.** Office of Environmental Health Hazard Assessment (OEHHA) staff concludes that the substitution of biodiesel for CARB diesel reduces the rate of carbon dioxide to the atmosphere and reduces the amount of particulate matter (PM), benzene, ethyl benzene, and polycyclic aromatic hydrocarbons (PAHs) released into the atmosphere, but may increase the emissions of oxides of nitrogen (NOx) and acrolein for certain blends.

Impact of biodiesel on public health was assessed by comparing the combustion emissions against that with petroleum based diesel fuels. Data show that there is a reduction in most of the primary pollutants from burning biodiesel, but a statistically significant increase in NOx. Since NOx is the main ingredient for ground level ozone, there should be a study on this secondary pollutant. Also, impact of feedstock collection, storage, transportation, and processing needs to be assessed if certain portion of the biodiesel is locally produced.

4. **Soil and Hazardous Waste Evaluation.** Department of Toxic Substances Control (DTSC) staff concludes that biodiesel aerobically biodegrades more readily than CARB diesel, has

potentially higher aquatic toxicity for a small subset of tested species, and generally has no significant difference in vadose zone infiltration rate.

Soil pollution and hazardous waste generation can occur during production, transportation, storage and use of biodiesel. It is very difficult to conduct a complete evaluation of the impact of biodiesel on soil and hazardous waste impact because there is a large variation in feedstock type, production method, composition (additives) and chemical properties (some of them are not yet known). The DTSC staff concludes that biodiesel is more readily to aerobically biodegrade, with higher potential aquatic toxicity for a small subset of tested species, and having a similar rate of vadose zone infiltration, compared to CARB diesel. However, the report also mentioned that biodiesel tends to move faster in the vertical than horizontal direction in subsurface soil, indicating a concern on potential deep soil and groundwater contamination. The transport and fate of chemicals in multimedia environment is also very strongly affected by meteorological and climatic variables. The studies conducted by UC researchers probably are sufficient for the purpose of impact evaluation. More research is called in the future for a better understanding of the multimedia transport and fate processes in biodiesel feedstock and fuel production, distribution, use and disposal.

Because the chemical composition of renewable diesel is similar to that of CARB diesel and renewable diesel has a lower content of aromatic hydrocarbons than CARB diesel, I agree with the DTSC staff on that the impacts on soil, surface water and groundwater of renewable diesel are similar to or less severe than that of CARB diesel. As pointed out by the DTSC Staff Report, the chemical composition and additives may vary with different feedstock and production processes. Large amount of biological feedstock also needs to be transported, stored, and processed should certain renewable diesel be produced locally. Therefore, additional studies may be needed in the future for regulatory purposes.