

# Chemical characterization and in vitro toxicity of diesel exhaust particulate matter generated under varying conditions

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**Abstract** Epidemiologic studies have linked diesel exhaust (DE) to cardiovascular and respiratory morbidity and mortality, as well as lung cancer. DE composition is known to vary with many factors, although it is unclear how this influences toxicity. We generated eight DE atmospheres by applying a  $2 \times 2 \times 2$  factorial design and altering three parameters in a controlled exposure facility: (1) engine load (27 vs 82 %), (2) particle aging (residence time  $\sim 5$  s vs  $\sim 5$  min prior to particle collection), and (3) oxidation (with or without ozonation during dilution). Selected exposure concentrations of both diesel exhaust particles (DEPs) and DE gases, DEP oxidative reactivity via DTT activity, and in vitro DEP toxicity in murine endothelial cells were measured for each DE atmosphere. Cell toxicity was assessed via measurement of cell proliferation (colony formation assay), cell viability (MTT assay), and wound healing (scratch assay). Differences in DE composition were observed as a function of engine load. The mean 1-nitropyrene concentration was 15 times higher

and oxidative reactivity was two times higher for low engine load versus high load. There were no substantial differences in measured toxicity among the three DE exposure parameters. These results indicate that alteration of applied engine load shifts the composition and can modify the biological reactivity of DE. While engine conditions did not affect the selected in vitro toxicity measures, the change in oxidative reactivity suggests that toxicological studies with DE need to take into account engine conditions in characterizing biological effects.

**Keywords** Air pollution · Diesel exhaust · Varying exposure conditions · Physicochemical properties · In vitro toxicity

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## Abbreviations

Ba	Barium
Ca	Calcium
CMD	Count median diameter
CO	Carbon monoxide
CO <sub>2</sub>	Carbon dioxide
Cr	Chromium
Cu	Copper
DE	Diesel exhaust
DEP	Diesel exhaust particulate
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EC	Elemental carbon
Fe	Iron
GE	Gasoline exhaust
HPM	Harvard personal environmental monitor
Mg	Magnesium
MMAD	Mass median aerodynamic diameter
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Ni	Nickel
1-NP	1-Nitropyrene
NO	Nitric oxide

NO <sub>x</sub>	Oxides of nitrogen
NO <sub>2</sub>	Nitrogen dioxide
OC	Organic carbon
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PBS	Phosphate-buffered saline
PM <sub>2.5</sub>	Fine particulate matter
PTFE	Polytetrafluoroethylene
SD	Standard deviation
Sn	Tin
TNB	3-Thio-6-nitrobenzoate
TRAP	Traffic-related air pollution
UV	Ultraviolet
UW	University of Washington

## Introduction

The world's vehicle fleet has been rapidly growing in concert with rising populations and improving economies (Health Effects Institute 2010). Diesel vehicle emission controls such as the use of reduced sulfur content in diesel fuel and catalyzed particle traps have been found to reduce several components of the complex mixture of diesel exhaust (DE) while increasing others (Kittelson et al. 2008; McDonald et al. 2004b). However, implementation of new technology will take time, and emissions from older engines are likely to dominate exposure for several years (Zhang et al. 2009). Correspondingly, there is a global trend of increasing airborne concentrations of markers of traffic-related air pollution (TRAP) (World Health Organization 2006). TRAP, which is attributed to both gasoline exhaust (GE) and DE, has been associated with numerous adverse health effects, including cardiovascular disease and inflammation of the respiratory system (Health Effects Institute 2010). The International Agency for Research on Cancer also recently classified DE as a human carcinogen (Benbrahim-Tallaa et al. 2012). The prevalence of vehicles with diesel engines relative to vehicles with gasoline engines has been increasing for the past 20 years and continues to rise in several countries (Zhang et al. 2009).

Application of numerous approaches to investigate health effects resulting from DE exposure—including epidemiology, animal toxicology, and controlled human exposure studies—is particularly useful due to the complex composition of DE. Controlled exposures are ideal for study of the mechanism of health effects caused by exposure because the concentration and duration of the exposure are well defined and it is possible to reduce the effects of confounding co-exposures (Ghio et al. 2012).

One challenge of selecting and designing a system for generation of DE exposures is that the DE composition is known to vary by many parameters including engine technology, fuel type, operating conditions, and ambient environment

(Hesterberg et al. 2009; Lloyd and Cackette 2001). The relative toxicity resulting from exposures to these different DE compositions is not well understood. Study of DE is further complicated by the fact that there has not been standardization in the reported characterization of the DE among existing exposure facilities (Birger et al. 2011; Fujitani et al. 2009; Laumbach et al. 2009; McDonald et al. 2004a, b; Rudell et al. 1994; Sawant et al. 2008; Sobus et al. 2008).

In the natural environment, engines operate at various loads depending on the grade of the road, vehicle type, carrying weight, and other conditions that place a draw on the engine. Controlled exposure facilities generally apply one of two options for mimicking the effect of engine load: a static load bank or a variable load dynamometer; alternatively, some facilities have traditionally run engines with no load. Investigations of generated DE to date have applied a wide range of engine loads as a standard operating condition ranging from 40 to 100 % (Birger et al. 2011; Laumbach et al. 2009; McDonald et al. 2004a; Rudell et al. 1994; Sobus et al. 2008). Our research group has usually applied 75 % load with our previous Cummins generator (Gould et al. 2008) and either 75 or 82 % load with our current Yanmar generator.

Photochemical aging of particles is not generally present in controlled exposure facilities, unless it is added to the diesel exhaust generation process by design. In the University of Washington (UW) facility, diluted diesel exhaust particulate (DEP) is typically aged for approximately 5 min. This is similar to other facilities, where residence times of DE prior to exposure range from 3 to 5 min (Birger et al. 2011; Fujitani et al. 2009; Sawant et al. 2008). Oxidation naturally occurs from the interaction of sunlight with DE in the environment, which leads to formation of secondary organic aerosols. The effect of exposing DE to ultraviolet (UV) light has previously been investigated with the use of smog chambers or UV lamps (Ebersviller et al. 2012b; Lichtveld et al. 2012; Robinson et al. 2007; Weitkamp et al. 2007). However, DE in the University of Washington (UW) facility, as in most other exposure facilities, is not UV-irradiated (Birger et al. 2011; Fujitani et al. 2009; Laumbach et al. 2009; McDonald et al. 2004a; Rudell et al. 1994; Sawant et al. 2008; Sobus et al. 2008). Ozone addition, which we applied in the current investigation, is a simplified way to represent the oxidation of near-road DE emissions.

The goal of this work was to evaluate the effects of altering operating condition (engine load), time since emission (residence time), and presence of ozone on DE composition, diesel exhaust particulate (DEP) reactivity, and DEP in vitro toxicity in an exposure facility. We selected these parameters to reflect compositions of DE in the natural environment. In vitro toxicity was assessed in murine endothelial cells with three relatively high-throughput cellular assays.

## Methods

### Study design

We investigated three parameters of DE generation: engine load, residence time, and ozonation of exhaust. For each parameter, we tested two contrasting conditions. Thus, we studied eight DE atmospheres in a  $2 \times 2 \times 2$  factorial design selected to maximize our statistical power to characterize each parameter. We collected measurements of physicochemical properties of DE composition (both particulate and gas components), DEP reactivity, and DEP in vitro toxicity.

We generated a single DE atmosphere on each day of sampling. Each of the eight unique atmospheres was run at least once for each type of measurement; for some measurements, atmospheres were run on multiple days. We averaged observational replicates from the day that the DE atmosphere was generated prior to analysis for all components and toxicity measurements. Summary statistics by condition along with the number of experimental replicates (samples collected on different days) and observational replicates (samples collected side by side or analyzed as laboratory replicates from the same day during DE generation) are shown in Table 1.

### Generation of diesel exhaust atmospheres

DE was generated within the UW facility using a two-step dilution process with dynamic control of fine particulate matter ( $PM_{2.5}$ ) mass concentration as previously described (Gould et al. 2008) (with DE parameters in Fig. 1). In 2010, the facility's engine was replaced with a 5.5-kW, single-cylinder generator (Yanmar model YDG5500EV-6EI) (Table 2). We used ultra-low sulfur diesel fuel with a maximum sulfur content of 15 ppm, the present highway grade diesel fuel obtained from local fuel distributors. The generator lubricating oil was Chevron DELO 400LE, SAE 15 W-40.

We selected the engine load by setting a load bank (Simplex Model Swift-E FT, Springfield, IL) to 1.5 kW (~27 % generator output, "low load") or 4.5 kW (~82 % generator output, "high load"). The ozonated condition was achieved by applying a series of adjustable UV generators (UV Pro 1100AT and UV Pro 4000, Crystal Air Marketing Inc., Langley, BC, Canada) to radiate the dilution air added to the exhaust, where the ozonated condition had a target excess ozone concentration of 100 ppb, measured by amount of excess nitrogen dioxide ( $NO_2$ ) relative to nitric oxide (NO) via direct reading measurement throughout the sample collection period. (Since oxides of nitrogen ( $NO_x$ ) levels are also altered by engine load, this was a more consistent approach for controlling the degree of oxidation between the DE atmospheres.) The non-ozonated condition was obtained with DE generated without the use of the UV lamps. Differences in the DE residence time since emission were tested by sampling at

different points in the exhaust train. "Fresh" exhaust was collected in the baffles of the exposure facility ~5 s after emission, while "aged" exhaust was collected downstream of the baffles within the exposure room ~5 min after emission (Table 2).

Our target concentration of  $PM_{2.5}$  during each of the 8 DE atmospheres was  $200 \mu\text{g}/\text{m}^3$ . During high engine load DE generation, we applied our automatic dynamic feedback system. The PM level was significantly reduced during low engine load conditions beyond the capability of the automated system, so we manually maintained the PM levels as close to  $200 \mu\text{g}/\text{m}^3$  as possible during the low load conditions by partially closing a slide gate in our second-stage dilution air supply.

### Sample collection and physicochemical characterization of particles and gases

Various components of diesel exhaust were collected and analyzed, as summarized in Table 3. Particle samples analyzed for  $PM_{2.5}$  and metal concentrations were collected on 37-mm-diameter polytetrafluoroethylene (PTFE, "Teflon") filters (Teflo with PMP support ring #R2PJ037, Pall Corporation, Port Washington, NY) within open-faced Harvard Impactors with a  $PM_{2.5}$  median cut-size.  $PM_{2.5}$  mass was measured in a humidity- and temperature-controlled room by gravimetric analysis before and after sample collection with a microbalance (UMT-2 microbalance, Mettler Toledo, Columbus, OH). Elements were analyzed on the Teflon filters via X-ray fluorescence (Cooper Environmental Services, Portland, OR). Among other components measured, comparisons of elements were limited to the six metals that were the most abundant across the average of the eight atmospheres: copper (Cu), zinc (Zn), calcium (Ca), magnesium (Mg), tin (Sn), and iron (Fe). (See Supplemental Material for mass fraction of other metals.)

Samples analyzed for elemental carbon (EC) and organic carbon (OC) were collected on 37-mm-diameter pre-fired quartz filters (Sunset Laboratory Inc., Tigard, OR) within Harvard Personal Environmental Monitors (HPEM) with a  $PM_{2.5}$  median cut-size, and a  $1.5\text{-cm}^2$  punch was analyzed using the IMPROVE-A thermal optical reflectance method (Sunset Laboratories, Tigard, OR). A second quartz filter placed downstream of the primary filter within the same HPEM housing served as a dynamic blank for OC results. By absorbing organic gases, this dynamic blank filter used for selected samples allowed the vapor phase artifact present in the primary quartz filter samples to be quantified and thereby separated from the contribution of only particulate matter OC. The dynamic blank OC concentration was found to vary with the OC concentration of the sample filter, so a derived correction factor was determined from pooling the dynamic blanks and paired samples using simple linear regression. We dropped two OC/EC samples from the analysis because of unexplained disparities between the observational replicates.

**Table 1** Exposure components, reactivity, and in vitro toxicity

	Exp Rep <sup>a</sup> (n)	Obs Rep <sup>b</sup>	Engine load			Residence time			Ozonation								
			Low		High	Aged		Fresh	No		Yes						
			Avg	Std Dev	Avg	Std Dev	Avg	Std Dev	Avg	Std Dev	Avg	Std Dev	<i>p</i> value				
<b>Exposure components</b>																	
EC (µg/µg)	15	2	0.94	0.12	1.04	0.06	0.051	1.01	0.08	0.99	0.12	0.68	0.98	0.11	1.04	0.06	0.30
OC (µg/µg)	15	2	0.18	0.04	0.11	0.02	<0.001	0.14	0.05	0.12	0.03	0.057	0.13	0.05	0.14	0.03	0.43
1-NP (pg/µg)	8	2	2.47	1.96	0.14	0.08	0.062	2.03	2.39	0.57	0.57	0.18	1.61	2.45	1.00	1.11	0.54
PAH (ng/µg)	19	c	13.3	3.8	17.5	3.8	<b>0.013</b>	15.0	5.0	16.4	3.6	0.53	17.7	3.4	14.0	4.4	0.024
Cu (ng/µg)	8	2	1.20	0.41	1.11	0.43	0.58	0.88	0.20	1.43	0.35	0.019	1.35	0.35	0.96	0.37	0.052
Zn (ng/µg)	8	2	0.66	0.08	0.90	0.18	0.031	0.85	0.20	0.71	0.16	0.12	0.71	0.08	0.84	0.25	0.15
Ca (ng/µg)	8	2	0.42	0.10	0.53	0.21	0.40	0.56	0.19	0.40	0.11	0.25	0.46	0.10	0.49	0.23	0.83
Mg (ng/µg)	8	2	0.53	0.18	0.43	0.08	0.10	0.46	0.13	0.49	0.16	0.54	0.37	0.01	0.58	0.12	<b>0.011</b>
Sn (ng/µg)	8	2	0.28	0.06	0.33	0.03	0.19	0.28	0.06	0.33	0.03	0.16	0.31	0.07	0.30	0.03	0.85
Fe (ng/µg)	8	2	0.19	0.08	0.31	0.06	0.060	0.27	0.11	0.22	0.08	0.31	0.22	0.07	0.28	0.11	0.26
NO <sub>2</sub> (ppb/µg/m <sup>3</sup> )	29	c	4.5	4.3	2.1	2.3	0.047	3.7	4.4	3.0	2.8	0.44	1.1	1.5	5.0	3.8	<b>0.002</b>
NO (ppb/µg/m <sup>3</sup> )	29	c	4.3	4.3	3.4	2.4	0.23	4.1	3.8	3.6	3.2	0.67	6.9	2.8	1.7	2.0	< <b>0.001</b>
CO <sub>2</sub> (ppm/µg/m <sup>3</sup> )	12	c	3.9	0.5	3.5	0.6	0.16	4.0	0.5	3.3	0.5	0.031	3.9	0.5	3.5	0.6	0.23
CO (ppb/µg/m <sup>3</sup> )	20	c	0.013	0.005	0.006	0.001	< <b>0.001</b>	0.010	0.006	0.009	0.005	0.38	0.01	0.006	0.009	0.005	0.21
MMAD (nm) (GSD)	8	1	79 (1.99)	8	65 (1.87)	24	0.33	80 (1.65)	6	64 (2.22)	23	0.25	68 (1.99)	1	76 (1.88)	18	0.57
<b>Reactivity</b>																	
DTT (ng/min/µg PM)	8	1-3	0.88	0.27	0.40	0.07	<b>0.010</b>	0.73	0.38	0.55	0.26	0.15	0.74	0.41	0.53	0.19	0.12
<b>In vitro toxicity</b>																	
MIT assay (5 µg/ml DEP) <sup>d</sup>	8	3	82	16	87	9	0.44	93	8	76	12	0.070	80	8	89	16	0.25
MIT assay (25 µg/ml DEP) <sup>d</sup>	8	3	60	17	50	5	0.28	60	17	50	6	0.29	49	6	60	16	0.25
MIT assay (100 µg/ml DEP) <sup>d</sup>	8	3	38	10	30	9	0.20	31	12	37	7	0.34	29	4	39	11	0.12
CFU assay (5 µg/ml DEP) <sup>d</sup>	8	3	82	8	68	5	0.046	78	12	72	5	0.32	76	6	74	12	0.80
Scratch assay (5 µg/ml DEP) <sup>d</sup>	8	4-5	87	5	89	4	0.54	89	4	88	5	0.75	87	5	90	3	0.47
Scratch assay (10 µg/ml DEP) <sup>d</sup>	8	4-5	86	11	95	6	0.28	93	14	89	5	0.61	89	13	93	6	0.59

Post hoc tests of contrasts from multivariate analysis are statistically significant for *p* values <0.0167

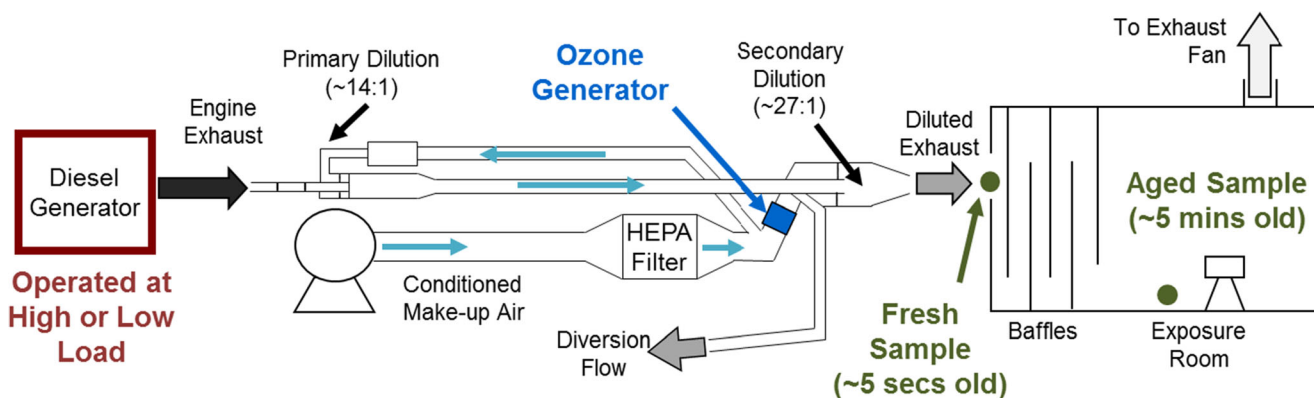
EC elemental carbon, OC organic carbon, PAH polycyclic aromatic hydrocarbon, MMAD mass median aerodynamic diameter, DEP diesel exhaust particulate

<sup>a</sup> Experimental replicates

<sup>b</sup> Observational replicates

<sup>c</sup> Direct reading measurement

<sup>d</sup> Measured as a % of controls



**Fig. 1** Schematic of UW DE exposure facility with generation parameters

Dithiothreitol (DTT) and 1-nitropyrene (1-NP) samples were collected on 37-mm-diameter Teflon membrane filters (Zefluor #P5PJ037, Pall Corporation, Port Washington, NY) within open-faced Harvard Impactors in which the impactor stage was removed. For DTT analysis, we adapted the procedure of Li et al. (2003b) and Kumagai et al. (2002) for use with a microplate reader. Briefly, DTT reactivity was analyzed by extracting particles from filters in methanol. The extract was then incubated with DTT and portions of the extract were removed at specific time points and allowed to react with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 3-thio-6-nitrobenzoate (TNB). The absorbance due to TNB at 412 nm was measured in a microplate reader (SpectraMAX plus, Molecular Devices Corporation, Sunnyvale, CA). The rate of consumption of DTT was calculated from the plot of absorbance versus time with a correction for atmospheric oxidation of DTT based on a reagent blank time series. 1-NP was analyzed by extraction of particles in dichloromethane, followed by evaporation and resuspension in a mixture of ethanol and sodium acetate buffer and analyzed via 2D-HPLC-MS/MS (Agilent 1100 HPLC, Agilent 6410 Triple Quad Mass Spectrometer, Santa Clara, CA), as previously described (Miller-Schulze et al. 2010).

The particle-bound fraction of polycyclic aromatic hydrocarbons (PAHs) was measured via direct reading of induced electrical charge (Ecochem PAS 2000, League City, TX).

The mass median aerodynamic diameter (MMAD) was measured via a 10-stage cascade impactor (MOUDI Model 110-NR, MSP, Shoreview, MN; stage cut-sizes 0.055–18  $\mu\text{m}$ ). The estimation of MMAD was restricted to mass collected on stages with cut-size  $\leq 0.55 \mu\text{m}$  to limit the analysis to a single mode representative of DEP. The impactor flow was operated at 30 l/min, and samples were collected on Zefluor substrates weighed before and after sampling via gravimetric analysis. The count median diameter (CMD) was measured with a P-Trak Ultrafine Particle Counter (Model 8525; TSI, Shoreview, MN) in combination with size-selective diffusion screens (cut-sizes 0.02–0.155  $\mu\text{m}$ ).

At least 20 % blanks and 20 % duplicates were collected during each day of DE generation for analysis of all particle samples on filters dedicated to DE composition analysis. Except for OC and EC, which were corrected as detailed above, analyte concentrations of samples were blank-corrected when deemed appropriate, which was generally when the blank concentrations were  $\geq 10 \%$  of the sample concentrations.

In the gas phase,  $\text{NO}_x$  and  $\text{NO}_2$  were measured continuously by chemiluminescence (Thermo Model 42C, Thermo

**Table 2** Specification of the University of Washington (UW) diesel exhaust (DE) exposure facility and the generation parameters tested

DE exposure facility specifications		
Generator type	5.5-kW, single-cylinder generator (Yanmar model YDG5500EV-6EI)	
Fuel type	Ultra-low sulfur diesel fuel, maximum sulfur content 15 ppm	
Lubricating oil	Chevron DELO 400LE, SAE 15 W-40	
Dilution system	2 step: immediate $\sim 14:1$ , delayed $\sim 27:1$ , overall $\sim 400:1$	
DE generation parameters		
Engine load (load bank)	Low: $\sim 27 \%$ generator output	High: $\sim 82 \%$ generator output
Residence time	Aged: sample collected at location where DE is $\sim 5$ min after emission	Fresh: sample collected at location where DE is $\sim 5$ s after emission
Ozone addition	No: no ozone added	Yes: ozone added to achieve $\text{NO}_2$ in excess of $\text{NO}$ by $\sim 100$ ppb

**Table 3** Sampling of DE components

Analysis	DE collection device	Collection media	Analytical procedure
Gravimetric mass	Harvard Impactor, 2.5 µm cut size	Teflon filter	Microbalance
Metals and other elements	Harvard Impactor, 2.5 µm cut size	Teflon filter	XRF
Organic/elemental carbon	Harvard Personal Environmental Monitor, 2.5-µm cut-size	Quartz filter	TOR
1-Nitropyrene	Open-Faced Aluminum Filter Holder (Harvard Impactor)	Teflon-coated fiber filter	HPLC-MS/MS
Polycyclic aromatic hydrocarbons	Ecochem photoelectric aerosol sensor	NA	NA
Oxidative potential (DTT reactivity)	Open-faced aluminum filter holder (Harvard Impactor)	Teflon-coated fiber filter	Absorbance Microplate Reader
Size distribution	Moudi Impactor	Teflon-coated fiber filter	Microbalance
Nitric oxides	Chemiluminescence Analyzer	NA	NA
Carbon dioxide	Infrared Detector	NA	NA
Carbon monoxide	Electrochemical Sensor	NA	NA

NA not Applicable

Fisher Scientific, Waltham, MA). Carbon dioxide (CO<sub>2</sub>) was measured by non-dispersive infrared detection (Telaire Model 1050, Telaire Systems). Carbon monoxide (CO) was measured by an electrochemical sensor (Langan Model T15n, Langan Products, San Francisco, CA).

We collected samples between April 2010 and May 2012. To allow for comparison across the varying environments, both gas and particle concentrations were normalized to PM<sub>2.5</sub> concentration.

#### Particle preparation, exposure of cells, and cell cultures

Particle samples for in vitro toxicity testing were collected on 3 µm pore size 8"×10" PTFE-coated fiber filter sheets (Zeflour #P5PI001, Pall Corporation, Port Washington, NY) using a hi-vol sample collector designed for total suspended particulate matter (Method 40 CFR 50, Appendix B, with a flow rate of 1.0 to 1.1 m<sup>3</sup>/min). Particles were extracted from filters by suspending in methanol, sonicating/shaking, and evaporating to dryness. The extracted particles were resuspended in phosphate-buffered saline (PBS) with <0.1 % dimethyl sulfoxide. A media control was prepared by applying the vehicle (PBS and dimethyl sulfoxide) to cells without extracted particles. A blank filter was processed using the same techniques to be used as a filter control.

SVEC4-10 murine endothelial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum plus antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin) at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>/95 % air. Cells were seeded at an assay appropriate starting density and grown for an additional 18–24 h prior to treatment. Dependent on assay type, suspensions of DEP were then administered at concentrations of 0, 5, 10, 25, or 100 µg/ml for periods of up to 24 h.

#### Quantification of cell viability, cell proliferation, and cell migration/repair

The MTT assay, a surrogate of cell viability, assesses metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide reagent (Sigma-Aldrich, St. Louis, MO) to formazan crystals. SVEC4-10 cells were plated and treated with DEP in 24-well microtiter plates. Subsequent to DEP treatment, medium was removed and cells were washed with PBS. MTT solution was added to the wells and then removed after 1-h incubation. Precipitated formazan was dissolved with 100 µl DMSO, and samples were centrifuged at 10,000×g to remove any residual DEP from the formazan suspension. Absorbance at 405 nm was read in a SpectraMax spectrophotometer (McConnachie et al. 2013).

For the colony formation assay, a measure of cell proliferation, 50 SVEC4-10 cells/well were introduced into a six-well tissue culture plate. After attachment, DEP was introduced at a concentration of 5 µg/ml. Seven days later, cell colonies were fixed and stained with 1 % Crystal Violet in 20 % methanol. Gray-scale images of the plates were then obtained using a BioRad Gel-Doc system (BioRad, Hercules, CA), and the change in relative optical density per well was used as an index of total colonies formed (Franken et al. 2006).

Wound healing was measured to indicate cell migration and repair by scratching a confluent monolayer of SVEC4-10 cells with a P200 pipette tip. Phase contrast images of the "wound" were obtained using an Olympus IMT2 inverted microscope (Olympus, Center Valley, PA). Time-dependent image analysis was utilized to assess cell migration into the "wound" induced into the monolayer (Liang et al. 2007). Results were calculated as the change in "wound" area over time.

Toxicity was assessed with a dose of 5 µg DEP/ml for all three cellular assays, and additional doses for the MTT (25 and 100 µg DEP/ml) and scratch assays (10 µg DEP/ml). All

toxicity effects are measured as the percentage difference in response of DEP dose compared to filter blank.

### Statistical analysis

All data were analyzed in Stata (StataCorp v 11.0, College Station, TX) using simple linear regression of the mean of replicate samples collected on a single day. (In Table 1 “Experimental Observations” represent the number of days of sampling, with a unique DE atmosphere each day). For estimates of the effect of each of the three DE generation factors on either the exposure composition or toxicity, a Bonferroni correction factor was applied to the multivariate analysis, leading to a statistical significance level of  $p=0.0167$ . To estimate the dose–response for the MTT assay, we applied a linear mixed model that accounted for the repeat MTT measures on individual filters, where filters correspond to unique conditions.

## Results

### Gas and particle characteristics of DE atmospheres

The average daily  $PM_{2.5}$  concentration in the exposure facility was  $233 \mu\text{g}/\text{m}^3$  (range 97 to  $376 \mu\text{g}/\text{m}^3$ ) across all tested conditions. Different sampling durations were applied based on the ideal PM loading for each component, varying between 1.5 and 6 h. The average daily temperature and relative humidity in the exposure facility during sample collection was  $24 \text{ }^\circ\text{C}$  (range of daily averages,  $20\text{--}27 \text{ }^\circ\text{C}$ ) and 36 %

(range, 0–11 %), respectively. Within a day of collection, the temperature increased by an average of  $4 \text{ }^\circ\text{C}$  and the relative humidity increased by an average of 3 % over a 6-h period.

Particulate-matter-normalized measurements were higher for low engine load versus high engine load by >15-fold for 1-NP (not statistically significant), approximately 2-fold for DTT ( $p=0.010$ ), approximately 2-fold for CO ( $p<0.001$ ), and approximately 1.5-fold for OC ( $p<0.001$ ) (Table 1 and Fig. 2). The mass fraction of particle-bound PAHs was approximately 2-fold higher for high engine load ( $p=0.013$ ).

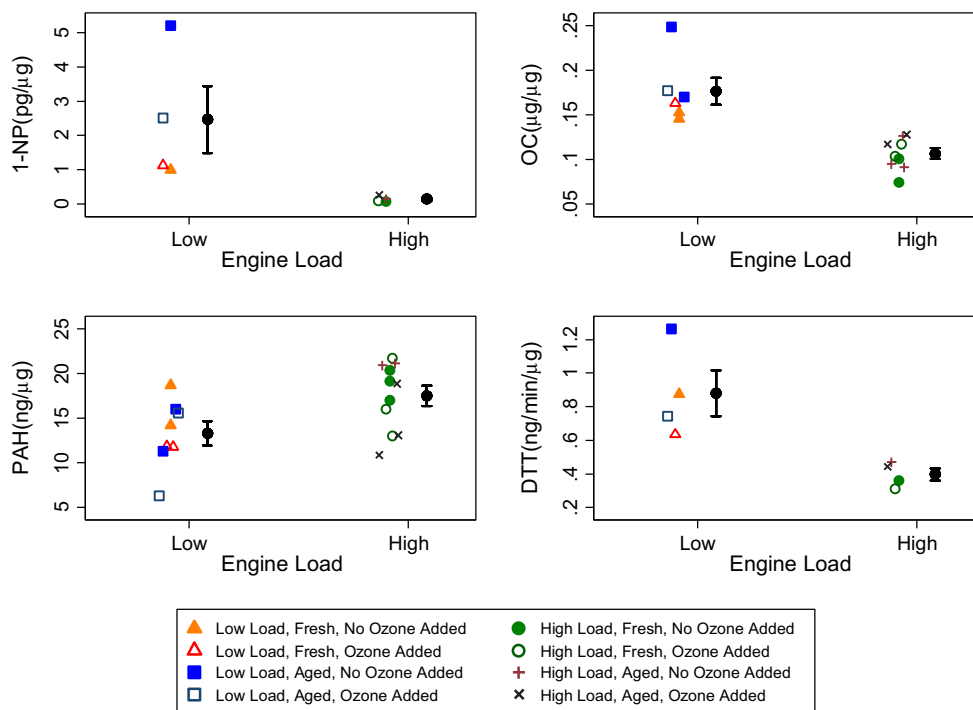
With the addition of ozone,  $\text{NO}_2$  was approximately 4-fold higher ( $p=0.002$ ), whereas NO was depleted by a similar magnitude (4-fold difference,  $p<0.001$ ), as would be expected due to titration. No exposure components differed statistically significantly by residence time.

The particle mass distribution of DEP, measured as the MMAD, was not found to vary by the three parameters. Although MMAD of DEP was estimated using a restricted particle size distribution, particles were collected with a median cut-size of up to  $18 \mu\text{m}$  in the MOUDI cascade impactor. In review of the full spectrum of particles collected in the MOUDI, during generation of DE from a typical condition, 97 % of particles by mass were less than a median  $1.8\text{-}\mu\text{m}$ -diameter cut-size, and 48 % of particles by mass were less than a median cut-size of  $0.1 \mu\text{m}$ .

### In vitro cytotoxicity

All cytotoxicity results were normalized with a corresponding cell culture medium-only control. Cell viability averaged across the DEP from the eight atmospheres was

**Fig. 2** Mass fraction of exposure components (1-NP, OC, and PAH) and reaction rate of reactivity (DTT) by engine load. Data points represent all experimental replicates; some DE atmospheres were repeated. (See Table 1 for number of experimental replicates.) Black point estimates and black error bars to the right of data points represent mean and standard error by load



84 % (SD 13) for a 5  $\mu\text{g}$  DEP/ml dose versus 99 % in filter control samples, 55 % (SD 13) for 25  $\mu\text{g}$  DEP/ml versus 95 % in filter controls, and 34 % (SD 10) for 100  $\mu\text{g}$  DEP/ml versus 85 % in filter controls. We observed a statistically significant linear decline in viability across the three doses ( $p < 0.001$ , Fig. 3). The average half maximal effective concentration ( $\text{EC}_{50}$ ) for a response in viability of the average of the eight DE atmospheres obtained from doses of 5, 25, and 100  $\mu\text{g}/\text{ml}$  is 13.6  $\mu\text{g}$  DEP/ml (SD 7.7). Average cell proliferation across the eight atmospheres was lower with a 5  $\mu\text{g}$  DEP/ml dose than in filter control samples (75 % (SD 9) vs 102 %, respectively). Average wound healing was lower across the eight atmospheres compared to filter controls for both 5  $\mu\text{g}/\text{ml}$  DEP (88 % (SD 4) vs 100 %) and 10  $\mu\text{g}/\text{ml}$  DEP (90 % (SD 10) vs 100 %).

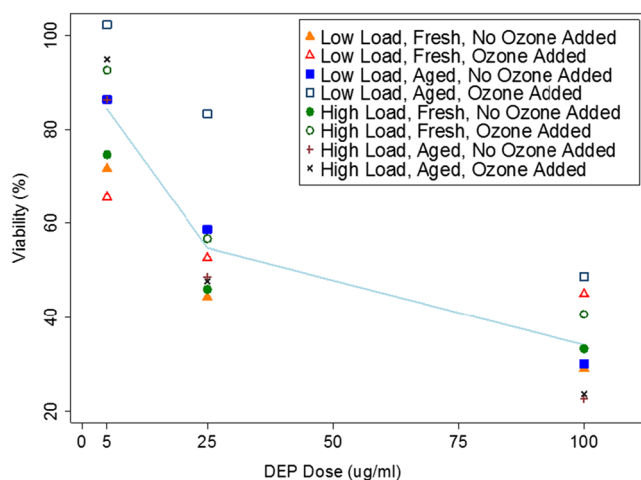
We report the relative toxicity by each of the DE generation parameters (engine load, residence time, and ozonation) in Table 1. There were no statistically significant differences in the *in vitro* toxicity of DE when varied by these parameters.

## Discussion

In this study of DE generated in a controlled exposure facility, we found that altering engine load had the greatest effect on exposure characteristics and oxidative reactivity. Ozonation of the exhaust stream and modification of the residence time prior to particle collection had less impact.

### Diesel exhaust atmospheres

The two-step design in our controlled facility allows for rapidly quenching particle conditions in the first dilution and adjustment of particle concentration to levels that are more ideal for study in the second step. With the application of these



**Fig. 3** Cell viability of DEP. The *light blue line* connects the mean response of each dose (Color figure online)

design features, we were able to compare the exposure components, reactivity, and toxicity of the DE generation parameters by adjusting for  $\text{PM}_{2.5}$  concentration.

Operating engines at low load generally results in less complete combustion—as demonstrated by the increase in the ratio of OC to EC with decreasing engine load that we and others have found (McDonald et al. 2011; Shi et al. 2000). The efficiency of engine operation also tends to alter emissions of PAHs, as the engine temperature is the main determinant of PAH emissions, and engine load is proportional to engine temperature (Scheepers and Bos 1992). In accordance, we measured more particle-bound PAH with high engine load. However, it is worth noting that our measurement of particle-bound PAH with the Ecochem aerosol monitor relies on selective photoionization of PAHs and cannot be compared directly with standard mass-spectrometry-based approaches.

In contrast with particle-bound PAHs, 1-NP decreased with increased load, which has similarly been reported by Schuetzle and Perez (1983). While many nitro-PAHs are formed following emissions in the atmosphere, the majority of 1-NP (>80 %) is formed in the exhaust stream (Scheepers and Bos 1992). Our finding of a >15-fold higher concentration of 1-NP in low versus high load conditions was not statistically significant; this may be attributed to increased variability caused by an interaction with one or both of the other DE generation parameters (residence time or ozonation), although we were unable to test for interactions in this small data set. 1-NP is particularly important among the components of DE because it is one of the largest contributors to mutagenicity, measured by the Ames test (Sjogren et al. 1996). At the same time, 1-NP does not appear to be related to cell proliferation, migration, or viability, since we observed no significant effects by engine load for these cytotoxicity measurements.

We found no general trends in metal content by the DE generation parameters, although we did observe a significant elevation of Mg with ozone addition. We do not have a hypothesis for this finding, and chance is a very plausible explanation. In contrast, Sharma et al. (2005) reported a modest decrease in several metals (Fe, Mg, Ca, Cr, Ni, Pb, Zn, and Ba) with increasing load on a larger four-cylinder truck engine, which they attribute to the increase of the engine's thermal efficiency (brake specific fuel consumption) at higher engine loads. However, our small engine is designed to produce a relatively constant electrical power output over a range of engine loads, and therefore, their results may not be directly comparable to ours.

As expected, DE in the UW facility has a high content of particles in the ultrafine size range. A measure of mass distribution (MMAD) was not found to vary substantially by load, in agreement with findings by McDonald et al. (2011).

The OC-to-EC ratio (0.10) in our facility with the Yanmar generator, under high load, in the aged location and without ozone addition (a typical operating condition) is lower than a



previous estimate from our facility with the Cummins engine (0.34), and that reported by McDonald et al. with a Yanmar generator (0.41) (Supplemental Material). This difference in the OC-to-EC ratio may be attributed to use of different engine models, differences in the applied engine loads, an update of the analytical method for carbon (IMPROVE-A vs NIOSH method), and the use of dynamic blanks to correct OC in the present study (correction reduces OC concentration by ~50 %) (Gould et al. 2008; McDonald et al. 2004a).

Our surrogates of two types of photochemical aging of particles, the time since emission and the presence or absence of ozonation, were simplified ways to represent the processes in the natural environment. Neither time since emission nor ozone addition was found to induce a significant change in the components of DEP.

### Oxidative reactivity

Oxidative reactivity represents an acellular approach providing information bridging toxicity and composition measurements, as it indicates the ability of particulate matter to produce reactive oxygen species (2008). Oxidative stress is thought to initiate a cascade of steps that lead to inflammation, which is hypothesized to play a role in more severe health outcomes such as cardiovascular morbidity, asthma, and diabetes (Brook et al. 2010; Li et al. 2003a; Lugrin et al. 2013; Moller et al. 2008; Yin et al. 2013). While the relationship to human health effects is not currently understood, DTT reactivity is a sensitive measurement that is relatively easy to obtain Ayres et al. (2008).

Our average DTT measurement for the eight conditions (0.64 ng/min/ $\mu\text{g}$  (SD 0.32), or 145 ng/min/ $\text{m}^3$  (SD 57)) is about 10 times higher than the average measurement our research group obtained from six urban areas in the USA as part of the National Particle Component Toxicity Initiative (NPACT, 11.04 ng/min/ $\text{m}^3$  (SD 3.62)), which applied a similar analysis method, other than the use of quartz filters (Vedal et al. 2013). Our DTT measurements are about five times less than those measured from urban ambient samples from California (3.2–5.5 ng/min/ $\mu\text{g}$ ) (Ntziachristos et al. 2007) and DE generated in another controlled exposure facility (2.7–3.5 ng/min/ $\mu\text{g}$ ) (Cheung et al. 2009). These differences may be partially explained by the collection of particles in a liquid impinger in both of these latter investigations rather than the use of particles extracted from filters in our study. Differences in extraction of particles and the applied analytical method may result in large differences in measured DTT activity values. Our research group uses methanol extraction, which is a mid-polarity solvent. Other researchers extract particles for DTT measurement with either dichloromethane, a non-polar organic solvent that would result in greater extraction of PAHs such as quinones or “quinone-like” species (Pan et al. 2004; Shinyashiki et al. 2009), or an aqueous solvent that

would result in extraction favoring metals (Biswas et al. 2009; Charrier and Anastasio 2012).

To our knowledge, this is the first report of a difference in DTT activity by engine load. Our finding suggests that low engine load produces a more reactive environment, indicative of greater toxicity, than high load.

### In vitro screening

To assess the relative toxicity of different diesel exhaust compositions, we measured the in vitro toxicity of diesel exhaust particulate (DEP) toward murine endothelial cells. Endothelial cells play an important role in the vasculature and have been previously investigated as relevant targets in DEP-induced cytotoxicity and oxidative stress (Bai et al. 2001; Hirano et al. 2003; Mattingly and Klinge 2012; Weldy et al. 2011). Murine SVEC4-10 endothelial cells, specifically, have also been used in several investigations of the vasculature and vascular reactivity (Choi et al. 2011; Kang et al. 2011; Wang et al. 2012; Weldy et al. 2011; You et al. 2012).

The MTT, colony formation, and scratch assays are in vitro toxicity assessments that provide insight regarding the gross impact of DE exposure on aspects of cell physiology, including cell viability, proliferation, and wound healing, respectively (Cory 2011; Katz et al. 2008; Peplow and Chatterjee 2013; Sylvester 2011). These toxicity assays were not intended to provide detailed mechanistic information about DE, but rather served as a screening tool to demonstrate which DE parameters from the different DE exposure atmospheres impact toxicity in general.

The MTT assay is generally interpreted as a measure of mitochondrial function and is one of the most common measurements of cell viability. The MTT assay has been applied in several investigations to measure toxicity in endothelial cells resulting from different pollutants (Hirano et al. 2003; Hsu et al. 2009; Liu et al. 2005; Mattingly and Klinge 2012; Napierska et al. 2009; Weldy et al. 2011). Comparison of cell viability attributed to different types of particles is limited by many factors, including differences in protocols and types of endothelial cell lines applied, yet the trend among these studies suggests that particle toxicity measured as cell viability is generally highest for wood smoke, followed by silica and DEP, and lowest for cigarette smoke (Hirano et al. 2003; Hsu et al. 2009; Liu et al. 2005; Napierska et al. 2009; Weldy et al. 2011).

The colony formation assay and the scratch assay are less standard techniques for assessing cytotoxicity but are similarly rapid and straightforward. The colony forming assay is considered a sensitive measure of cytotoxicity, since the formation of a visible colonies over a 7-day period from single, plated cells requires both maintenance of cell viability and replication through multiple cell cycles in the presence of the toxicant. The scratch assay is a surrogate for a wound healing

response following the introduction of a scratch across a cell monolayer with a pipette tip. The culture is then monitored over time for how long it takes to “repair” the wound as cells fill in the opened area left by the scratch. This involves both migration of cells from the leading edge of the wound as well as replication of cells.

These three *in vitro* toxicity assays have been previously used to demonstrate DEP-induced cytotoxicity (Danielsen et al. 2008; LaGier et al. 2013; Mattingly and Klinge 2012; Okayama et al. 2006; Poss et al. 2013; Tau et al. 2013), which we also observed. Based on the mean toxicity compared to controls, our results suggest that the order of sensitivity of these assays to a low dose of DEP (5 µg/ml) is highest for the colony formation assay (75 %, SD 9 %), followed by the MTT assay (84 %, SD 13 %), and lowest for the scratch assay (88 %, SD 4 %). Although differences between these assays may be specific to cell type and toxicants, the crystal violet assay (similar to the colony formation assay) was similarly found to be more sensitive than the MTT assay when applied to cancer cells (Martin and Clynes 1993).

Few published studies have investigated the differential effects of DEP with varying composition. Although we found no differences in toxicity by the three DE generation parameters tested, an overall effect of DEP versus controls was observed for all three *in vitro* toxicity assays studied, suggesting that these are appropriate models. In contrast with our analysis of the effect of DEP on MTT toxicity, which did not result in a meaningful difference by the three DE generation parameters, Liu et al. exposed epithelial cells to gaseous extracts of DE and observed a trend of increasing toxicity in the MTT assay with decreasing engine load (Liu et al. 2009). Perhaps this difference is explained by Liu et al.’s focus on the gaseous fraction of DE versus our focus on particles for toxicity testing.

#### Limitations

The goal of this study was to generally explore the influence of DE generation parameters (engine load, residence time, and presence of ozone). Applying a factorial design provided greater statistical power to investigate these three parameters for the number of samples tested. We used the statistical analysis as a tool to highlight exposure components, reactivity, and toxicity measures of particular interest for future study. With a small data set, we are not able to also consider other factors, such as measurement error, another controlled factor, or even one of the other measured parameters that have affected our data.

In the natural environment, the effects of residence time and ozonation are generally intertwined, resulting in complex particle growth, particle and gas phase interactions, and formation of secondary organic aerosols. The ozone generators inserted in the DE train for this investigation did not achieve

the degree of oxidation that occurs with sunlight or smog chambers, for which an increase in markers of inflammation and cytotoxicity has been reported (Doyle et al. 2007; Ebersviller et al. 2012b; Sexton et al. 2004).

While the introduced parameters were intended to mimic real-world differences in ambient DE in the UW facility’s generated DE, they were not perfect representations. Engine load was likely the best rendition in the facility, as the application of the load bank on the engine is directly comparable to load changes induced by driving vehicles. This may also partially explain why the effects of engine load on composition and reactivity were the most pronounced.

While using a toxicity screening approach with efficient laboratory procedures is arguably appropriate for the purpose of this study, there is considerable concern about measuring toxicity with exposures from extracted particles. DEP extracts exclude gases, which are thought to be an important contributor to DE toxicity, and the process of extraction is thought to remove important components from the surface of particles. These issues likely led to reduced toxicity from exposure to DEP compared to complete DE (Ebersviller et al. 2012a; Lichtveld et al. 2012; Totlandsdal et al. 2012). A smaller overall effect for DEP would likely reduce the ability to detect differences between the eight atmospheres. Our research group is currently working on exposing cell cultures to native DE without extraction. Further, the toxicological impacts of the gas/particle DE mixture in the environment can be more comprehensively evaluated in human and animal inhalation studies such as those typically conducted in our facility. The study described in this paper was designed to inform decision-making for future inhalation studies.

#### Conclusions

Alteration of engine load, compared to addition of ozone or changes in residence time, resulted in the largest compositional differences: 1-NP, DTT activity, and OC were elevated with low load, while PAHs were elevated with high load. All three high-throughput assays selected to screen for gross *in vitro* toxicity in murine endothelial cells demonstrated an effect of DEP. However, these effects on cytotoxicity of particle exposures were not modified by alteration of engine load, residence time, and ozonation.

Studies involving controlled DE exposures are generally limited to comparison of effects following exposure to a single mixture (though sometimes at varying concentrations) versus a filtered air exposure. Given that natural DE composition can shift widely with different vehicle parameters and conditions of the environment, future work is needed to identify which components are associated with the numerous toxic effects. Our findings, though limited, suggest that important

compositional aspects and biological reactivity may be altered by engine operating characteristics.

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