Preliminary differences in composition and cytotoxicity between diesel and biodiesel emissions

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Introduction

Biodiesel is considered an environmentally friendly, sustainable alternative fuel to petroleum diesel. Many studies have been done to date on the toxicology of diesel particulate matter (PM); however, literature regarding the toxicology of biodiesel PM is limited. While it is well known that biodiesel blends reduce tailpipe emissions of PM mass compared to petroleum diesel (Graboski et al. 1998; Lapuerta et al. 2007; Yanowitz et al. 2009), less is known regarding the impact of biodiesel PM on toxicological responses, especially from PM generated in 'real world' operations using biodiesel fuel. The need for improved understanding of the impact of biodiesel fuel on real world emissions and exposures is particularly important since the U.S. plans to increase production of biodiesel from 9 billion to 36 billion gallons by the year 2022 (110^{th} Congress 2007).

Exposure to PM from diesel engines poses a significant risk to public health. Exposure to fine PM (PM <2.5 µm in diameter) is associated with a range of health effects including but not limited to: reduced lung function, exacerbation of asthma, and an increase in mortality rates (Pope et al. 2000; Lippmann et al. 2003; USEPA 2004).

Pope et al. (2010) discuss that even low to moderate levels of exposure to fine PM may result in a linear exposure-response relationship, suggesting that humans may not have a 'safe' exposure threshold to PM. Diesel engines are an important source of this fine and ultrafine (<0.1 µm diameter) PM (U.S. EPA 2002). Exploration of the potential of alternative fuels such as biodiesel to reduce PM emissions and associated health impact is an area of active research (Cheung et al. 2010; Hemmingsen et al. 2011; Gerlofs-Nijland et al. 2013)**.**

Commercial B20 (20% biodiesel/80% petroleum diesel blend) is considered a renewable fuel, and is produced mainly from soybeans and canola oil (Kinast et al. 2003). Biodiesel is synthesized through transesterification in which triglycerides in oil or fats are catalyzed by an acid or base with the addition of methanol. Transesterification yields two distinct products: glycerol (a byproduct used commercially in cosmetics) and fatty acid methyl esters (Zhou et al. 2006). Biodiesel fuels are mainly composed of long chain fatty acid methyl esters (FAMEs) compared to hydrocarbons in petroleum diesel (Hoekman et al. 2012).

Because biodiesel is manufactured from plant feedstocks, it is considered a fuel that reduces greenhouse gas emissions, and is thus more environmentally friendly. Under the Energy Independence and Security Act of 2007 (EISA), performance threshold standards for the lifecycle of greenhouse gas performance were implemented. EISA promotes the use of renewable fuels, which release a net 50% less greenhouse gas emissions compared to fossil fuels. For example, under EISA, biodiesel should reduce greenhouse gases emitted by at least 50% in comparison to the petroleum diesel which it aims to replace (EPA 2010). This is due to biodiesel having a greater turnover rate, since it is coming from plant feedstocks versus oil reserves in the Earth's crust.

Petroleum diesel PM has a high surface area and contains semi-volatile organic compounds (SVOC's), polycyclic aromatic hydrocarbons (PAH's), and nitro-polycyclic aromatic hydrocarbons (Mauderly et al. 2000; Cho et al. 2005; Hemmingsen et al. 2011). The aforementioned compounds possess carcinogenic properties (Angerer et al. 1997). The combustion of biodiesel reduces PM mass in tailpipe emissions compared to petroleum diesel, but less is known about worker exposure concentrations to PM from use of biodiesel in real world operations. Recent work by Traviss et al. (2012), demonstrated a significant reduction in worker exposure to PM from use of a B20 blend. However, if biodiesel PM's chemical composition is more toxicologically active, than the reduced emissions profile may not necessarily result in reduced health impact.

The goal of this study was to compare biodiesel vs. petroleum diesel PM characteristics, including mass concentration, metals composition and cytotoxicity of PM collected in a 'real world' occupational setting. We examined the differences in PM generated by non-road heavy duty diesel engines using petroleum diesel fuel and commercially purchased B20 blend. . We collected PM from within the operator cabin of the same construction type equipment, hypothesizing that there would be differences in particle mass concentration, chemical composition, and cytotoxicity between fuel types. The goal is to increase general understanding of potential health effects from the combustion of biodiesel as an alternative to petroleum diesel.

Overall study approach

Particulate matter was collected from the operator cabins of non-road construction vehicles and an interior work area located at the Keene Recycling Center (KRC). The KRC is a municipal materials recovery facility in Keene, NH. At this location, waste collection trucks from Keene and surrounding towns drop off recyclable and non-recyclable materials. KRC non-road equipment, listed in Figure 1 below, moves the trash or recyclables around the site, staging it for processing or eventual offsite transport. The site is ideal for this study due to the isolated location and consistent outside traffic patterns. Onsite KRC vehicles are the main source of diesel/biodiesel PM. A schematic layout of the KRC and the PM sampling locations are shown in Figure 1. PM was collected inside the cabin of a John Deere 624K large front loader (six cylinder diesel engine, 198 horsepower at 1800 rpm), near the worker's breathing zone over the entire 8 hour work shift. This location is designated as P1B. PM was collected from June to August 2011, with nine days each collected for diesel and biodiesel operation. A three week fuel transition period was allowed between the diesel and biodiesel collection periods so that the equipment could fully transition from diesel fuel to B20 fuel use before B20 sampling took place.

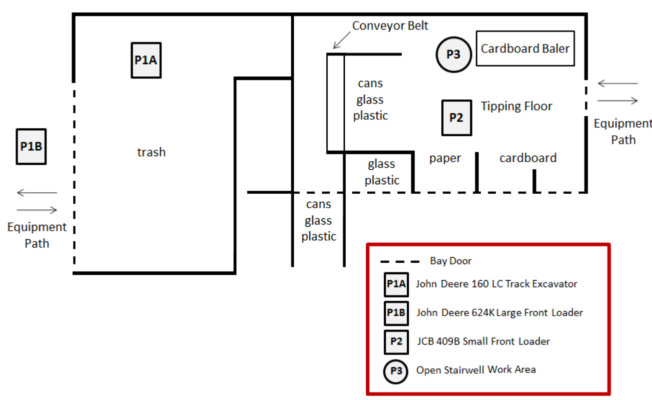

Figure 1: Study site layout. Keene Recycling Center, Keene, New Hampshire

PM Collection and Chemical Analysis

PM was collected using Sioutas impactors containing 25 mm and 37 mm Teflon filters (PALL corporation Zeflour 25 mm filters Product Number: P5PQ025; SKC 37 mm filters Catalog Number: 225-1709) located inside the equipment cabin, within the employee's breathing zone, for each piece of equipment in Figure 1. However, this paper reports only on the PM collected for P1B, the John Deere 624K. The Sioutas impactors size segregate PM across five size cuts: >2.5 µm, 2.5 to 1.0 µm, 1.0 to 0.5 µm, 0.5 to 0.25 µm, and <0.25 µm. Air was collected through the impactor using a Leland Legacy pump calibrated to a flow rate of nine liters per minute (LPM). Pumps were pre and post calibrated daily using a Bios Defender (#530-H) primary calibrator. Mass concentration data are reported for the smallest size cut (<0.25 µm) only. At this size cut, the predominant source of PM is fuel combustion, not dust or other sources. Tailpipe raw exhaust samples were also collected from P1B on 47 mm Tissuquartz filters (SKC 47 mm filters Catalog Number: 225-1823) that were pre-baked to 550 degrees Celsius and placed in a stainless steel filter holder. The tailpipe filters were subsequently extracted and used in the toxicological experiments, as described in the section below.

The Teflon filters were pre and post weighed in a temperature and humidity controlled environment using a Mettler Toledo Microbalance XP-6 (readability of 1 µg) to determine mass concentration. The metals concentrations and analysis (for Teflon filters collected at P1B) were completed by the Dartmouth College Trace Elements Laboratory (Hanover, NH) using an Inductively Coupled Mass Spectrometer (ICP-MS). The metals analyzed in this study include: sodium, aluminum, chromium, manganese, iron, nickel, copper, zinc, molybdenum, barium, and lead.

PM extraction for cytotoxicity assay

The tailpipe 47 mm filter sample was cut in half for ethanol extraction. The filter was placed in a 40 mL amber glass bottle and soaked overnight in 5 mL ethanol. To prevent photolytic reactions, the bottles were wrapped in in aluminum foil. The filter was then sonicated for 60 minutes (ice was added to ensure temperature did not exceed 35^o C). Extracts were filtered using a 10 mL flat-bottom syringe with a Whatman 1.5 cm filter. The sample was vortexed for 30 seconds on medium setting, and three 100 µL aliquots were removed and evaporated to determine the mass of PM in solution. The ethanol was allowed to evaporate and a PM stock solution of approximately 1 mg/mL in cell culture media (with <0.1 % v/v ethanol) was prepared from which treatment concentrations of 300, 150, and 50 µg/ml were generated. A blank Tissuquartz filter with no PM was also extracted with ethanol through the same process to act as a method blank. PM suspensions at the 300, 150, and 50 µg/ml concentrations were vortexed for 30 seconds before adding to BEAS-2B human bronchial epithelial lung cells.

Cell culture and cytotoxicity assay

BEAS-2B human bronchial epithelial lung cells were used in the cytotoxicity assay. This cell line has been used in other *in vitro* studies of biodiesel and diesel PM (Swanson et al. 2009; Totlandsdal et al. 2010). The cells were cultured in 75 cm^2 cell culture flasks in Bronchial Epithelial Growth Medium (BEGM). Cellular culture flasks were coated with Vitrogen Plating Medium (VPM) and incubated (37^o C, 5% CO_2) for 24 hours. Cells were fed by replacing the media (BEGM) every second or third day. Cells were passaged once >75% confluence was reached. Cells were removed from the flask by first washing with phosphate-buffered saline (PBS) without calcium or magnesium, then dislodging with 0.25% Trypsin EDTA solution.

Cytotoxicity at 300, 150, and 50 µg/ml was measured by the lactose dehydrogenase (LDH) assay following the manufacturer's instructions in a Promega CytoTox 96 kit. A blank

Tissuquartz filter with no PM was also extracted with ethanol through the same process to act as a method blank, and water was implemented as the control (0 µg/mL treatment concentration). In summary, LDH is a stable enzyme present in cells, which is released into the culture medium upon damage of the plasma membrane. LDH converts lactate to pyruvate and NAD^+ to NADH. The resulting NADH is converted back to NAD^+ by an oxidation reaction involving the conversion of diaphorase to formazin. Formazin can then be measured spectrophotometrically via absorbance at 490nm wavelength on a plate reader (BioTek Synergy HT). The amount of diaphorase to formazin conversion can be used as a measurement of cellular death.

Data Analysis

PASW/SPSS (version 18.0) was used to examine differences in concentrations of metals between fuels (Mann Whitney-U Test) and to perform correlation analysis (Spearman's rho) among metals per fuel type. Cytotoxicity data was analyzed using ANOVA with Tukey's post-hoc analysis to compare differences in percent LDH released per fuel type in treated versus untreated (control) cells.

Results and Discussion

Table 1 summarizes the PM (<0.25 µm) mass concentration of in cabin samples of P1B. Both the geometric and arithmetic mean PM mass concentrations were higher during biodiesel operation. In contrast to previous studies (Traviss et al. 2012), biodiesel use in this study resulted in higher in cabin mass concentrations compared to diesel operation.

Fuel	Geometric Mean	Arithmetic Mean	Geometric Standard Deviation	Standard Error
Diesel	26.39	28.12	1.48	2.49
Biodiesel	33.47	37.15	1.63	5.32

Table 1: Comparison of average mass concentrations (µg/m^3) for petroleum diesel and B20 PM (smaller than 0.25 µm in diameter)

Figures 2 and 3 represent the concentrations of metals in the $PM_{<0.25}$ size fraction on a normalized basis, or ng of metal per mg of total $PM_{<0.25}$ mass. Copper is significantly higher in B20 PM, and lead is significantly higher in diesel PM. While zinc and manganese concentrations are higher in diesel PM, these differences were not significant. Copper may be associated with food sources in the waste grease feedstock used to manufacture biodiesel (Betha et al. 2011). Lead is typically associated with diesel traffic.

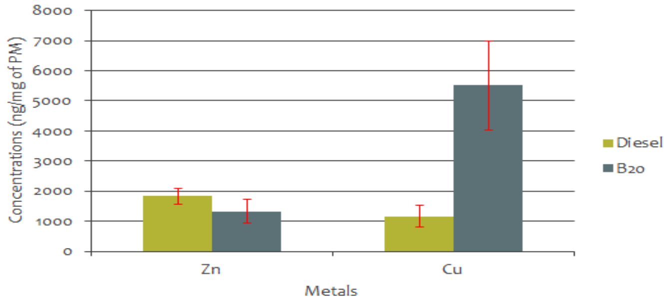

Figure 2: Metal concentrations (ng/mg of PM) of zinc and copper in petroleum diesel and B20 PM. Copper is significantly higher in B20 PM ($p < 0.05$).

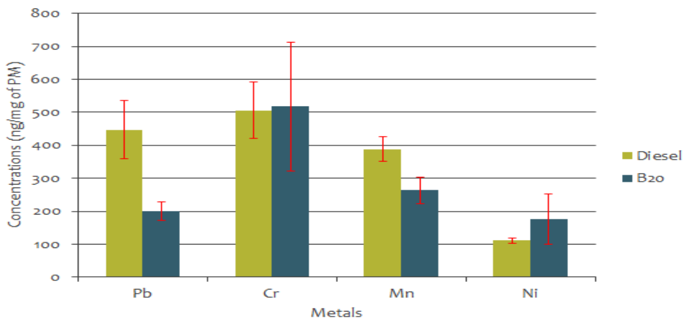

Figure 3: Metal concentrations (ng/mg of PM) of lead, chromium, manganese, and nickel in petroleum diesel and B20 PM. Lead is significantly higher in petroleum diesel PM ($p < 0.05$).

Table 2 presents correlations among metals in PM per fuel type. Statistically significant correlations ($p < 0.05$) are highlighted in blue.

B20 AF	Na	Al	Cr	Mn	Fe	Ni	Cu	Zn	Mo	Ba	Pb
Na	1	0.6	0.9	0.7	-0.7	1	0.9	0.7	1	0.9	0.5
Al		1	0.7	0.9	-0.5	0.6	0.7	0.9	0.6	0.3	0.7
Cr			1	0.6	-0.9	0.9	1	0.6	0.9	0.8	0.3
Mn				1	-0.3	0.7	0.6	1	0.7	0.4	0.9
Fe					1	-0.7	-0.9	-0.3	-0.7	-0.6	0.1
Ni						1	0.9	0.7	1	0.9	0.5
Cu							1	0.6	0.9	0.8	0.3
Zn								1	0.7	0.4	0.9
Mo									1	0.9	0.5
Ba										1	0.3
Pb											1
Diesel AF	**Na**	**Al**	**Cr**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Mo**	**Ba**	**Pb**
Na	1	-0.8	0	-0.5	-0.1	-0.7	0.1	0.1	0	0.5	0.9
Al		1	0.3	0.7	0	0.7	0.4	0	0.3	-0.6	-0.9
Cr			1	0	-0.9	0.7	0.9	0.9	1	0.5	0.1
Mn				1	0.4	0.3	0.1	-0.4	0	-0.5	-0.6
Fe					1	-0.6	-0.7	-1	-0.9	-0.7	-0.3
Ni						1	0.5	0.6	0.7	0.1	-0.5
Cu							1	0.7	0.9	0.2	0
Zn								1	0.9	0.7	0.3
Mo									1	0.5	0.1
Ba										1	0.8
Pb											1

Table 2: Correlation coefficients (Spearman's rho), r, among metals species in PM (<0.25 µm) per fuel type. Significant correlations are highlighted in blue ($p < 0.05$)

In biodiesel fuel, sodium is highly correlated with chromium, manganese, nickel, copper, molybdenum, and barium which are likely due to the use of sodium in the biodiesel manufacturing process. Correlations between Cr and Cu, Cr and Ni, Cr and Mo, and Cr and Zn were elevated for both fuels, indicating a similar, non-fuel related source for chromium such as engine wear. A similar pattern is noted for Mo. In summary, highly correlated metals tend to be from the equipment source (engine wear – [Cr, Mo]), the manufacturing process (Na), or emissions sources (fuel related - [Cu] or lube oil related - [Zn, Mo]).

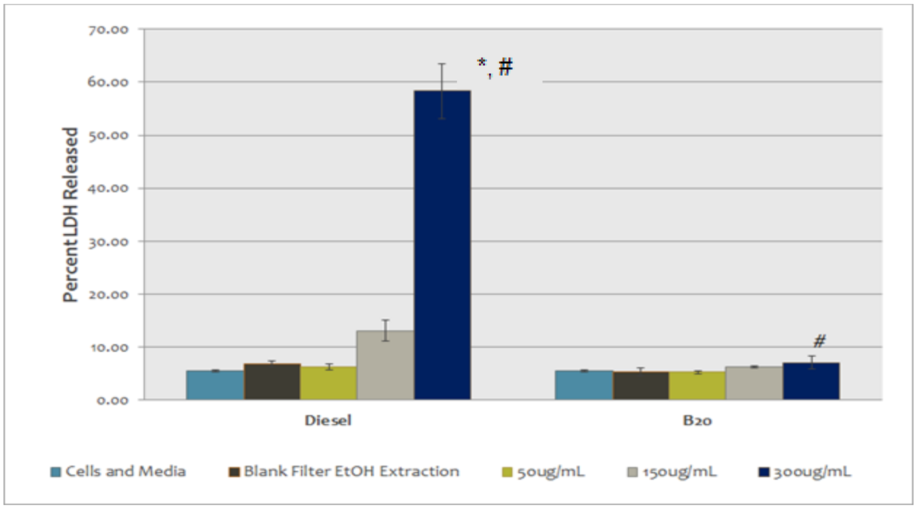

Figure 4: Cytotoxicity as represented by mean % LDH released from BEAS-2B epithelial lung cells in response to increasing concentrations of petroleum diesel and B20 PM. Asterisk (*) indicates significant difference from control ($p<0.05$). Pound (#) indicates a significant difference between fuel types at the 300 µg/ml treatment concentration ($p<0.05$)

Cytotoxicity in cells treated with petroleum diesel PM at 300 µg/mL was significantly elevated compared to that of the control and compared to B20 PM at the same treatment concentration. B20 PM did not appear to have cytotoxic effects at any dose. These results suggest exposure to diesel fuel PM may have increased cytotoxic effects compared to B20 PM. However, other studies have yielded conflicting results; Steiner et al. (2013) determined the highest cytotoxic effect from B100 PM yet Swanson et al. (2009) reported no cytotoxicity from biodiesel PM. Thus more experiments/analysis are recommended in order to explore the relationships between PM composition and other biological endpoints

Conclusion

Biodiesel and diesel PM have different physical, chemical and toxicological characteristics but more research is needed to understand the implications for human health. While B20 PM mass concentration inside the front loader was higher than during petroleum diesel operation, the difference was not statistically significant. Biodiesel and diesel fuel have different chemical structures, implying that PM composition generated by fuel combustion may differ. In this study, B20 PM and diesel PM differed with respect to metals composition with higher levels of lead, zinc and manganese in diesel PM, although only lead was significantly higher. Copper was significantly higher in B20 PM, and may be related to the waste grease feedstock. Differences in PM metals composition between fuels have been noted by other researchers (Cheung et al. 2010; Betha et al. 2011). Our results indicate an important difference in PM metals composition between fuels. Metals composition, and PAH composition, may play a role in the difference in cytotoxicity between fuels, with petroleum diesel PM significantly more cytotoxic at the highest dose. PAH and other organic species composition was not measured in this study and would be recommended for future work. Another recommendation would be to evaluate if the differences in PM metals lead to differences in cellular reactive oxygen species (ROS) generated between fuel types. While ROS is integral to many cellular processes, increased exposure to ROS has been shown to cause damage to intracellular components: DNA, lipids, and proteins (Droge et al. 2002). Hemmingsen et al. (2011) for example found that diesel PM generated more cellular ROS compared to biodiesel blends. Previous studies such as Verma et al. (2010), have evaluated biodiesel toxicology using PM collected from an engine lab in a controlled setting; this study was one of the first to evaluate PM collected in a 'real world' setting from an occupational environment.

Additional insight of differences in PM toxicological impact to compare against other studies in the literature could be provided by use of the dithiothreitol assay (DTT). This assay can cross reference the results of the ROS assay. The difference between the two assays is that the DTT assay is performed in a non-cellular environment. In addition, evaluation of the production of inflammatory cytokines by BEAS-2B cells exposed to PM from both fuel types is recommended. This will add more information to evaluate the potential health effects of use of biodiesel fuels compared to petroleum diesel fuels in 'real world' applications.

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