Health effects of soy-biodiesel emissions: bioassay-directed fractionation for mutagenicity

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Abstract

Context: Soy biodiesel is the predominant biodiesel in the USA, but there is little understanding of the classes of chemicals responsible for the mutagenicity of its emissions. Objective: We determined some of the chemical classes responsible for the mutagenicity of the particulate matter (PM) of the emissions from petroleum diesel (B0) and biodiesel containing increasing concentrations of soy methyl esters (B20, B50, and B100). Materials and methods: We subjected organic extracts of the PM to bioassay-directed fractionation by sequential elution on silica gel with solvents of increasing polarity to produce four fractions per fuel. We injected these onto high performance liquid chromatography to produce 62 sub-fractions per fraction based on chemical polarity and evaluated all fractions and sub-fractions for mutagenicity in Salmonella. We correlated the results with the concentrations of 32 polycyclic aromatic hydrocarbons (PAHs) in the fractions. Results: The mutagenicity-emission factors of the fractions generally decreased with increasing concentrations of soy in the fuel. Despite the different chemical compositions of the fuels, the extractable organics of all four emissions had similar features: ~60% of the mass was nonpolar, non-mutagenic compounds; most of the PAHs were polar; and most of the mutagenicity was due to weakly polar and polar compounds. Some of the mutagenicity of B20 was due to highly polar compounds. Conclusions: The PM from soy biodiesel emissions was less mutagenic than that from petroleum diesel, and this reduction was associated with reduced concentrations of various weakly polar, polar, and highly polar mutagens, including PAHs, aromatic amines, nitroarenes, and oxy-PAHs.

Introduction

Biodiesel is a fuel composed of fatty acid methyl esters made by transesterification of fatty acids (triglycerides) from animal fats or plant oils, typically soy or rapeseed (canola). In the USA, plant-based biodiesel is composed of 2, 5, or 20% soybean oil, whereas in Canada and Europe rapeseed oil is the primary source of biodiesel, typically at 30 or 100% (Swanson et al., 2007).

As reviewed by Bünger et al. (2012), the emissions from biodiesel relative to petroleum diesel generally have lower concentrations of carbon monoxide, hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and particulate matter (PM) but higher concentrations of nitrogen oxides and aldehydes. Approximately half of the studies found that the emissions from biodiesel fuels were less mutagenic than those from petroleum diesel (Bünger et al., 2012). Although engine load and other engineering parameters can influence the mutagenicity of the emissions, there are other technical issues that may account for this lack of coherency in the literature (Mutlu et al., 2015a).

We evaluated the PM of emissions from petroleum diesel (B0) and soy biodiesel containing 20% (B20), 50% (B50), or 100% (B100) soy methyl esters and found that the fuel-energy mutagenicity-emission factors (revertants/megajoulethermal rev/MJth) were consistently reduced with increasing amounts of biodiesel in the fuel (Mutlu et al., 2015b). These results are consistent with the chemical analyses, engineering parameters, and toxicity in mice exposed in parallel with these studies that we report elsewhere (Madden, 2015; Mutlu et al., 2015a,b).

Here we have extended our study by performing bioassay-directed fractionation and chemical analysis of the whole extracts of B0, B20, B50, and B100 soy biodiesel emissions.
The purpose of this study was to use these techniques to identify some of the main classes of chemicals responsible for the various types of mutagenicity of diesel and biodiesel emissions. Using a procedure we have applied recently to a standard set of diesel particles (Mutlu et al., 2013), we fractionated the PM extract into four fractions based on polarity and then sub-fractionated the fractions into 62 sub-fractions also by polarity by high performance liquid chromatography (HPLC) to create mutagenicity profiles (mutagrams) (Lewtas et al., 1990). Combined with analysis for 32 PAHs in the four fractions, we then correlated the PAH-emission factors with the mutagenicity-emission factors to assess the role of certain chemical classes in the mutagenicity of diesel versus biodiesel emissions.

Methods

Combustion conditions and PM collection

Details of the engineering and combustion conditions associated with this study are described in Mutlu et al. (2015 a,b). Briefly, we generated emissions from four blends of soy biodiesel: B0 (pure petroleum diesel), B20 (20% soy biodiesel, 80% petroleum diesel), B50 (50% soy biodiesel, 50% petroleum diesel), and B100 (100% soy biodiesel) using a Yanmar L70 diesel engine (Adairville, GA, USA) and Pramac E3750 generator (Marietta, GA, USA). We maintained a load of 3 kW on the engine using two electric heaters, measured a variety of engine parameters in real time, and diluted the PM ~1:10 prior to capture on 90 mm Teflon® filters at 20 L/min and fractionated these extracts to chemical analysis for 32 PAHs (Kooter et al., 2011). We performed the

Organic extractions, fractionation, and chemical analyses

We prepared dichloromethane (DCM) extracts of the PM collected on Teflon® filters at 20 L/min and fractionated these as described by Mutlu et al. (2013) on silica gel by sequential elution with solvents of increasing polarity: hexane, 75% hexane:25% DCM, DCM, methanol. We determined the percentage of extractable organic material (% EOM) by gravimetric analysis (Mutlu et al., 2013) and subjected the extracts to chemical analysis for 32 PAHs (Kooter et al., 2011).

For all but the nitro-PAHs, samples were analyzed using GC-MS (Agilent Technologies, model 5975C inert MSD), which was tuned in EI+ mode using device-specific automated protocols. The MS analysis was performed in selected ion-monitoring (SIM) conditions with a capillary column AT-5 ms (30 m × 0.25 mm × 0.25 μm) with a pulsed splitless injection of 2 μl of extract. The oven was programmed as follows: 60 °C for 1 min; 10 °C/min; 100 °C (0 min); 20 °C/ min; 320 °C (8 min). PAHs were quantified by an isotope-dilution method and related the concentrations to the added extraction standard. All 16 Environmental Protection Agency (EPA) Priority PAHs were present in the deuterated spiking solution; thus, we quantified each PAH relative to its own deuterated form.

The nitro-PAHs were analyzed by GC-NCI-MS (Agilent Technologies, model 5975B inert XL MSD), with chemical ionization performed by methane as the reaction gas tuned with device-specific automated protocols. The MS analysis was performed in SIM conditions. A capillary column AT-5 ms (30 m × 0.25 mm × 0.25 μm) with a pulsed splitless injection of 1 μl of extract was used. The oven was programmed as follows: 60 °C for 1 min; 20 °C/min; 320 °C (8 min), and the quantification of nitro-PAHs was based on an isotope-dilution method. The chemistry results are reported in full in Mutlu et al. (2015a); here we re-report the PM-emission factors from Mutlu et al. (2015a) to enhance understanding of this study. We sub-fractionated all but the hexane fraction (because it was not mutagenic) from each set of emissions by HPLC and compared the elution profiles of the same set of chemical standards to that of the mutagenicity as described by Mutlu et al. (2013).

Mutagenicity assays

We performed the Salmonella plate-incorporation mutagenicity assay with the whole and four fractions as described (Mutlu et al., 2013), which also describes the strains and positive controls. We generated dose-response data for the extracts by testing each at one plate per dose in at least two independent experiments due limited amounts of sample. We calculated the mutagenic potencies of the whole extracts (average of four plates/dose) and four fractions (average of two plates/dose) from each of the four PM emission samples based on the slopes of linear regressions of the dose-response data. We then calculated the percent distribution of mutagenicity across the four fractions by multiplying the number of rev/μg of EOM for each fraction by the number of micrograms of EOM recovered for each fraction (Supplementary Material). We then summed these values (rev/fraction) for all four fractions and expressed each as a percentage relative to the sum (Σ) (Supplementary Material). We calculated the mutagenicity-emission factors (revertants/ megajoule of fuel thermal energy consumed, rev/MJth) of the
fractions by distributing the rev/MJ\text{th} of the whole extract from Mutlu et al. (2015b) among the four fractions according to the percent distribution of the mutagenic activity shown in Supplementary Material. We determined the mutagenicity of the HPLC sub-fractions using a micro-suspension assay to generate mutagrams (Lewtas et al., 1990) as described by Mutlu et al. (2013).

**Statistical analysis**

We calculated correlations between the PAH and mutagenicity-emission factors to produce a heat map as described previously (Mutlu et al., 2013). Briefly, PAH and mutagenicity values that were missing were coded as 0s. We calculated Pearson product–moment correlations using SAS v.9.3 Proc Corr within each fuel for each strain–PAH combination across the five values: the four fractions and the whole extract. If a correlation was not defined, as when all five values were the same for the strain or PAH, then we coded the correlation coefficient as 0. We visualized the resulting correlation matrix using two-way hierarchical clustering in SAS/IMPRO v.10, where clusters were chosen using Ward’s minimum-variance method.

**Results**

**Mutagenic potencies of EOM of fractions**

The average rev/plate from two experiments for the fractions from all four fuels are shown in Tables 1–4, and the slopes of the linear regressions of these dose-response curves, which are the mutagenic potencies of the fractions (rev/\mu g of EOM), are shown in Tables 5–8. The fractions containing the highest mutagenic potencies were the weakly polar Fraction 2 for B0 and B20, and the polar Fraction 3 for B50 and B100. As the concentration of biodiesel in the fuel increased, polar compounds contributed increasingly to the mutagenic activity of the emissions, and the mutagenic potencies of the fractions decreased as did those of the whole extract.

The reconstituted mixture of the fractions of EOM had similar mutagenic potencies among most of the strains relative to the whole extracts for B0 and B20. In contrast, the reconstituted mixture of the fractions of EOM from B50 and B100 had no mutagenic activity at all in the majority of the strains, whereas the original whole extracts were mutagenic in all of the strains. Thus, the fractionation procedure altered considerably the chemical matrix of the EOM of the emissions from B50 and B100 such that the activity of the whole extracts could not be reconstituted for most of the strains.

**Distribution of mass and mutagenicity- and PAH-emission factors among the fractions**

We next distributed the eluted mass and mutagenic potencies (rev/\mu g EOM) based on percentages across the four fractions for each strain for all four fuels by multiplying the mutagenic potency of the EOM (rev/\mu g EOM) for each fraction from Tables 5–8 by the number of micrograms of EOM recovered for each fraction as noted in the second column of Tables 5–8. We expressed these values (rev/fraction) as a percentage relative to the sum of the recovered mass of the fractions noted in Tables S1–S4 in Supplementary Material. The resulting percent distributions of mutagenic activity are shown in Supplementary Material. Using these percent distributions, we then apportioned across the fractions the mutagenicity-emission factor (rev/MJ\text{th}) of each whole extract, which is reported in Mutlu et al. (2015b) and also shown in the top row of numbers in Tables 5–8. This resulted in a distribution of mutagenicity-emission factors (rev/MJ\text{th}) among the fractions for the four fuels in a variety of strains of *Salmonella* (Tables 9–12).

For B0 and B20, the highest rev/MJ\text{th} values were induced by Fraction 3 in strain YG1042 – S9, indicating that polar, base-substitution mutagens, such as modified PAHs, were a predominant cause of the mutagenicity of the emissions from these fuels (Tables 9 and 10). In general, the second highest rev/MJ\text{th} values across the strains for B0 and B20 were induced by the weakly polar Fraction 2, suggestive of unsubstituted PAHs.

For B50 the highest rev/MJ\text{th} values in all strains were also induced by the polar Fraction 3, but the highest value overall was in the frameshift strain YG1041 – S9 and its homologous base-substitution strain YG1042 – S9, indicating that nitroarenes played a more dominant role than PAHs in the mutagenicity-emission factor for B50 (Table 11). For B100 the highest rev/MJ\text{th} value was induced by the weakly polar Fraction 2 in the base-substitution strain YG1042 + S9, indicative of substituted nitroarenes and aromatic amines, again indicating a shift away from PAHs as the predominant cause of the mutagenicity as the percentage of biodiesel increased in the fuel (Table 12).

Figure 1 shows the distribution across the four fractions for all four fuels of the (a) mass of the EOM, (b) total PAH-emission factor (\mu g PAH/MJ\text{th}) reported in Mutlu et al. (2015a), and (c) mutagenicity-emission factors for TA100 + S9 and TA98 – S9 from Tables 9–12. The distribution patterns were similar for any one metric across all four fuels. Thus, most of the mass (~60%) of the EOM from the emissions from all four fuels eluted in the nonpolar Fraction 1, ~20% eluted in Fraction 4, with the remaining 20% eluting in Fractions 2 and 3. In contrast, most of the PAH-emission factors eluted in Fraction 3 for all four fuels. Again in contrast, most of the mutagenicity-emission factors for PAH-type mutagenicity (strain TA100 + S9) eluted in Fraction 2, whereas most of the nitro-PAH-type mutagenicity (strain TA98 – S9) eluted in Fraction 3. The sum of the mutagenicity-emission factors of the four fractions of any one whole extract generally equaled the mutagenicity-emission factor of the whole extract. Exceptions to these occurred among B50 and especially B100 where the mutagenicity-emission factor of the whole extract was low, in which cases the sum of the activity of the fractions for some strains, especially the nitro-PAH-detecting ones, was less than that of the whole extract.

**Correlation between PAH- and mutagenicity-emission factors**

For each fuel we used hierarchical clustering to determine the correlations among each of the PAH-emission factors (Table 13) in each fraction and the mutagenicity-emission factors for that fraction in each of the strain/activation combinations of
Table 1. Mutagenicity of fractions of organic extract of B0 in *Salmonella*.

| Strain                  | µg EOM per plate | Hexane | Hexane/DCM | DCM | Methanol | Reconstituted |
|-------------------------|------------------|--------|------------|-----|----------|--------------|
|                         |                  | +S9    | −S9        | +S9| −S9      | +S9          | −S9        | +S9      | −S9      |
| TA100                   | 0                | 109    | 101        | 115| 101      | 109          | 96         | 109      | 90       |
|                         | 0.15             |        |            | 194|          |              |            |          |          |
|                         | 0.3              |        |            | 274|          |              |            |          |          |
|                         | 0.625            |        |            | 489|          |              |            |          |          |
|                         | 1.25             |        |            | 960|          |              |            |          |          |
|                         | 2.5              |        |            | 239|          |              |            |          |          |
|                         | 5                |        |            | 128|          |              |            |          |          |
|                         | 10               |        |            | 123|          |              |            |          |          |
|                         | 20               |        |            | 125|          |              |            |          |          |
|                         | 40               |        |            | 48  |          |              |            |          |          |
| TA98                    | 0                | 42     | 34         | 40 | 34       | 42           | 34         | 42       | 35       |
|                         | 0.625            |        |            | 87 |          |              |            |          |          |
|                         | 1.25             |        |            | 132|          |              |            |          |          |
|                         | 2.5              |        |            | 209|          |              |            |          |          |
|                         | 5                |        |            | 44  |          |              |            |          |          |
|                         | 10               |        |            | 38  |          |              |            |          |          |
|                         | 20               |        |            | 48  |          |              |            |          |          |
| TA98 NR                 | 0                | 42     | 42         | 40 | 42       | 42           | 42         | 42       | 42       |
|                         | 5                |        |            | 24  |          |              |            |          |          |
|                         | 10               |        |            | 19  |          |              |            |          |          |
|                         | 20               |        |            | 19  |          |              |            |          |          |
| YG1021                  | 0                | 37     | 32         | 57 | 32       | 37           | 32         | 37       | 40       |
|                         | 0.125            |        |            | 57b |          |              |            |          |          |
|                         | 0.25             |        |            | 105b|          |              |            |          |          |
|                         | 0.5              |        |            | 291 |          |              |            |          |          |
|                         | 1                |        |            | 384 |          |              |            |          |          |
|                         | 2.5              |        |            | 973 |          |              |            |          |          |
|                         | 5                |        |            | 42  |          |              |            |          |          |
|                         | 10               |        |            | 37  |          |              |            |          |          |
|                         | 20               |        |            | 39  |          |              |            |          |          |
| YG1024                  | 0                | 46     | 29         | 46 | 29       | 46           | 29         | 46       | 29       |
|                         | 5                |        |            | 46  |          |              |            |          |          |
|                         | 10               |        |            | 108 |          |              |            |          |          |
|                         | 20               |        |            | 50  |          |              |            |          |          |
| YG1041                  | 0                | 37     | 34         | 37 | 34       | 37           | 34         | 37       | 40       |
|                         | 0.25             |        |            | 72  |          |              |            |          |          |
|                         | 0.5              |        |            | 72  |          |              |            |          |          |
|                         | 2.5              |        |            | 72  |          |              |            |          |          |
|                         | 5                |        |            | 52  |          |              |            |          |          |
|                         | 10               |        |            | 52  |          |              |            |          |          |
|                         | 20               |        |            | 53  |          |              |            |          |          |
| YG1042                  | 0                | 148    | 22         | 148| 22       | 148          | 22         | 148      | 354      |
|                         | 0.25             |        |            | 315 |          |              |            |          |          |
|                         | 0.5              |        |            | 474 |          |              |            |          |          |
|                         | 2.5              |        |            | 911 |          |              |            |          |          |
|                         | 5                |        |            | 70  |          |              |            |          |          |
|                         | 10               |        |            | 87  |          |              |            |          |          |
|                         | 20               |        |            | 72  |          |              |            |          |          |
| TA104                   | 0                | 352    | 269        | 352| 269      | 352          | 269        | 352      | 269      |
|                         | 0.625            |        |            | 597 |          |              |            |          |          |
|                         | 1.25             |        |            | 597 |          |              |            |          |          |
|                         | 2.5              |        |            | 597 |          |              |            |          |          |
|                         | 5                |        |            | 352 |          |              |            |          |          |
|                         | 10               |        |            | 352 |          |              |            |          |          |
|                         | 20               |        |            | 352 |          |              |            |          |          |
|                         | 40               |        |            | 352 |          |              |            |          |          |

aData not used in the linear regressions because they were outside of the linear portion of the dose-response curves.

bData from a single plate from a single experiment; other data are the average of two or more plates, each from an independent experiment. Positive control data (average rev/plate, range) are 2-aminoanthracene + S9: TA100 (670, 233-1175), TA98 (463, 239-964), YG1021 (411, 102-831), YG1024 (1373, 780-2364), YG1041 (968, 450-1620), YG1042 (556, 223-1166), TA104 (654, 572-751); sodium azide −S9: TA100 (683, 501-862), YG1042 (830, 750-960); 2-nitrofluorene −S9: TA98 (387, 270-516), TA98NR (62, 41-93), TA98/1, DNP6 (83, 60-112), YG1021 (1572, 787-2341), YG1024 (1992, 1332-2706), YG1041 (1558, 1307-1864); methylglyoxal −S9: TA104 (526, 427-605).
Table 2. Mutagenicity of fractions of organic extract of B20 in *Salmonella*.

| Strain      | µg EOM per plate | Hexane   | Hexane/DCM | DCM     | Methanol | Reconstituted |
|-------------|------------------|----------|------------|---------|----------|--------------|
|             |                  | +S9      | −S9        | +S9     | −S9      | +S9          | −S9          |
| TA100       |                  | 104      | 89         | 109     | 101      | 104          | 89           |
|             | 0.3              | 115      | 101        | 110     | 101      | 109          | 101          |
|             | 0.625            | 183      | 101        | 113     | 113      | 142          |              |
|             | 1.25             | 235      | 160        | 236     | 113      | 178          | 109          |
|             | 2.5              | 727      | 190        | 320     | 133      | 324          | 143          |
|             | 5                | 124      | 356        | 242     | 110      | 463          | 186          |
|             | 10               | 105      | 639        | 363     | 127      | 1038         | 253          |
|             | 20               | 114      | 975        | 624     | 153      |              |              |
|             |                  | 118      | 160        | 483     | 156      |              |              |
|             | 40               | 104      | 110        | 324     | 253      |              |              |
|             | 80               | 109      |            | 143     |          |              |              |
| TA98        |                  | 42       | 34         | 46      | 35       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| TA98NR      |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| TA98/1,8-DNP6 |                | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| YG1021      |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| YG1024      |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| YG1041      |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| YG1042      |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |
| TA104       |                  | 42       | 31         | 42      | 34       | 42           | 34           |
|             | 2.5              | 149      | 34         | 164     | 87       | 52           | 49           |
|             | 5                | 46       | 72         | 73      | 44       | 53           |              |
|             | 10               | 50       | 101        | 95      | 51       | 39           |              |
|             | 20               | 52       | 249        | 61      | 73       | 68           |              |
|             | 40               | 114      | 190        | 486     | 62       |              |              |
|             | 80               | 113      |            | 58b     |          |              |              |

aData not used in the linear regressions because they were outside of the linear portion of the dose-response curves.

bData from a single plate from a single experiment; other data are the average of two or more plates, each from an independent experiment.
Table 3. Mutagenicity of fractions of organic extract of B50 in *Salmonella*.

| Strain | µg EOM per plate | Hexane | Hexane/DCM | DCM | Methanol | Reconstituted | Rev/plate |
|--------|------------------|--------|------------|------|----------|--------------|-----------|
|        |                  | +S9    | −S9        | +S9  | −S9      | +S9          | −S9       |
| TA100  | 0                | 109    | 101        | 109  | 101      | 107          | 90        |
|        | 2.5              | 170    | 116        | 172  | 126      |              |           |
|        | 5                | 119    | 106        | 241  | 101      | 243          | 130       |
|        | 10               | 111    | 100        | 401  | 102      | 391          | 127       |
|        | 20               | 125    | 116        | 546  | 112      | 638          | 157       |
|        | 40               |        |            |      |          | 169          | 125       |
|        |                  |        |            |      |          | 260          |           |
| TA98   | 0                | 42     | 34         | 42   | 34       | 42           | 34        |
|        | 2.5              | 49     | 49         | 56   | 58       |              |           |
|        | 5                | 44     | 38         | 66   | 41       | 67           | 39        |
|        | 10               | 47     | 49         | 56   | 58       |              | 55        |
|        | 20               | 37     | 46         | 59   | 58       |              |           |
|        | 40               |        |            |      |          | 58           |           |
| TA98NR | 0                | 42     | 42         | 42   | 36       |              | 36        |
|        | 2.5              | 30     | 52         |      |          |              |           |
|        | 5                | 44     | 40         | 65   | 42       | 47           |           |
|        | 10               | 47     | 49         | 56   | 40       | 41           |           |
|        | 20               | 37     | 46         | 59   | 35       | 38           |           |
|        | 40               |        |            |      |          | 41           |           |
| TA98/1,8DNP6 | 0             | 34     | 34         | 34   | 28       |              | 34        |
|        | 2.5              | 34     | 34         | 34   | 28       |              |           |
|        | 5                | 27     | 30         | 34   | 30       | 21           |           |
|        | 10               | 35     | 38         | 45   | 29       | 31           |           |
|        | 20               | 30     | 43         | 54   | 26       | 22           |           |
| YG1021 | 0                | 37     | 32         | 37   | 32       |              | 37        |
|        | 0.5              | 32     | 37         | 37   | 32       |              | 32        |
|        | 1                | 37     | 25         | 37   | 51       |              |           |
|        | 2.5              | 37     | 25         | 37   | 51       |              |           |
|        | 5                | 40     | 35         | 45   | 55       | 39           |           |
|        | 10               | 42     | 35         | 45   | 55       | 39           |           |
|        | 20               | 43     | 32         | 419  | 74       | 39           |           |
|        | 40               |        |            |      |          | 234          | 94        |
| YG1024 | 0                | 37     | 25         | 46   | 29       | 38           | 25        |
|        | 0.5              | 32     | 25         | 46   | 29       | 38           | 25        |
|        | 1                | 37     | 25         | 46   | 29       |              |           |
|        | 2.5              | 37     | 25         | 46   | 29       |              |           |
|        | 5                | 40     | 35         | 45   | 55       | 39           |           |
|        | 10               | 42     | 35         | 45   | 55       | 39           |           |
|        | 20               | 43     | 32         | 419  | 74       | 39           |           |
|        | 40               |        |            |      |          | 234          | 94        |
| YG1041 | 0                | 37     | 25         | 46   | 29       |              | 38        |
|        | 2.5              | 37     | 25         | 46   | 29       |              | 38        |
|        | 5                | 40     | 35         | 45   | 55       | 39           |           |
|        | 10               | 42     | 35         | 45   | 55       | 39           |           |
|        | 20               | 43     | 32         | 419  | 74       | 39           |           |
|        | 40               |        |            |      |          | 234          | 94        |
| YG1042 | 0                | 37     | 25         | 46   | 29       |              | 38        |
|        | 2.5              | 37     | 25         | 46   | 29       |              | 38        |
|        | 5                | 40     | 35         | 45   | 55       | 39           |           |
|        | 10               | 42     | 35         | 45   | 55       | 39           |           |
|        | 20               | 43     | 32         | 419  | 74       | 39           |           |
|        | 40               |        |            |      |          | 234          | 94        |
| TA104  | 0                | 37     | 25         | 46   | 29       |              | 38        |
|        | 2.5              | 37     | 25         | 46   | 29       |              | 38        |
|        | 5                | 40     | 35         | 45   | 55       | 39           |           |
|        | 10               | 42     | 35         | 45   | 55       | 39           |           |
|        | 20               | 43     | 32         | 419  | 74       | 39           |           |
|        | 40               |        |            |      |          | 234          | 94        |

aData not used in the linear regressions because they were outside of the linear portion of the dose-response curves.

bData from a single plate from a single experiment; other data are the average of two or more plates, each from an independent experiment.
Table 4. Mutagenicity of fractions of organic extract of B100 in Salmonella.

| Strain  | µg EOM per plate | Hexane | Hexane/DCM | DCM | Methanol | Reconstituted |
|---------|------------------|--------|------------|-----|----------|---------------|
|         |                  | +S9    | −S9        | +S9| −S9      | +S9          |
| TA100   | 0                | 109    | 101        | 109| 101      | 109          |
|         | 2.5              | 34     | 34         | 34 | 34       | 34           |
|         | 5                | 51     | 53         | 51 | 53       | 51           |
|         | 10               | 63     | 59         | 63 | 59       | 63           |
|         | 20               | 59     | 59         | 59 | 59       | 59           |
| TA98    | 0                | 42     | 42         | 42 | 42       | 42           |
|         | 2.5              | 34     | 34         | 34 | 34       | 34           |
|         | 5                | 52     | 50         | 52 | 50       | 52           |
|         | 10               | 52     | 50         | 52 | 50       | 52           |
|         | 20               | 50     | 50         | 50 | 50       | 50           |
| TA98NR  | 0                | 42     | 42         | 42 | 42       | 42           |
|         | 2.5              | 34     | 34         | 34 | 34       | 34           |
|         | 5                | 52     | 50         | 52 | 50       | 52           |
|         | 10               | 52     | 50         | 52 | 50       | 52           |
|         | 20               | 50     | 50         | 50 | 50       | 50           |
| TA98/1,8DNP6 | 0  | 34     | 34         | 34 | 34       | 34           |
|         | 2.5              | 34     | 34         | 34 | 34       | 34           |
|         | 5                | 28     | 28         | 28 | 28       | 28           |
|         | 10               | 41     | 41         | 41 | 41       | 41           |
|         | 20               | 43     | 43         | 43 | 43       | 43           |
| YG1021  | 0                | 37     | 37         | 37 | 37       | 37           |
|         | 0.5              | 32     | 32         | 32 | 32       | 32           |
|         | 1                | 34     | 34         | 34 | 34       | 34           |
|         | 2.5              | 42     | 42         | 42 | 42       | 42           |
|         | 5                | 52     | 52         | 52 | 52       | 52           |
|         | 10               | 52     | 52         | 52 | 52       | 52           |
|         | 20               | 52     | 52         | 52 | 52       | 52           |
| YG1024  | 0                | 46     | 46         | 46 | 46       | 46           |
|         | 0.5              | 46     | 46         | 46 | 46       | 46           |
|         | 1                | 42     | 42         | 42 | 42       | 42           |
|         | 2.5              | 49     | 49         | 49 | 49       | 49           |
|         | 5                | 52     | 52         | 52 | 52       | 52           |
|         | 10               | 52     | 52         | 52 | 52       | 52           |
|         | 20               | 52     | 52         | 52 | 52       | 52           |
| YG1041  | 0                | 62     | 62         | 62 | 62       | 62           |
|         | 0.5              | 62     | 62         | 62 | 62       | 62           |
|         | 2.5              | 62     | 62         | 62 | 62       | 62           |
|         | 5                | 72     | 72         | 72 | 72       | 72           |
|         | 10               | 129    | 129        | 129| 129      | 129          |
|         | 20               | 150    | 150        | 150| 150      | 150          |
| YG1042  | 0                | 81     | 81         | 81 | 81       | 81           |
|         | 0.5              | 81     | 81         | 81 | 81       | 81           |
|         | 2.5              | 81     | 81         | 81 | 81       | 81           |
|         | 5                | 84     | 84         | 84 | 84       | 84           |
|         | 10               | 104    | 104        | 104| 104      | 104          |
|         | 20               | 105    | 105        | 105| 105      | 105          |
| TA104   | 0                | 392    | 392        | 392| 392      | 392          |
|         | 0.5              | 353    | 353        | 353| 353      | 353          |
|         | 2.5              | 374    | 374        | 374| 374      | 374          |
|         | 5                | 374    | 374        | 374| 374      | 374          |
|         | 10               | 374    | 374        | 374| 374      | 374          |
|         | 20               | 351    | 351        | 351| 351      | 351          |

aData not used in the linear regressions because they were outside of the linear portion of the dose-response curves.

bData from a single plate from a single experiment; other data are the average of two or more plates, each from an independent experiment.
### Table 5. Mutagenic potencies (rev/µg of EOM) of fractions of B0.

| Strain          | Whole | Hexane | Hexane/DCM | DCM  | Methanol | Reconstituted |
|-----------------|-------|--------|------------|------|----------|---------------|
| TA100 +S9       | 26.8  | 0.0    | 682.6      | 63.6 | 0.0      | 21.1          |
| TA100 – S9      | 6.8   | 0.0    | 46.6       | 46.7 | 0.0      | 0.0           |
| TA98 +S9        | 3.7   | 0.0    | 85.1       | 14.9 | 0.0      | 3.2           |
| TA98 – S9       | 2.7   | 0.0    | 14.3       | 15.3 | 0.0      | 1.8           |
| TA98NR – S9     | 1.4   | 0.0    | 7.7        | 9.2  | 0.0      | 0.0           |
| TA98/DCM – S9   | 1.2   | 0.0    | 9.1        | 0.0  | 0.0      | 0.0           |
| YG1021 +S9      | 23.1  | 0.0    | 376.4      | 53.7 | 3.3      | 20.7          |
| YG1021 – S9     | 14.9  | 0.0    | 148.2      | 66.6 | 4.1      | 12.6          |
| YG1024 +S9      | 11.6  | 0.0    | 137.3      | 38.1 | 4.4      | 10.2          |
| YG1024 – S9     | 20.3  | 0.0    | 187.3      | 125.3| 8.3      | 16.8          |
| YG1041 +S9      | 18.3  | 0.0    | 310.7      | 50.5 | 4.0      | 18.2          |
| YG1041 – S9     | 42.7  | 0.0    | 596.9      | 271.1| 9.3      | 35.5          |
| YG1042 +S9      | 28.7  | 0.0    | 321.4      | 33.0 | 4.5      | 18.2          |
| YG1042 – S9     | 33.0  | 0.0    | 23.6       | 31.3 | 0.0      | 4.1           |
| TA104 + S9      | 13.2  | NES    | 306.1      | 27.6 | 0.0      | 9.4           |
| TA104 – S9      | 4.1   | NES    | 0.0        | 0.0  | 0.0      | 0.0           |

Data are slopes of linear regressions; Whole from Mutlu et al. (2015b); others calculated from the data in Table 1. NES, not enough sample to test.

### Table 6. Mutagenic potencies (rev/µg of EOM) of fractions of B20.

| Strain          | Whole | Hexane | Hexane/DCM | DCM  | Methanol | Reconstituted |
|-----------------|-------|--------|------------|------|----------|---------------|
| TA100 +S9       | 13.7  | 0.0    | 249.1      | 25.3 | 0.0      | 11.6          |
| TA100 – S9      | 6.1   | 0.0    | 53.6       | 22.0 | 0.0      | 2.1           |
| TA98 +S9        | 2.1   | 0.0    | 43.6       | 12.0 | 0.0      | 1.9           |
| TA98 – S9       | 2.4   | 0.0    | 10.5       | 12.8 | 1.7      | 1.6           |
| TA98NR – S9     | 1.3   | 0.0    | 6.3        | 0.0  | 0.0      | 0.0           |
| TA98/DCM – S9   | 1.2   | 0.0    | 194.1      | 32.6 | 3.1      | 12.2          |
| YG1021 +S9      | 11.6  | 0.0    | 108.9      | 48.0 | 4.8      | 7.5           |
| YG1021 – S9     | 8.9   | 0.0    | 75.9       | 34.5 | 3.8      | 4.3           |
| YG1024 +S9      | 6.9   | 0.0    | 112.6      | 109.0| 7.2      | 11.1          |
| YG1024 – S9     | 15.0  | 0.0    | 138.4      | 44.2 | 4.1      | 10.5          |
| YG1041 +S9      | 10.7  | 0.0    | 356.7      | 214.6| 10.4     | 24.6          |
| YG1041 – S9     | 26.8  | 0.0    | 207.1      | 14.2 | 0.0      | 10.4          |
| YG1042 +S9      | 24.0  | 0.0    | 12.4       | 0.0  | 0.0      | 0.0           |
| TA104 + S9      | 5.7   | NES    | 128.6      | 14.5 | NES      | 6.1*          |
| TA104 – S9      | 3.8   | NES    | 12.9*      | 0.0  | NES      | 0.0           |

Data are slopes of linear regressions; Whole from Mutlu et al. (2015b); others calculated from the data in Table 2. NES, not enough sample to test.

### Table 7. Mutagenic potencies (rev/µg of EOM) of fractions of B50.

| Strain          | Whole | Hexane | Hexane/DCM | DCM  | Methanol | Reconstituted |
|-----------------|-------|--------|------------|------|----------|---------------|
| TA100 +S9       | 3.0   | 0.0    | 29.5       | 26.7 | 0.0      | 4.8           |
| TA100 – S9      | 2.9   | 0.0    | 0.0        | 6.6  | 0.0      | 0.0           |
| TA98 +S9        | 0.6   | 0.0    | 2.2        | 4.3  | 0.0      | 0.0           |
| TA98 – S9       | 1.0   | 0.0    | 0.0        | 7.6  | 0.0      | 0.0           |
| TA98NR – S9     | 0.7   | 0.0    | 0.0        | 0.0  | 0.0      | 0.0           |
| TA98/DCM – S9   | 0.6   | 0.0    | 0.0        | 0.0  | 0.0      | 0.0           |
| YG1021 +S9      | 4.2   | 0.0    | 14.2       | 19.0 | 1.8      | 4.8           |
| YG1021 – S9     | 3.7   | 0.0    | 5.0        | 15.8 | 2.2      | 3.4           |
| YG1024 +S9      | 3.6   | 0.0    | 6.0        | 13.8 | 2.9      | 3.3           |
| YG1024 – S9     | 6.8   | 0.0    | 3.5        | 22.7 | 3.7      | 4.4           |
| YG1041 +S9      | 4.4   | 0.0    | 15.3       | 22.4 | 0.0      | 5.0           |
| YG1041 – S9     | 9.1   | 0.0    | 15.6       | 47.5 | 6.9      | 8.1           |
| YG1042 +S9      | 7.4   | 0.0    | 25.9       | 27.1 | 0.0      | 0.0           |
| YG1042 – S9     | 7.1   | 0.0    | 0.0        | 4.5  | 0.0      | 0.0           |
| TA104 + S9      | 2.3   | NES    | 0.0        | 0.0  | 0.0      | 0.0           |
| TA104 – S9      | 1.7   | NES    | 0.0        | 0.0  | 0.0      | 0.0           |

Data are the slopes of linear regressions; Whole from Mutlu et al. (2015b); others calculated from the data in Table 3. NES, not enough samples to test.
the correlation (gray color). A few of these PAHs showed a correlation or had insufficient data to permit assessment of PAHs. For the other fuels these PAHs exhibited weak associations, and even then the correlation did not include the nitro- PAHs. A strong correlation with mutagenicity only for B50 emissions. In contrast to B0, B50 showed a gray block for the other PAHs for approximately half of the strains/conditions. This indicates that the oxy-PAHs played a smaller role in the mutagenicity of B20 emissions than they did in that of the other fuels. For the last 12 PAHs, which contain mostly the remaining EPA Priority PAHs along with 3 of the nitro-PAHs, there was a strong correlation with mutagenicity only for B50 emissions, and even then the correlation did not include the nitro-PAHs. For the other fuels these PAHs exhibited weak correlation or had insufficient data to permit assessment of the correlation (gray color). A few of these PAHs showed a strong negative correlation (blue) with the mutagenicity of B0 and to some extent with B20 and B100.

### Mutagens of sub-fractions of fractions of EOM

Figure 3 shows the elution profiles of the mutagenicity (mutagrams) of 62 sub-fractions of Fractions 2, 3, and 4 of B0, B20, and B50 generated by HPLC compared with the elution profile of a set of 10 chemical standards. We could not sub-fractionate any of the four fractions of B100 due to inadequate amounts of EOM of each fraction. Also, we did not sub-fractionate Fraction 1 from B0, B20, or B50 because Fraction 1 was not mutagenic for any of the emissions from these fuels, and we have found with other combustion emissions that sub-fractionation of a non-mutagenic fraction does not produce any peaks of mutagenicity in the mutagram (D.M. DeMarini, unpublished data).

The mutagrams of Fraction 2 of all three fuels had peaks of mutagenic activity that eluted in similar fractions. The prominent class of mutagenicity was in strain TA100 + S9 and, thus, likely due to PAHs; it showed a broad and high peak at sub-fractions 33–36 for B0, a narrower but similarly high peak for B20, and a much narrower and lower peak for B50. These sub-fractionation data confirm the reduced role of unsubstituted PAHs in the mutagenic activity of the fuels with increasing concentrations of biodiesel to the fuel. Although B0 and B20 also exhibited peaks of mutagenicity at sub-fraction 36 due to nitro-PAH activity (higher in TA98 relative to TA98NR), more of this nitro-PAH-type activity eluted in B20 than in B0. In contrast, almost no such activity eluted in Fraction 2 of B50. Thus, along with standard PAHs, mutagenicity due to nitro-PAHs accounted for less of the mutagenicity of the emissions as the percentage of biodiesel in the fuel increased.

In contrast to Fraction 2, the mutagrams of Fraction 3 had predominant peaks of mutagenicity in strain TA98 rather than TA100 and also showed two distinct peaks of mutagenicity, with the pattern being similar for B0 and B20. In both B0 and B20 the predominant peaks were of nitroarene-associated

| Strain     | Whole | Hexane | Hexane/DCM | DCM | Methanol | Reconstituted |
|------------|-------|--------|------------|-----|----------|---------------|
| TA100 + S9 | 2.1   | 0.0    | 15.0       | 0.0 | 0.0      | 0.0           |
| TA100 – S9 | 1.2   | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| TA98 + S9  | 0.3   | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| TA98 – S9  | 0.5   | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| TA98NR – S9| 0.3   | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| TA98/DNP6 – S9 | 0.3 | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| YG1021 + S9 | 1.9   | 0.0    | 8.8        | 2.5 | 2.9      | 3.2           |
| YG1021 – S9 | 1.7   | 0.0    | 3.3        | 6.5 | 2.4      | 1.1           |
| YG1024 + S9 | 1.7   | 0.0    | 4.2        | 3.6 | 2.6      | 1.5           |
| YG1024 – S9 | 2.8   | 0.0    | 6.2        | 9.1 | 5.3      | 3.9           |
| YG1041 + S9 | 2.1   | 0.0    | 8.0        | 5.3 | 4.6      | 0.0           |
| YG1041 – S9 | 4.2   | 0.0    | 12.3       | 26.4| 5.3      | 6.7           |
| YG1042 + S9 | 3.4   | 0.0    | 8.6        | 0.0 | 0.0      | 0.0           |
| YG1042 – S9 | 3.7   | 0.0    | 0.0        | 0.0 | 0.0      | 0.0           |
| TA104 + S9  | 1.1   | 0.0    | NES        | NES | 0.0      | 0.0           |
| TA104 – S9  | 0.8   | 0.0    | NES        | NES | NES      | 0.0           |

Data are the slopes of linear regressions; Whole from Mutlu et al. (2015b); others calculated from the data in Table 4. NES, not enough sample to test.
Table 9. Distribution of mass and mutagenicity-emission factors (rev/MJ\textsubscript{th}) among fractions of B0.

| Sample     | EOM (µg) | Recovery (%) | Recovered mass (%) | TA100 \(+S9\) | TA100 \(-S9\) | TA98 \(+S9\) | TA98 \(-S9\) | TA98/\textsubscript{DNP6} | YG1021 \(+S9\) | YG1021 \(-S9\) | YG1024 \(+S9\) | YG1024 \(-S9\) | YG1041 \(+S9\) | YG1041 \(-S9\) | YG1042 \(+S9\) | YG1042 \(-S9\) | TA104 \(+S9\) | TA104 \(-S9\) |
|------------|----------|--------------|--------------------|----------------|---------------|---------------|---------------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Whole      | 105 790  |              |                    | 2.9            | 0.8           | 0.4           | 0.3           | 0.2             | 0.1            | 2.5            | 1.6            | 1.3            | 2.2            | 2.0            | 4.6            | 3.1            | 3.6            | 1.4            | 0.5            |
| 1 Hexane   | 71 600   | 68.0         | 67.0               | 0.0            | 0.0           | 0.0           | 0.0           | 0.0             | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            |
| 2 Hexane/DCM | 3180     | 3.0          | 3.0                | 2.4            | 0.2           | 0.3           | 0.1           | 0.1             | 0.1            | 1.8            | 0.7            | 0.7            | 0.8            | 1.3            | 2.1            | 2.3            | 0.9            | 1.0            | 0.0            |
| 3 DCM      | 7550     | 7.1          | 7.0                | 0.5            | 0.6           | 0.1           | 0.2           | 0.1             | 0.0            | 0.6            | 0.7            | 0.4            | 1.2            | 0.5            | 2.3            | 0.6            | 2.7            | 0.2            | 0.0            |
| 4 MeOH     | 24 750   | 23.4         | 23.0               | 0.0            | 0.0           | 0.0           | 0.0           | 0.0             | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            |
| \(\sum^a\) | 107 080  | 101.5        | 100.0              | 2.9            | 0.8           | 0.4           | 0.3           | 0.2             | 0.1            | 2.5            | 1.6            | 1.3            | 2.3            | 1.9            | 4.7            | 3.2            | 3.6            | 1.4            | 0.0            |

\(^a\)Sum of the fractions; values for mass and rev/MJ\textsubscript{th} for the whole extract for each strain are from Mutlu et al. (2015a,b).

Table 10. Distribution of mass and mutagenicity-emission factors (rev/MJ\textsubscript{th}) among fractions of B20.

| Sample     | EOM (µg) | Recovered (%) | Recovered mass (%) | TA100 \(+S9\) | TA100 \(-S9\) | TA98 \(+S9\) | TA98 \(-S9\) | TA98/\textsubscript{DNP6} | YG1021 \(+S9\) | YG1021 \(-S9\) | YG1024 \(+S9\) | YG1024 \(-S9\) | YG1041 \(+S9\) | YG1041 \(-S9\) | YG1042 \(+S9\) | YG1042 \(-S9\) | TA104 \(+S9\) | TA104 \(-S9\) |
|------------|----------|--------------|--------------------|----------------|---------------|---------------|---------------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Whole      | 80 750   |              |                    | 1.1            | 0.5           | 0.2           | 0.2           | 0.1             | 0.1            | 0.9            | 0.7            | 0.5            | 1.2            | 0.8            | 2.1            | 1.4            | 1.9            | 0.5            | 0.3            |
| 1 Hexane   | 53 500   | 66.3         | 68.1               | 0.0            | 0.0           | 0.0           | 0.0           | 0.0             | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            | 0.0            |
| 2 Hexane/DCM | 2500     | 3.1          | 3.2                | 0.9            | 0.3           | 0.1           | 0.0           | 0.0             | 0.0            | 0.1            | 0.6            | 0.3            | 0.2            | 0.4            | 0.4            | 0.9            | 1.2            | 0.0            | 0.5            |
| 3 DCM      | 5000     | 6.2          | 6.4                | 0.2            | 0.2           | 0.1           | 0.1           | 0.1             | 0.0            | 0.2            | 0.3            | 0.2            | 0.7            | 0.3            | 1.1            | 0.2            | 1.9            | 0.0            | 0.0            |
| 4 MeOH     | 17 500   | 21.7         | 22.3               | 0.0            | 0.0           | 0.0           | 0.1           | 0.0             | 0.0            | 0.1            | 0.1            | 0.1            | 0.2            | 0.1            | 0.2            | 0.0            | 0.0            | 0.0            | 0.0            |
| \(\sum^a\) | 78 500   | 97.3         | 100.0              | 1.1            | 0.5           | 0.2           | 0.2           | 0.1             | 0.1            | 0.9            | 0.7            | 0.5            | 1.3            | 0.8            | 2.2            | 1.4            | 1.9            | 0.5            | 0.0            |

\(^a\)Sum of the fractions; values for mass and rev/MJ\textsubscript{th} for the whole extract for each strain are from Mutlu et al. (2015a,b).
Table 11. Distribution of mass and mutagenicity-emission factors (rev/MJth) among fractions of B50.

| Sample          | EOM (µg) | Recovery (%) | Recovered mass (%) | TA100 + S9 | TA100 − S9 | TA98 + S9 | TA98 − S9 | TA98NR | TA98/ DNP6 | YG1021 + S9 | YG1021 − S9 | YG1024 + S9 | YG1024 − S9 | YG1041 + S9 | YG1041 − S9 | YG1042 + S9 | YG1042 − S9 | TA104 + S9 | TA104 − S9 |
|-----------------|----------|--------------|--------------------|------------|------------|-----------|-----------|---------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Whole           | 67 160   |              |                    | 0.40       | 0.3        | 0.1       | 0.1       | 0.1     | 0.5        | 0.5        | 0.5        | 0.8        | 0.5        | 1.1        | 0.9        | 0.9        | 0.3        | 0.2        |
| 1 Hexane        | 36 500   | 54.3         | 61.6               | 0.0        | 0.0        | 0.0       | 0.0       | 0.0     | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        |
| 2 Hexane/DCM    | 1500     | 2.2          | 2.5                | 0.1        | 0.0        | 0.0       | 0.0       | 0.0     | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.1        | 0.0        | NES        | NES        |
| 3 DCM           | 10 000   | 14.9         | 16.9               | 0.3        | 0.3        | 0.1       | 0.1       | 0.0     | 0.4        | 0.4        | 0.4        | 0.7        | 0.5        | 0.9        | 0.8        | 0.9        | 0.0        | NES        |
| 4 MeOH          | 11 250   | 16.8         | 19.0               | 0.0        | 0.0        | 0.0       | 0.0       | 0.0     | 0.1        | 0.1        | 0.1        | 0.0        | 0.1        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        |
| Σα              | 59 250   | 88.2         | 100                | 0.40       | 0.3        | 0.1       | 0.1       | 0.0     | 0.4        | 0.5        | 0.5        | 0.8        | 0.5        | 1.0        | 0.9        | 0.9        | 0.0        | 0.0        |

αSum of the fractions; values for mass and rev/MJth for the whole extract for each strain are from Mutlu et al. (2015a,b). NES, not enough sample to test.

Table 12. Distribution of mass and mutagenicity-emission factors (rev/MJth) among fractions of B100.

| Sample          | EOM (µg) | Recovery (%) | Recovered mass (%) | TA100 + S9 | TA100 − S9 | TA98 + S9 | TA98 − S9 | TA98NR | TA98/ DNP6 | YG1021 + S9 | YG1021 − S9 | YG1024 + S9 | YG1024 − S9 | YG1041 + S9 | YG1041 − S9 | YG1042 + S9 | YG1042 − S9 | TA104 + S9 | TA104 − S9 |
|-----------------|----------|--------------|--------------------|------------|------------|-----------|-----------|---------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Whole           | 98 730   |              |                    | 0.4        | 0.2        | 0.1       | 0.1       | 0.1     | 0.30       | 0.30       | 0.30       | 0.50       | 0.40       | 0.80       | 0.6        | 0.7        | 0.2        | 0.2        |
| 1 Hexane        | 46 500   | 47.1         | 54.3               | 0.0        | 0.0        | 0.0       | 0.0       | 0.0     | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        | 0.0        |
| 2 Hexane/DCM    | 10 000   | 10.1         | 11.7               | 0.4        | 0.0        | 0.0       | 0.0       | 0.0     | 0.16       | 0.06       | 0.10       | 0.12       | 0.14       | 0.18       | 0.6        | 0.0        | NES        | NES        |
| 3 DCM           | 13 250   | 13.4         | 15.5               | 0.0        | 0.0        | 0.0       | 0.0       | 0.0     | 0.06       | 0.16       | 0.11       | 0.23       | 0.13       | 0.50       | 0.0        | 0.0        | NES        | NES        |
| 4 MeOH          | 15 900   | 16.1         | 18.5               | 0.0        | 0.0        | 0.0       | 0.0       | 0.0     | 0.08       | 0.07       | 0.09       | 0.16       | 0.13       | 0.12       | 0.0        | 0.0        | 0.0        | NES        |
| Σα              | 85 650   | 86.7         | 100                | 0.4        | 0.0        | 0.0       | 0.0       | 0.0     | 0.30       | 0.30       | 0.30       | 0.50       | 0.40       | 0.80       | 0.6        | 0.0        | 0.0        | 0.0        |

αSum of the fractions; values for mass and rev/MJth for the whole extract for each strain are from Mutlu et al. (2015a,b). NES, not enough sample to test.
mutagenicity (TA98−S9) that eluted with weakly and moderately polar standards (sub-fractions 14–18, 34–39, and 43). In B50 the peaks of weakly polar mutagenicity were greatly diminished and replaced by a high peak of mutagenicity in TA98−S9, which was greatly reduced in TA98NR−S9. This is most likely due to highly polar compounds, likely polar dinitroarenes, which appear as more biodiesel is present in the fuel.

The amount of mutagenic activity that eluted in the mutagrams of Fraction 4 was considerably less than the activity that eluted in Fractions 2 and 3. For B0 there was a notable peak of highly polar mutagenicity in TA100 + S9 that co-eluted with the oxy-PAH 1,3-dihydroxynaphthalene (standard 10). However, little of this activity was present in the mutagram of B20, and essentially no mutagenicity eluted across any of the 62 sub-fractions of B50.

Discussion

Comparison of petroleum diesel samples B0 and C-DEP

The data in this paper are from the composite of extracts from two separate experiments, which produced mutagenicity data that were generally not significantly different (Mutlu et al., 2015b). To our knowledge, this is the first report to show that the results of replicate experiments in terms of chemical and mutagenicity endpoints. As discussed below, the results for petroleum diesel PM (B0) reported here were similar to those from a standard set of compressor diesel-exhaust particles (C-DEP) that we evaluated previously (Mutlu et al., 2013), even though the two sets of emissions were produced by different engines. The mutagenic potencies for B0 and C-DEP were within twofold of each other for each strain/condition. Thus, the B0 generated here has similar mutagenic potencies as C-DEP, which we consider a standard for diesel-exhaust studies (Mutlu et al., 2013), supporting the validity of our biodiesel studies here, which were conducted under the identical conditions as B0.

Comparison of biodiesel to petroleum diesel

The EOM from the four fuels assessed here had highly disparate chemical compositions, especially when considering petroleum diesel (B0) versus 100% soy-based biodiesel (B100) fuels (Mutlu et al., 2015a). These differences were also reflected in the % EOM of the emissions: B0 (26%), B20 (21%), B50 (32%), and B100 (60%) (Mutlu et al., 2015a). Nonetheless, there were many similarities between the organic compositions of the extractable organics of the emissions from all four fuels. For the emissions from all
Table 13. Average measured and calculated PM mass emissions and emission factors from Mutlu et al. (2015a).

| Fuel | Chamber PM mass concentration mg/m³ | Dilution factor | Exhaust PM mass concentration mg/m³ | PM mass emission rate mg/h | PM mass emission factor mg/kg fuel | PM mass emission factor mg/MJth | PM mass emission factor mg/MJₑ |
|------|-----------------------------------|-----------------|-------------------------------------|---------------------------|----------------------------------|-------------------------------|----------------------------------|
| B0   | 2.320 ± 0.060                     | 12.05 ± 0.35    | 27.956 ± 1.535                      | 1999.5 ± 111.2            | 1913.4 ± 152.2                   | 41.81 ± 3.33                  | 191.34 ± 10.64                   |
| B20  | 2.275 ± 0.095                     | 10.55 ± 0.35    | 24.001 ± 1.798                      | 1796.2 ± 149.3            | 1663.2 ± 138.2                   | 37.26 ± 3.10                  | 170.75 ± 14.19                   |
| B50  | 2.355 ± 0.035                     | 10.55 ± 0.15    | 24.845 ± 0.722                      | 1826.8 ± 83.5             | 1638.4 ± 82.2                    | 38.14 ± 1.91                  | 175.54 ± 8.03                    |
| B100 | 2.075 ± 0.035                     | 9.50 ± 0.20     | 19.713 ± 0.747                      | 1414.7 ± 82.8             | 1209.2 ± 70.7                    | 30.11 ± 1.76                  | 138.55 ± 8.11                    |

Average values ± standard errors presented for measured data during the separate 2-d collection of filter samples used to determine PM emission factors and subsequently extracted for chemistry and mutagenicity analyses. Values calculated from measurements include propagated standard errors. Chamber PM mass concentrations are measured from PM filters. Dilution factors are based on continuous nitric oxide (NO) measurements. Other parameters are calculated from measured values (see Table 1).

* Dilution factors are determined by the ratio of continuous NO measurements made in the engine exhaust and exposure chamber.

Figure 2. Heat map of correlations between PAH (µg/MJₑ) and mutagenicity-emission factors (rev/MJₑ) in various strains among the whole extract and four fractions for each of the fuels. Red represents a positive correlation; blue a negative one. Numbers 1–16 are the 16 EPA Priority PAHs, 17–25 are 9 oxy-PAHs, and 26–32 are 7 nitro-PAHs; each number corresponds to the PAH as noted in Mutlu et al. (2013). The strain abbreviations with (+) or without (−) S9 are TA100 (0), TA98 (98), TA98NR (NR), TA98/DNP6 (P6), YG1021 (21), YG1024 (24), YG1041 (41), YG1042 (42), TA104 (4). The chemical abbreviations are defined in Table 13.
The limited correlations among the PAH- and mutagenicity-emissions, we cannot make a direct comparison of the two data sets.

As illustrated in Figure 1, the distributions of the mass of the EOM, and the mutagenicity- and PAH-emission factors across the fractions were rather similar for all four fuels. Nonetheless, the chemical matrices of B50 and B100 were clearly quite different based on the heatmap (Figure 2) and the mutagrams (Figure 3), along with the reduced mutagenicity of B50 and B100 relative to that of B0 and B20. Thus, despite some similarities, the chemical matrix was altered by high concentrations of biodiesel, preventing a high recovery of mutagenicity among the reconstituted samples. The small amount of mutagenic EOM from each of the fractions of B100 prevented us from sub-fractionating the fractions to produce mutagrams for B100.

Despite evaluating the mutagenicity of the whole extract (Mutlu et al., 2015b) and four fractions for each whole extract in 16 Salmonella strain/S9 combinations, we were able to evaluate all samples at multiple doses in at least two independent experiments. This was possible by using only one plate/dose, as is common when evaluating limited amounts of complex, environmental samples. This approach, we and others have used for nearly three decades, provided for a more reliable assessment of the mutagenicity of the samples compared to using two plates/dose in only one experiment. By being able to perform a repeat experiment, the results can be replicated and, if necessary, the doses modified to produce linear dose-response curves. In our case, nearly all of the dose-response curves have $R^2$ values of 0.9 or greater. Thus, given the limited amounts of sample available, the use of one plate/dose was a strength, not a weakness, of this study for the reasons noted above.

Although there was a decline in the mutagenicity-emission factors of the fuels as the percentage of biodiesel increases, that decline was not linear. As shown with the whole extracts (Mutlu et al., 2015b), there was a sharp decline in mutagenicity-emission factors when going from B0 to B20; however, this reduction was not as large as the amount of biodiesel increased from 50% and then to 100%. The chemistry of this is unclear to us, but the presence of a relatively small (20%) amount of biodiesel, combined with the complex matrix of petroleum diesel, results in a fuel whose emissions are considerably less mutagenic than those of B0, whereas adding more biodiesel results in only an incremental further reduction in mutagenicity.
Limitations of the study

We used a small-diesel generator run at a constant load, which does not necessarily reflect normal diesel engine operation in a truck or other such vehicle. As noted by Bünger et al. (2012), adding a load can increase the mutagenicity of the emissions. Thus, our results do not take into account this aspect of diesel engines as used in vehicles.

Our small generator did not have emission controls, whereas some diesel engines in current use and many newer engines have a variety of emission controls. However, there is evidence that the EOM from some engines with emission controls can have higher mutagenic potencies than the EOM from the same engines without controls (IARC, 2014). Although the mutagenicity-emission factors for engines with emissions-control devices have not yet been published, we...
would expect that such controls would reduce considerably (by orders of magnitude) the mutagenicity-emission factors relative to those from engines without such controls. Emission controls would also likely reduce the contribution of PAHs to the mutagenicity of the emissions.

Conclusions
The majority of the mass of the extractable organics of the emissions from petroleum diesel and soy biodiesel fuel of various compositions was composed of predominantly nonpolar, non-mutagenic compounds; however, most of the PAHs were weakly polar, and most of the mutagenicity was due to a mixture of weakly polar and polar compounds. Some of the mutagenicity of B20 was due to highly polar compounds. Despite these similarities, there were sufficient chemical differences such that the PAH- and mutagenicity-emission factors declined as the concentration of biodiesel in the fuel increased. These results demonstrate that the emissions from soy biodiesel are less mutagenic than those from petroleum diesel and that this is due partly to reduced concentrations of a variety of weakly polar, polar, and highly polar mutagens in the emissions with increasing biodiesel content of the fuel.

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Declaration of interest
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Supplementary material available online