Supporting Information

Manuscript Title: Soy Biodiesel and Petrodiesel Emissions Differ in Size, Chemical Composition and Stimulation of Inflammatory Responses in Cells and Animals

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SUPPORTING INFORMATION

Thermal Desorption Gas Chromatography Mass Spectrometry (TD-GCMS)

Nonpolar compounds in the particulate matter were extracted using a solvent mixture of dichloromethane (DCM)/Hexanes (1:1, v/v), while the polar compounds were extracted using methanol. All extractions were performed three times in series and replicate extracts were combined for subsequent processing and analysis by TD-GCMS.

A ¼" diameter punch was removed from each filter and inserted into a 180 µL TD-vial. For nonpolar compound extraction, 70 µL of DCM/Hexanes solvent was added to the vial, followed by 5 min sonication. This was done three times, and the extracts were combined in another glass TD-vial. The nonpolar fraction was concentrated using a gentle stream of N_2 gas to 10 µL final volume, then covered with a 30 mL glass vial in the fume hood until all the solvent evaporated. The TD-vial was then inserted into the TD-GCMS for analysis after adding 1 µL of a 2 ppm solution of phenanthrene-d10 and perylene-d12 as internal standards.

The polar fraction was also extracted three times, but with 70 µL of methanol and sonicating for 5 min each time. The three methanol extracts were also combined in another glass TD-vial, blown down to 7 µL, and 1 µL of a 2.4 ppm solution of 6-fluoro-4-chromanone quantitation standard was added to the extract, followed by 1.5 µL of a 25 mg/mL O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA; Sigma-Aldrich) solution. Acetonitrile/DCM (9:1, v/v) was then added to the vial to achieve a final volume of 30 µL and a PFBHA concentration of 5 mM. The sample derivatization reaction proceeded at room temperature for 24 hours, then excess PFBHA was quenched by adding 11 µL of acetone, waiting for at least 1 hour at room temperature for the oxime to form. The sample was then blown down to near dryness before sitting for 2 hours covered by a 30 vial to evaporate remaining PFBHA-acetone oxime. The sample was then analyzed on the TD-GCMS using 1 µL of a 2 ppm solution of phenanthrene-d10 and perylene-d12 internal standards.

Chemical analysis of the filter extracts was carried out using a Hewlett Packard Gas Chromatograph(GC)/Mass Spectrometer (5890GC/5972MSD) equipped with a thermal desorption (TD) injector (Lavigne Laboratories, Storrs, CT). The GC conditions were: Restek (Rxi-XLB) capillary GC column (30 m long, 0.25 mm i.d, and 0.25 µm film thickness), 99.999% helium carrier gas at 1 mL/min, and 295^{o} C injector operated in splitless mode, 290^{o} C detector. The oven temperature was held at 65^{o} C for 12 min to allow thermal desorption (10 min at 295^{o} C) of the sample, then ramped to 186^{o} C at a 10^{o} C/min and held for 3 min, and then finally ramped to 300^{o} C at 2.5^{o} C/min and held for 15 min. The analytes were ionized using electron impact ionization, and the mass spectra were obtained using scan mode for ions with m/z ranging from 50 to 650 amu.

Quantification of individual compound mass was based on total ion peak areas normalized to the phenanthrene-d10 internal standard, assuming unit response factors. PAHs were quantified based on extraction ion peak areas. Compound identifications were based on the NIST08 Library and authentic standards for n-alkanes, 16 PAHs, 26 carbonyls and 10 FAMES.

Stein, S. Y. Mirokhin, D. Tchekhovskoi, G. Mallard (2008) The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library. Version 2.0 f, build Jul 23 2008.

Dithiothreitol (DTT) Assay

An abiotic DTT assay was used to measure the B0- and B20-dependent DTT consumption. 0.5 ml of DTT (100 μM) was added to duplicates of each tube containing 40 μg particles and incubated at 37° C in a dry bath for 0, 15, 30, and 45 min. One set of 0.5 ml DTT without particles was used as a blank control. (Net reaction: DTT + 2 O_2 èDTT-disulfide + 2 $O_2^{\cdot-}$). 0.5ml of 10% trichloroacetic acid was added to the incubation mixture to stop the reaction at each designated time until all incubations were completed. A 0.5 ml aliquot of the reaction mixture was then mixed with 25 μl of 10mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and reacted for 5 min, followed by addition of 1 ml of 0.4 M Tris–HCl, pH 8.9 with 20 mM EDTA. The concentration of the formed 5-mercapto-2-nitrobenzoic acid was measured by spectrophotometry at absorption 412 nm.

Cell Culture and Biological Assays

Human THP-1 monocytes (ATCC) were grown in RPMI 1640 medium (Invitrogen) containing 10% Fetal Bovine Serum; 100 U/ml Penicillin-Streptomycin; 10mM HEPES and 1mM Sodium pyruvate at 37.0° C in 5% CO_2. Cells require differentiation into a macrophage-like cell line using 100 nM phorbol-12-myristate-13-acetate (PMA) overnight and then refreshing the cells with medium without PMA prior to exposure to PM.

Human BEAS-2B bronchial epithelial cell line (ATCC) were seeded into flasks or plates pre-coated with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin in medium. The cells were grown in DMEM/ F12 medium (Invitrogen) containing 10% Fetal Bovine Serum; 100 U/ml of Penicillin-Streptomycin, 1μg/ml Insulin-Transferrin-Na selenite (Sigma), 1μg/ml Hydrocortisone (Sigma), and 10 mM HEPES at 37.0°C in 5% CO_2.

C57BL/6 mice were anesthetized with inhaled isoflurane (to effect) and suspended by their incisors on a 60° incline board. The tongue was gently extended and 50 μl of the stock solution particles delivered into the distal part of the oropharynx and aspirated into the lower respiratory tract. Mice were allowed to aspirate the material for approximately 15 seconds, after which they were returned to the cage and monitored until *fully* recovered. The dose of particles delivered was determined as the mean gravimetric mass of triplicate 50 μl aliquots of the stock suspension on the day of use.

Cytokines: The culture medium in which the cells were grown and treated, as described above, was collected and centrifuged twice at 14000 rpm and the supernatants stored at -80° C until analysis. Bronchoalveolar lavage fluid (BALF) obtained from the mice at necropsy were frozen at -80° C until analysis. Lung tissue lysates were prepared by T-PER Tissue Protein Extraction Reagent complemented with Halt Protease and Phosphatase Inhibitor (Thermo Scientific). Cytokines and chemokines in the above culture medium, BALF and lung tissue lysates were analyzed using both the Bio-Plex Pro™ Assay (Bio-Rad) and Milliplex Map® Assay (Millipore). Bio-Plex-27 or Milliplex 26 human cytokine kits were used in screening of human cytokine responses. Mouse samples were analyzed using the Milliplex-22 murine cytokine kit.

All samples were prepared according to the manufacturer's instructions and ran on the Bio-Plex suspension array system (Bio-Rad). Standard curves were calculated and samples were analyzed using the Bio-Plex Manager Software Version 6 (Bio-Rad).

Cytokine Binding Assay: To determine whether variation in cytokine levels might be attributable to adsorption of the particles themselves, B0 and B20 particles used in the *In Vitro* and *In Vivo* experiments were added to cell-free medium at final concentrations of 0, 5, 10, 20, or 40 μg/ml. Human G-CSF (Novus Biologicals) was added to the medium containing different concentrations of particles and mixed well. After incubation for 24 h, cytokine levels were measured using the Milliplex cytokine assay.

Cytotoxicity: Lactate Dehydrogenase (LDH) concentrations in the culture medium from exposed cells were measured using the Cytotox96 Assay (Promega) and used as an index of toxicity.

OxiSelect™ Intracellular ROS: The intracellular ROS was measured by OxiSelect™ Intracellular ROS Assay Kit (Cell Biolabs INC), which is a cell-based assay for measuring antioxidant or ROS activity. The kit included H_2O_2 that was diluted to 0.1 and 1 mM and served as the positive control. Cells were seeded in a 96-well cell culture plate at $3.2x10^4$/well overnight and then pre-incubated with DCFH-DA (cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate). The biodiesel and petrodisel extracts were then added to the cells. After a selected period time of incubation, the cells were read on a Synergy HT microplate reader (BioTek Instruments) at 480 nm/530 nm.

Oxidized Protein Detection: Protein carbonyl modifications were detected and quantified using the OxyBLOT™ assay (Millipore) according the manufacturer's protocol. Briefly, protein lysates from mouse lung tissues were incubated with 2,4-Dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by SDS-PAGE followed by Western blotting. The same membranes were stripped and then reimmunoblotted with β-actin or stained with India ink as a loading control.

Western-blot: The antibodies for total or phospho-ERK1/2, caspase-1, total or phospho-EGFR (Tyr1068) were purchased from Cell Signaling; the antibody for TLR4 and Nrf2 was from Santa Cruz; the antibodies for GCLC were from Abnova and β-actin was from Abcam Inc. 20 μg of each total protein was electrophoresed on 10% or 7.5% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) and then electroblotted onto nitrocellulose or PVDF membranes. After blocking of membranes with 1% BSA blocking/dilution buffer, the membranes were incubated with the appropriate primary antibody at the recommended concentration overnight with shaking at 4° C and then rinsed. After incubation with corresponding secondary antibodies, the protein bands were visualized using SuperSignal™ West Pico Trial Kit (PIERCE) and exposed to radiographic films. The images and densities were captured with a GS-700 Imaging Densitometer (Bio-Rad, Richmond, CA) and analyzed with Quantity One Software Version 4.2 (Bio-Rad, Richmond, CA). The membranes were stripped and then reimmunoblotted with another antibody or β-actin antibody as a loading control.

OxiSelect™ Total Glutathione (GSSG/GSH) Assay: The total glutathione content was measured by OxiSelect™ Total Glutathione (GSSG/GSH) Assay kit (Cell Biolabs INC). The serum samples from treated and untreated mice were added to equal volumes of ice-cold 5% metaphosphoric acid, mix thoroughly and stored on ice for 10 min. The suspension was centrifuged at 12,000 rpm for 10 min at 4º C. 40 µl of supernatant then was collected and added to 96-well plate with 160 µl mixture of glutathione reductase, NADPH and chromogen. Glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. The absorbs were immediately recorded at 405 nm at 1 min intervals for 10 min on a BioTek Synergy H4 (BioTek Instruments) under perform kinetics. The GSSG/GSH was finally assessed by GSSG standard curve.

Murine plasminogen activator inhibitor type 1 (PAI-1) ELISA: Murine PAI-1 Total Antigen Assay kit (Molecular Innovations, Inc) was used for the quantitative determination of total PAI-1 in mouse plasma or BALF. Lungs were lavaged with PBS and then centrifuged at 3000 rpm for 3 min. Duplicates of supernatants without the cell pellets were measured according manufacturers' procedures. PAI-1 present in BALF reacted with the captured antibody-coated microtiter plate followed by the anti-PAI-1 primary and secondary antibodies, TMB was then used for color development. The plate was finally read on a Synergy HT microplate reader at 450nm. The concentration of PAI-1 protein in the BALF was determined against PAI-1 standard curve.

Armfield CM-12 Light-duty Diesel Engine Specifications, Test Cycle and PAH Concentrations

The CM-12 engine is an industrial engine with a non-road emission certification EC 97/68 Stage IIIA rating. (http://ec.europa.eu/enterprise/sectors/mechanical/documents/legislation/emissions-non-road/, stated to be "aligned with the U.S. standards.)

For each test, the fuel tank and fuel lines were purged with the test blend the day prior to testing to ensure that no residual fuel was in the lines. Different fuel tanks are used for petrodiesel and biodiesel fuel types. Our commercial B100 biodiesel fuel met the U.S. certification by the American Society for Testing and Materials (ASTM) D6751.

A maximum of two test cycles were run per day, but particles from the 9-modes collected after the CM-12 engine coolant reached a stable temperature of 92^{o}C (end of Warm-Up) were used for subsequent chemical and biological tests.

Stainless steel filter holders and Teflon impingers were cleaned with triplicate hexane/acetone rinses between tests.

Labview logged all engine operating parameters. QA/QC procedures included collection of instrument blanks (SPMP inlet fitted with HEPA filter) and tunnel blanks (10 minutes, engine not running; collect pre-and post- all engine tests). Blank runs involved all steps of a routine run without starting the CM-12 engine.

Table S1. Armfield CM-12 Engine Dynamometer Specifications

Engine	
Manufacturer	Volkswagen
Identification code	ARD
Charge air	Naturally aspirated
Capacity	1896 cm^3
Cylinders	4
Bore	79.5 mm
Stroke	95.5 mm
Compression ratio	19.5:1
Nominal output	44 kW @ 3600 RPM
Max torque	130 Nm @ 2000–2400 RPM
Fuel	Diesel DIN EN590
Control system	Bosch EDC
Exhaust gas recirculation (EGR)	None
Retarder	
Manufacturer	Klam
Model number	K40
Max power	60 kW
Max torque	145 Nm

Table S2. Armfield CM-12 Engine Operating Conditions

	Torque	RPM setpoint	Throttle position	% Load
Warm up idle	0	idle	0	0
Mode 1	23	3300	40	19
Mode 2	2	3300	30	2
Mode 3	5	3000	30	4
Mode 4	10	2700	30	8
Mode 5	16	2400	30	13
Mode 6	25	2000	30	19
Mode 7	37	2000	35	29
Mode 8	51	2000	40	40
Mode 9	11	2700	30	9
Cool down idle	0	idle	0	0

Note: Samples were collected after the warm-up (engine coolant stabilized at 92^{o}C). Each mode's duration was 7.5 min to enable triplicate SMPS scan collection.

Table S3. Polycyclic Aromatic Hydrocarbon (PAH) Mass Concentrations (ng/m^3)

PAH	blank	B0	B20	error (%)[a]
naphthalene	ND	319	142	2
acenaphthylene	ND	237	93	0.0
acenaphthene	63	189	77	1.1
fluorene	51	171	68	0
phenanthrene	38	152	49	1.6
anthracene	ND	218	100	6.5
fluoranthene	40	135	76	6.3
pyrene	ND	191	137	5.4
benzo[a]anthracene	ND2	177	84	1.6
chrysene	42	139	64	0.6
benzo[b]fluoranthene	46	142	77	0
benzo[k]fluoranthene	48	168	61	4.0
benzo[a]pyrene	ND	175	103	10.4
indeno[1,2,3-cd]pyrene	56	184	74	1.0
benzo[ghi]perylene	43	160	70	1.8
dibenz[a,h]anthracene	65	207	ND	0.3
total PAH	*492*	*2964*	*1277*	

[a]Error based on extractions of duplicate punches of biodiesel filter as percent relative standard deviation.

Other Cytokine Concentrations in Cells and BALF

Table S4 THP-1 Cells - Cytokine/Chemokine Concentrations (pg/ml)

Treatment	Eotaxin	IL-6	IL-7	IFNA2	IP-10	MCP-1
Con	63.6 ± 23	6.1 ± 1	59.1 ± 1	14.9 ± 1	12685 ± 563	11508 ± 2086
Vehic	68.0 ± 32	5.2 ± 0	55.2 ± 8	20.3 ± 9	10312 ± 526	11885 ± 2707
Amorp	65.2 ± 25	5.0 ± 1	52.2 ± 3	19.7 ± 6	7336 ± 3050	11147 ± 542
Cristob	67.0 ± 29	6.9 ± 0	56.1 ± 12	14.5 ± 0	17803 ± 5213	11518 ± 3262
B20-10	66.0 ± 30	2.5 ± 1	61.0 ± 6	14.8 ± 5	756 ± 168	1863 ± 550
B20-20	58.6 ± 19	1.8 ± 0	45.6 ± 4	11.0 ± 3	158 ± 45	201 ± 58
B0-10	58.4 ± 22	3.1 ± 1	46.5 ± 10	15.5 ± 7	392 ± 64	458 ± 217
B0-20	65.6 ± 36	1.7 ± 1	56.3 ± 21	10.2 ± 5	187 ± 24	185 ± 42

Levels of other cytokines/chemokines detected by the MilliPlex-26 kit in medium of THP-1 cells exposed to control medium (Con), vehicle (Vehic, ethanol), amorphous silica (Amorp), cristobalite (Cristob), dose of 10 μg/ml B20 particles (B20-10), dose of 20 μg/ml B20 (B20-20), dose of 10 and 20 μg/ml B0 (B0-10 and B0-20). Values are mean ± SE.

Table S5 BEAS-2B cells - Cytokines/Chemokines Concentrations (pg/ml)

Treatment	Eotaxin	G-CSF	IL-6	IP-10	MIP-1a
Con	11.0 ± 6.2	6.2 ± 3.1	10.5 ± 1.8	5.7 ± 1.3	5.8 ± 2.3
Vehic	8.9 ± 3.0	3.0 ± 0.7	8.7 ± 1.0	4.5 ± 0.3	6.0 ± 2.1
Amorp	11.6 ± 5.9	5.9 ± 0.7	12.7 ± 2.0	6.0 ± 3.7	7.6 ± 2.0
Cristob	9.4 ± 4.4	4.4 ± 1.2	14.0 ± 2.6	4.5 ± 0.3	6.5 ± 2.0
B20-10	9.6 ± 6.5	6.5 ± 1.4	10.8 ± 1.3	3.6 ± 1.7	6.4 ± 2.8
B20-20	7.3 ± 3.5	3.5 ± 0.9	11.1 ± 2.0	3.5 ± 1.9	5.6 ± 2.5
B0-10	7.4 ± 3.3	3.3 ± 0.2	10.7 ± 2.4	3.1 ± 1.4	5.7 ± 2.4
B0-20	10.1 ± 4.5	4.5 ± 0.8	10.1 ± 1.6	3.5 ± 1.9	6.2 ± 2.0

Levels of other cytokines/chemokines detected by the MilliPlex-26 kit in medium of BEAS-2B cells exposed to control medium (Con), vehicle (Vehic, ethanol), amorphous silica (Amorp), cristobalite (Cristob), dose of 10 μg/ml B20 particles (B20-10), dose of 20 μg/ml B20 (B20-20), dose of 10 and 20 μg/ml B0 (B0-10 and B0-20). Values are mean ± SE.

Table S6 Cytokines/chemokines Concentrations in BALF (pg/ml) and Lung Tissue Homogenates (pg/mg protein)

Cytokine Group:	Con	B20	B0
A. Lung BALF			
IL-5	6.39 ± 2.3	53.77 ± 10.2	24.18 ± 9.9
KC	4.46 ± 1.2	5.48 ± 2.3	1.68 ± 1.2
B. Lung Tissues			
KC	343.1 ± 52.0	415.7 ± 68.9	300.5 ± 58.3
MCP-1	425.4 ± 98.7	652.6 ± 111.9	537.9 ± 120.0
IFNg	39.8 ± 16.3	48.9 ± 7.6	31.2 ± 9.7
IL-1A	103.2 ± 32.0	95.3 ± 14.1	72.4 ± 16.7
IL-1B	355.9 ± 76.3	368.1 ± 74.6	271.5 ± 56.3
IL-10	61.9 ± 15. 5	58.2 ± 12.7	37.3 ± 8.4
IL-13	83.9 ± 30.5	128.3 ± 26. 7	55.6 ± 11.3
IL-15	101.9 ± 25.9	101.2 ± 23.3	55.4 ± 15.6
MIP-1A	204.8 ± 38.1	222.8 ± 31.4	151.4 ± 28.7
TNF-A	22.3 ± 3.8	22.5 ± 4.7	16.3 ± 2.8

Levels of other cytokines/chemokines detected in BALF (A) and lung tissue (B) by the MilliPlex-22 kit. Values are mean ± SE**.**

SMPS Particle Distributions – Number, Surface Area and By Engine Mode

Figure S1. Number and surface area weighted particle distributions for two fuel types, petrodiesel (B0) and 20% biodiesel (B20).

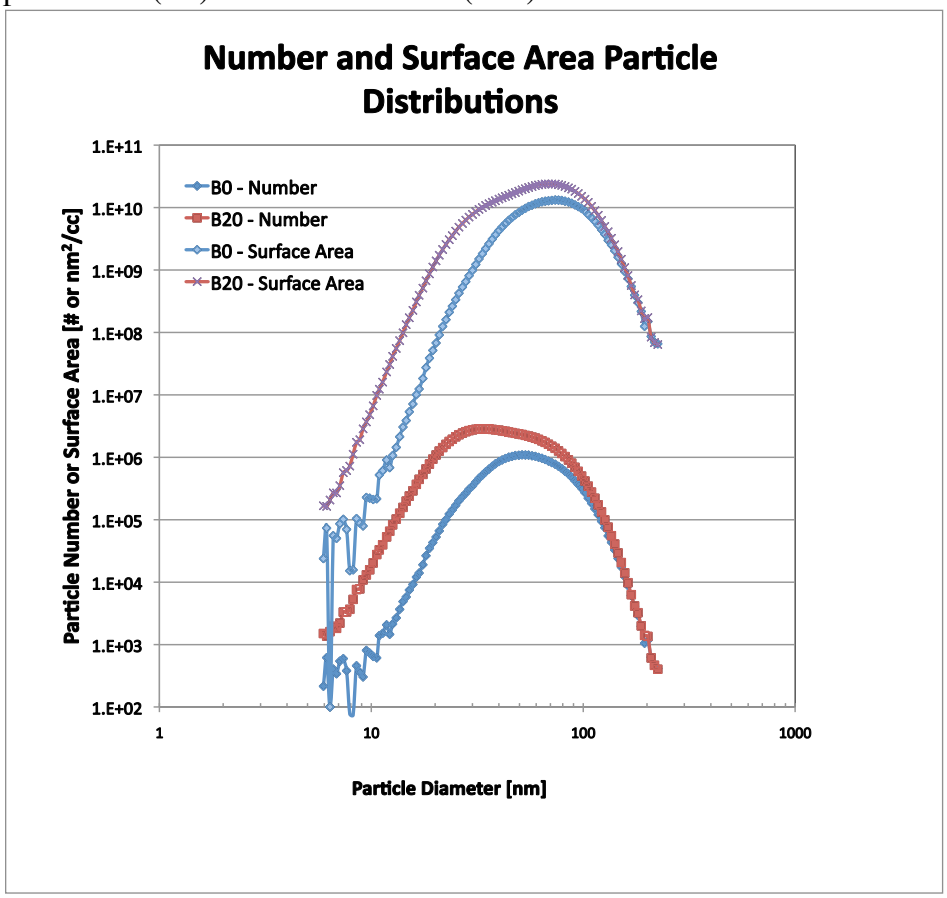
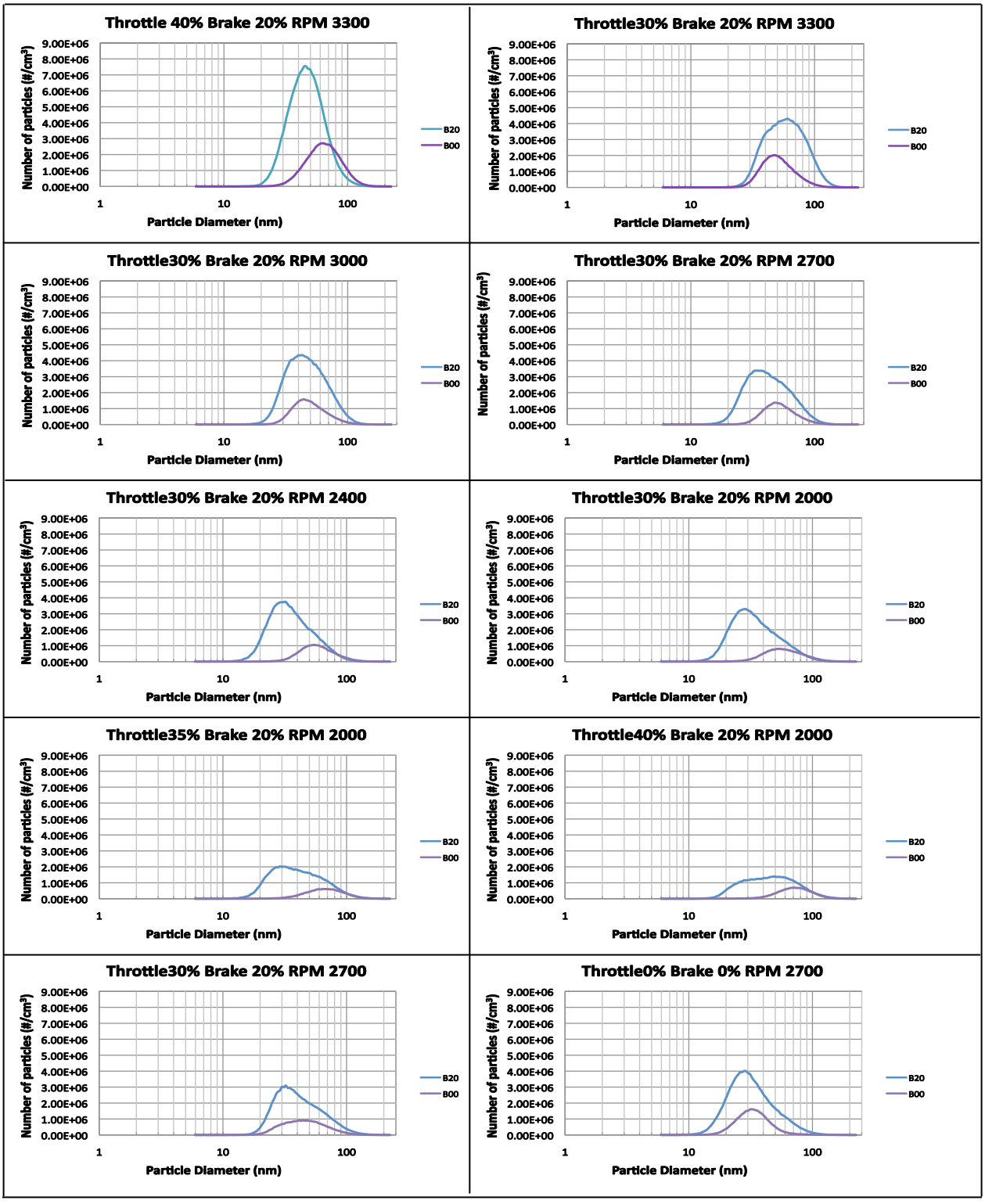


Figure S2. Individual operating mode mean SMPS particle number distributions for petrodiesel (B0) and 20% soy biodiesel fuel (B20). The last mode is cool-down. Warm-up data not shown.



Relative Particle Composition by Functional Groups

Figure S3. Relative functional group organic composition by fuel type based on TD-GCMS analysis of filter punches. Data are means of single punches for filters #18 and #40 and two punch extractions for filters 27, 33.

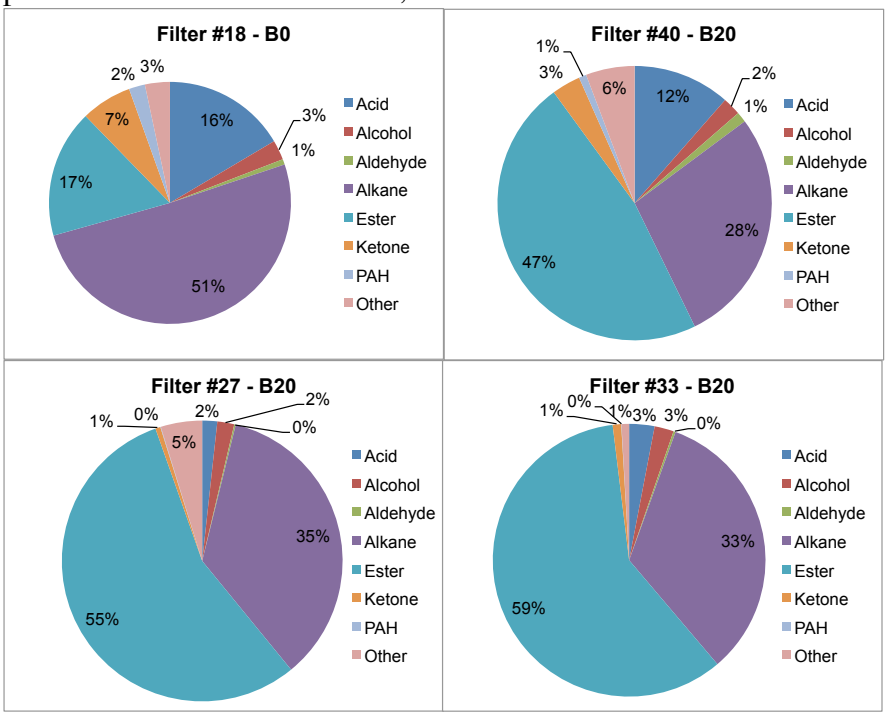
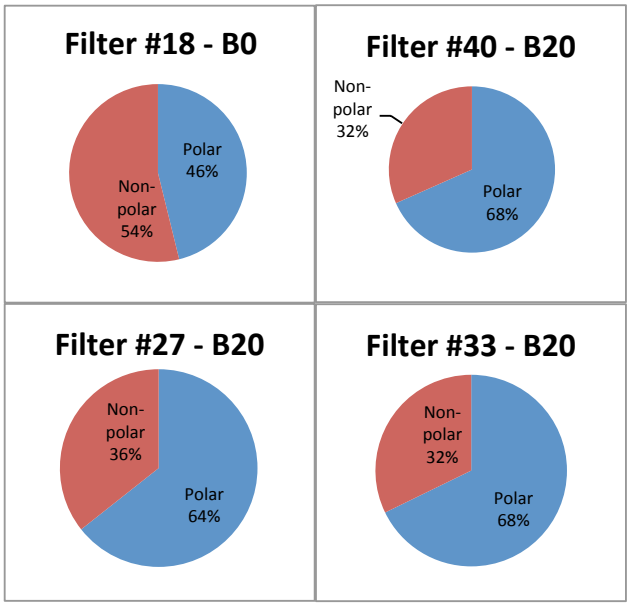
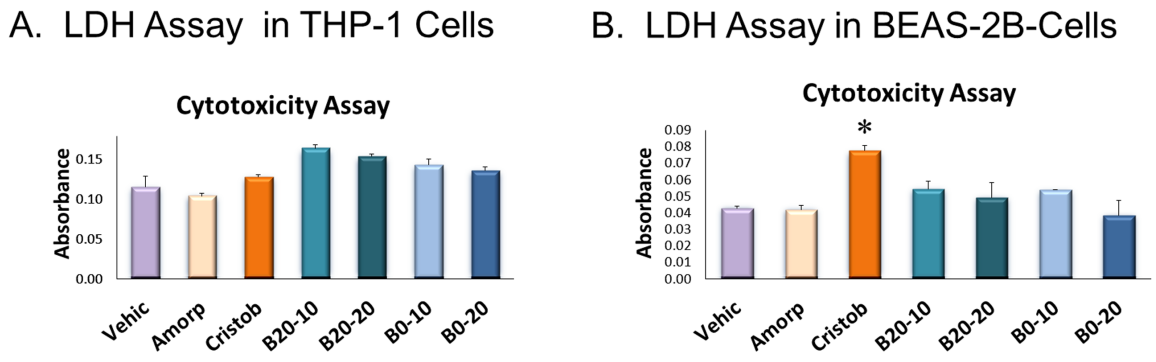


Figure S4. Filter punch polar and nonpolar composition based on GCMS analysis and functional group assignments from NIST library search. Data are means of single punches for filters #18 and #40 and two punch extractions for filters 27, 33.



Cytotoxicity Results

Figure S5 Particles derived from both types of fuel had minimal cytotoxicity in the THP-1 cells (A) and BEAS-2B cells (B). B20-10, dose of 10 μg/ml B20 particles ; B20-20, dose of 20 μg/ml B20; B0-10 and B0-20, dose of 10 and 20 µg/ml B0, respectively. Only cristobalite resulted in significantly more cytotoxicity in BEAS-2B cells (*P<0.05).



Cytokine Adsorption to Particles in Cell-free Medium

Figure S6 Cytokine binding assay in cell-free medium containing B0 and B20 exhaust particles. The same concentration of G-CSF were added to a series of doses of particles. After 24 h incubation, G-CSF concentrations in the medium were measured by the Milliplex Cytokine kit. The actual measured G-CSF concentrations are shown in the graph for the different particle concentrations (mean ± SE). No significant differences were found between measured G-CSF concentrations at all doses of particles, suggesting that the dose of particles had no effect on the measured values of G-CSF.

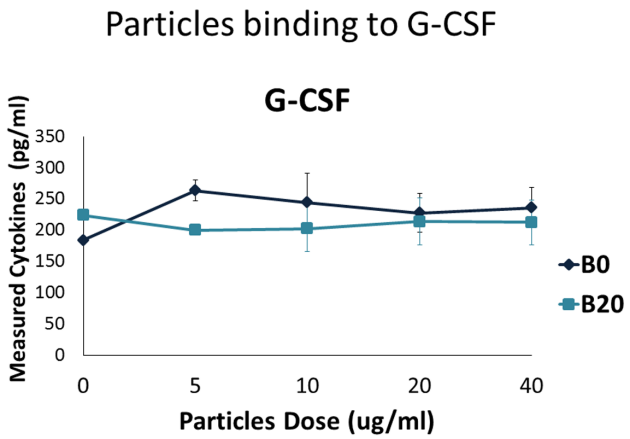
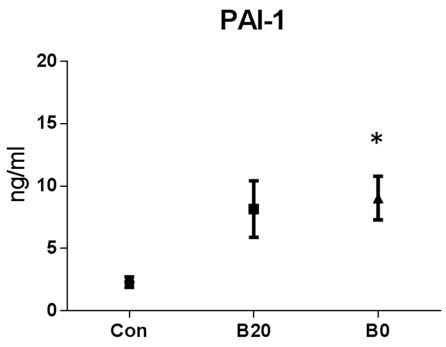


Figure S7. Both B20 and B0 particles induced an increase of PAI-1 in BALF (A) of mice treated for 3 days (*P<0.05 compared to control), but not in lung tissues (B). Values are mean±SE.

A. BALF



B. Lung Tissues