Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells.

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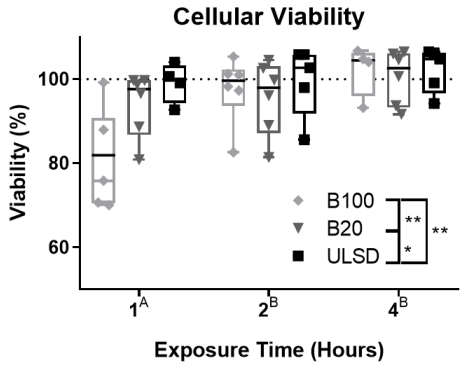
ABSTRACT: As global biodiesel production increases, there are concerns over the potential health impact of exposure to the exhaust, particularly in regards to young children who are at high risk due to their continuing lung development. Using human airway epithelial cells obtained from young children, we compared the effects of exposure to exhaust generated by a diesel engine with Euro V/VI emission controls running on conventional diesel (ULSD), soy biodiesel (B100) or a 20% blend of soy biodiesel with diesel (B20). The exhaust output of biodiesel was found to contain significantly more respiratory irritants, including NO_x, CO and CO_2 and a larger overall particle mass. Exposure to biodiesel exhaust resulted in significantly greater cell death and a greater release of immune mediators compared to both air controls and ULSD exhaust. These results have concerning implications for potential global health impacts, particularly for the pediatric population.

ABSTRACT ART:



INTRODUCTION:

Since limited battery storage capability decreases the feasibility of electrical engines in long distance transport and goods shipping[1], and the inefficiency of natural gas storage limits natural gas engine capabilities in long distance haulage[2], combustion engines are likely to be used for the foreseeable future. However, as the world pushes for cleaner, renewable energy, and fossil fuels become more difficult and expensive to extract, replacements for diesel fuel are currently being explored. Created through the transesterification of lipids into fatty acid methyl esters[3], biodiesel is gaining popularity as a renewable, sustainable fuel due to its ability to directly replace diesel fuel in many engines[4]. However, as biodiesel usage is predicted to increase worldwide[5, 6], concerns have been raised over the health impact of exposure to its exhaust emissions[7].

Most previous studies comparing mineral diesel and biodiesel combustion have found that biodiesel exhaust contains more toxic gases such as nitrogen oxides and a greater proportion of smaller particles which, when inhaled, penetrate deeper into the lungs[3, 4, 8, 9]. Despite the potentially more toxic effects of biodiesel exhaust, most studies comparing biodiesel to commercial mineral diesel instead focus on fuel economy and engine wear, or the physico-

chemical differences between the exhausts[4, 9]. Few compare health effects to exhaust exposure[7, 10, 11]. Those that do primarily use the Ames mutagenic assay[12, 13] or immortalized cell lines[8, 14] and the majority only focus on the cytotoxic and mutagenic potential of the particulate matter, ignoring the effects of the gaseous components of the exhaust entirely[7, 15]. Particle concentrations are also rarely relevant to real world exposure levels, often being far too concentrated to simulate a realistic dosage[15]. In addition, in *in vitro* based studies, the cell lines used are not always human, or even derived from respiratory tissue[3, 16]. This brings into question their relevance in human exposure studies where the main exposure route through inhalation of the exhaust means that the respiratory epithelium is among the first tissue exposed and thus likely to be among the most effected. Immortalized cell lines also negate genetic variability, and are limited in how accurately they can model normal human tissue[17].

As exhaust is typically inhaled, health complications can occur in the respiratory[18, 19], circulatory[20] and immune systems[21]. Of concern, inhalation of ultrafine exhaust particles has been correlated with exacerbation of childhood asthma[22], and associations between air pollution from major roads and decreased lung function in children have been identified[23, 24]. This suggests children may be at greater risk from adverse health effects caused by exhaust exposure. This is unsurprising as children breathe faster than adults and have higher ventilation to lung surface area/body weight ratios[25], meaning that over the same period of time, they are exposed to a larger dosage of exhaust than adults[25, 26]. In addition, the respiratory and immune systems of children are still developing and insults, such as exposure to large concentrations of exhaust, are known to have lifelong consequences[23, 27, 28]. Despite this, the effect of exposure to biodiesel exhaust has not yet been studied in children.

Due to paucity of information in this setting we tested the hypothesis that soy biodiesel exhaust would contain a greater proportion of ultrafine particles and more oxides of nitrogen and thus exposure would result in more pronounced effects on the airway epithelium. To test this, we exposed primary human airway epithelial cells from young healthy volunteers to whole exhaust from a diesel engine fueled by either pure mineral diesel, a 20% blend of soy biodiesel with mineral diesel or pure soy biodiesel. Physico-chemical exhaust properties were recorded and 24 hours' post exposure, cells were analyzed for a variety of health effect endpoints.

MATERIALS AND METHODS:

Fuel Types and Control: Three different fuels were used in this study; pure soy biodiesel (B100) created using high quality, food grade, commercial soybean oil (MOI International (AUS.)) converted via a sodium methoxide transesterification process[29], a 20% blend (B20) of soy biodiesel in commercial ultra-low-sulfur mineral diesel (SHELL, WA, AUS) and finally, commercial ultra-low-sulfur mineral diesel (ULSD). To negate background effects, HEPA filtered air was used as a control exposure.

Subjects: This study was approved by the St John of God Hospital Human Ethics Committee (901) and written consent was obtained from each participant's legal guardian after being fully informed about the nature and purpose of the study. Here, airway epithelial cells were derived from 12 healthy, typical volunteers (aged 2.7-11.2yrs, 8 males) undergoing elective surgery for non-respiratory related conditions. Children with existing bacterial or viral chest infections were excluded as was the diagnosis or chronic respiratory diseases including asthma and those with atopy determined by a positive radioallergosorbent test (RAST) to a panel of common childhood allergens.

100 **Sampling & tissue culture:** Airway epithelial cells were derived via trans-laryngeal, non
101 bronchoscopic brushing of the tracheal mucosa of children through an endotracheal tube as
102 previously described[17, 30]. Primary cell cultures were established as previously described [31], and
103 grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. Cultures were
104 maintained in Basal Epithelial Basal Media (BEBM® ; LONZA, Switzerland) supplemented
105 with growth additives (termed BEGM). Cells were passaged weekly and used before passage 6
106 in all experiments. Twenty four hours prior to experimentation, cells were placed in starvation
107 media, consisting of BEGM minus epithelial growth factor. Volunteers were age and gender
108 matched and split into 2 groups (n=6) whereupon the cells were exposed to either the control or
109 the exhausts. After exposure, supernatants were collected and stored at -80°C for cytokine
110 analysis. Cells were collected and stained with Annexin-V (BD Biosciences, CA, USA) before
111 being analyzed with flow cytometry.

112 **Exposure Methodology:** (See supplementary materials, Figure S1, for Exposure Diagram) All
113 exposures used exhaust generated from a Yanmar L100V engine (Yanmar, Italy). The engine is a
114 single cylinder, 435cc design coupled with a dynamometer and fitted with Euro V/VI after
115 treatment equipment consisting of an oxidation catalyst and diesel particulate filter (Daimler,
116 Germany). The engine was run at a constant load of 40% and speed of 2000rpm. Exhaust was
117 diluted 1 in 10 with HEPA filtered air inside a dilution/mixing chamber attached to the engine
118 exhaust pipe and then extracted at a rate of 10L per minute through an isokinetic sampling point,
119 leading to a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) containing the
120 cells. The incubator was kept at 36-37°C and exhaust was injected into each cell containing dish
121 via a manifold arrangement. The exhaust then passed through a baffleplate before extraction at
122 the base of the incubator. A vacuum pump (Part No. D50819, JAVAC, VIC, AUS) and flow

controller (10L/min Rotameter (TSI, MN, USA)) were used to ensure a continuous flow of exhaust over the cells. Exhaust removed from the incubator chamber was analyzed for physico-chemical properties.

Gas Measurements: Exhaust removed from the incubator was analyzed for quantities of combustion gas types using a multi-gas analyzer (TESTO 350, Testo, Lenzkirch, Germany). Measurements of O_2, CO, CO_2, NO_x, NO, NO_2 and SO_2 were taken every 10 minutes.

Particle Analysis: Exhaust was analyzed for fine particle concentrations between the sizes of 3 and 340 nm using a Universal Scanning Mobility Particle Sizer set up (U-SMPS 1700, Palas, Karlsruhe, Germany). Readings were taken every 10 minutes, starting 5 minutes into the exposure to ensure adequate sampling. Mean particle size was calculated using the number of particles mean. Particles were either analyzed as the total number of particles or as particles separated into 2 fractions: particles below 23 nm in size and solid particles above 23 nm[32].

Cell Viability: Cell viability was analyzed 24 hours after exposure using Annexin V staining methodologies (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Thermo Fisher Scientific, MA, USA). Briefly, cells were suspended in 1x Annexin staining buffer and incubated for 15 minutes with a 1/40 dilution of Annexin V, Alexa Fluor™ 488 conjugate solution and 1µg/ml propidium iodide before undergoing flow cytometry analysis. Annexin positive cells were counted as apoptotic, annexin negative/PI positive were counted as necrotic and the double negative population was included as viable cells. The 24 hour incubation time was chosen based on previous research showing that the effect of exhaust exposure was most evident 24 hours after the exposure event[8]. Exhaust exposures were normalized to controls before any statistical analysis on viability occurred.

Mediators: Cytokine release was analyzed in duplicate 24 hours after exposure using a BioRad 27plex human cytokine kit (BioRad, CA, USA). Cytokine release was then calculated using Bio-Plex manager (v6.1.1, BioRad, Tokyo, Japan) and results normalized to cell viability. Readings below detection limits were replaced with a value equal to half the concentration of the lowest standard for ease of statistical analysis.

Statistical Analyses: The majority of biological results contains data for all patients (n=6), excluding B100 4 hour and ULSD 1 hour exposures (n=4) and ULSD 2 and 4 hours (n=5). Data are presented as mean ± standard deviation where indicated. Gas measurements were analyzed using general additive model methodologies. All other statistical analysis was performed using multivariate linear regression methodologies. All statistical analyses were completed using R statistical software (v3.4.3) and p-values less than 0.05 were considered significant.

RESULTS:

Gas Analysis: Combustion gas measurements were taken every 10 minutes over a period of 4 hours (figure 1). All fuels show similar trends in combustion gas production over the 4 hour sampling period, with CO_2, NO, NO_2 and SO_2 all increasing rapidly within the first ~60 minutes before plateauing, while O_2 decreased from atmospheric levels to ~19% during this period. Carbon monoxide production peaked rapidly within the first 10 minutes before dropping below detectable limits within 60 minutes. There were, however, significant differences identified between fuels. Compared to ULSD, the B100 exhaust contained significantly higher production of CO, CO_2 and NO_2 and significantly lower levels of O_2 over the exposure period ($p<0.05$, figures 1a-c and 1e) and B20 contained significantly higher NO ($p<0.01$; figure 1d). Over the entire exposure period, B100 combustion also produced a greater amount of CO_2 and NO_2

compared to B20 combustion ($p<0.0001$; figure 1c and 1e), whereas B20 combustion produced greater amounts of NO ($p<0.001$; figure 1d).

Particle Analysis: An average fine particle size spectrum was obtained for each exhaust at each timepoint (figure 2). The particle spectra for the B100 exhaust was significantly different to the particle spectra for both the B20 and ULSD exhausts ($p<0.05$ and $p<0.01$ respectively). Both B100 and B20 showed peaks in particle number concentration around the ultrafine particle size of 100 nm. This peak was largest in B100 and not present in ULSD. In addition, the B100 exhaust showed a peak in particle number concentration at approximately 20 nm that was not present in the other 2 exhausts. The 1 hour timepoint was also significantly different to the 2 and 4 hours ($p<0.001$).

Particle size and concentrations were obtained for all three exhausts and timepoints (table 1). The difference in particle mass and number concentration between the 1 and 4 hour exhausts was smallest in ULSD and largest in B100. In addition, over 90% of the total number of particles were below the size of 23 nm in all 3 exhaust for the 1 hour timepoint. Comparing within particle sizes, the B100 exhaust contained the largest particle number concentration both above and below 23 nm in size. Thus, the B100 exhaust, particularly in the 1 hour, had the highest particle mass concentration, the largest particle number concentration and the smallest median particle size, as well as the largest differences in particle mass, number and the shape of the particle spectra between the 1 and 4 hour timepoints.

Cellular Viability: The B100 exhaust demonstrated the highest toxicity with significantly lower viability compared with the B20 and ULSD exposures (figure 3, $p<0.001$ and $p<0.0001$

respectively). The largest differences in mean viability occurred in the 1 hour exposure, with B100 demonstrating 14.2% and 19.2% more cell death than B20 and ULSD respectively.

Comparing timepoints, the 1 hour exposure consistently demonstrated the lowest viability in all fuels, followed by the 2 hour exposure with the second lowest viability. The 1 hour exposure was significantly different to both the 2 and 4 hour exposures ($p<0.001$ and $p<0.0001$ respectively). Up to 5% cell death was observed in the 2 hour exposure, however this was not significantly different to the 4 hour exposure timepoint, which consistently demonstrated the highest viability in all fuels. The difference in viability between the 1 and 4 hour timepoints was largest in the B100 fuel, with a 22.3% mean difference, and smallest in ULSD, which demonstrated only a 3.0% mean difference. The mean difference in cell death for B20 is 6.5%, which is 18.1% more toxic than ULSD when comparing the overall toxicity of ULSD and B100.

Mechanisms of cell death changed significantly after exhaust exposure, with all fuels showing significantly increased necrotic cell death in comparison to the controls (figure 3b, $p<0.001$), but not each other. Significant differences also occurred between timepoints, with the 4 hour exposure timepoint demonstrating significantly lower levels of necrotic cell death in comparison to the 2 hour timepoint ($p<0.05$).

Cytokine Release: Of the 27 cytokines tested, only 11 were released at measurable concentrations; IL-1β, IL-1RA, IL-6, IL-8, G-CSF, GM-CSF, IP-10, MIP-1β, IP-10 and RANTES. The results for these 11 cytokines have been displayed in graphical form (figure 5). Both IL-6 and GM-CSF were produced at significantly increased amounts after most exhaust exposures in comparison to the controls ($p<0.05$ in all cases) with the B20 inducing release of GM-CSF, although this elevated production was not significant ($p=0.06$). Both RANTES and IP-

10 release was significantly decreased after most exhaust exposures ($p<0.05$ in all cases) with the B100 release of IP-10 decreasing, although not significantly ($p=0.06$). Exposure to B100 exhaust resulted in significantly increased production of IL-1RA, IL-8, G-CSF and MIP-1β ($p<0.05$ in all cases) while ULSD exposure resulted in significantly increased production of IL-1RA, IL-1β and VEGF ($p<0.05$ in all cases). Comparing timepoints, the 1 hour exposures had significantly increased release of IL-1β, IL-6, G-CSF, MIP-1β and GM-CSF ($p<0.05$ in all cases) and after 4 hours of exposure there was significantly increased release of IL-1RA, VEGF, TNF-α and RANTES ($p<0.05$ in all cases).

DISCUSSION:

The results of this study show that exposure to mineral diesel, pure soy biodiesel or a 20% blend of soy biodiesel in mineral diesel induced airway epithelial cell death, increased the percentage of necrotic cell death mechanisms and increased the release of immune modulating cytokines compared to control cells. Exhaust characteristics varied significantly between all three fuel types, with B100 containing significantly higher levels of respiratory irritants including NO_2, CO, CO_2 and ultrafine particulate matter at a smaller median particle size, in comparison to both B20 and ULSD. The B20 exhaust contained significantly higher levels of NO in comparison to both B100 and ULSD and more particles than ULSD. Correspondingly, B100 exhaust was significantly more toxic than both B20 and ULSD, resulting in a higher percentage of cell death and the increased release of the largest number of cytokines, particularly in the first hour of exposure. The B20 exhaust was second most toxic with significantly more cell death than ULSD. In contrast, ULSD exposure resulted in a higher release of cytokines than the B20 exposure, suggesting that mineral diesel is more immunogenic. Thus, exposure to the exhaust of all 3 fuels resulted in toxic effects on human airway epithelial cells.

Combustion of both diesel and biodiesel results in the generation of toxic gases and respiratory irritants such as carbon dioxide, carbon monoxide, nitrogen oxides and sulfur dioxide[8]. The results of previous studies comparing the gaseous outputs of the two exhausts vary considerably[4, 9, 33, 34]. The majority have found that biodiesel exhaust contains more NO_x as well as a decrease in the average size of the particulate matter [4, 7, 8]and this study has found similar results. Long term exposure to NO_x, (made primarily of NO and NO_2) is associated with decreased lung volume, increased allergen response and increased risk of respiratory infections[35]. Nitrogen monoxide readily oxidizes into nitrogen dioxide at atmospheric conditions[36] and nitrogen dioxide interacts with moisture to form nitric acid[35]. In addition, NO_x is a major contributor to photochemical smog and ozone pollution[36]. Even at environmental levels, NO_x exposure is implicated with increased health risks, including ischemic stroke and cardiovascular disease[35, 37]. In children, daily exposure to NO_2 is associated with increased asthmatic symptoms[35, 38], which is of concern as both B100 and B20 exhaust contained significantly higher levels of NO_2, either as the pure gas or as NO with the potential to oxidize. As the exhaust used in this study was first diluted with HEPA filtered air in order to simulate a realistic exposure dosage, changes in the sampled exhaust are over 10 fold greater in undiluted exhaust, making increased environmental NO_x levels a potential health threat as biodiesel usage continues to increase.

Carbon monoxide oxidizes slowly at atmospheric conditions[39] and exposure to low levels of CO for an extended period of time is associated with adverse neurological impacts including emotional instability and difficulty concentrating[40]. The 0.28% increase in CO_2 levels between B100 and ULSD at the end of the 4 hour exposure translates to approximately a 28000 ppm difference in the undiluted exhausts, and represents a considerable increase in total CO_2 production. Short-term exposure to only 1500 ppm is associated with cognitive impairment[41] and

negative effects on bone formation and blood pressure have been observed at 12000 ppm[42, 43]. This makes increased biodiesel usage a concern in terms of increased atmospheric CO and CO_2 levels, which will in turn have potential impacts on population health and climate change.

The particulate matter phase of exhaust is defined as the inert elemental carbon particles created during combustion and the potentially toxic chemicals adhered to their surface[44]; including polyaromatic hydrocarbons, aldehydes, ketones and heavy metals[33, 34]. Despite the fact that the elemental carbon particles within the particulate matter itself are relatively inert, inhalation causes respiratory irritation[19] and the inhalation of ultrafine particulate matter is associated with worsening health outcomes including pulmonary inflammation[45], exacerbation of existing respiratory diseases[46], increased blood pressure and increased risk of heart failure as the small size of the particles allows entrance directly into the cardiovascular system, bypassing the lung barrier function entirely[47, 48]. Asthma sufferers are particularly susceptible to adverse effects from inhalation, and exacerbation of childhood asthma has been associated with ultrafine particulate pollution[22].

In addition, inhalation of the particulate matter allows the toxic chemical adhered to the surface to deposit within the lungs and cardiovascular system. Smaller particles have an increased surface to volume ratio, meaning that a greater amount of harmful substances can adhere to the surface for a given mass[8, 49]. Smaller particles are also able to penetrate deeper into the lungs, causing an irritant effect as well as potentially depositing the harmful chemicals over a greater percentage of the total lung tissue[45]. In this study, biodiesel combustion was found to generate a greater amount of particles, with a smaller mean diameter, making the adverse effects from the inhalation of these chemicals a greater concern, alongside the potential health effects of increased ultrafine particulate pollution. Observing the change in particle spectra over time, the

greatest number concentration of ultrafine particles was found in the first hour of the exhaust for all three fuels. The 1 hour B100 exhaust contained both the greatest particle number concentration, above and below 23 nm in size, and the smallest median particle diameter. In addition, it was the only exhaust where the majority of particles below 23 nm in size persisted into the 2nd hour. This change in fine particles over time is likely due to diesel particulate filter loading, as well as possible condensation effects[32].

Comparing exhaust characteristics between the 1, 2 and 4 hour timepoints, two characteristics standout in the first hour; the carbon monoxide readings and the particulate matter. The difference in these two characteristics can be attributed to the cold start effect, where the engine is started at below optimum working temperature, and thus the devices such as the catalytic converter and diesel particulate filter are also working at below optimum temperature. This helps to explain why these readings decrease as exposure time, and thus engine temperature, increases. All other characteristics are higher in the 4 hour timepoint, excluding oxygen.

The effects of exposure to all exhausts can be observed in the increased cell death for both B100 and B20 and the increased release of cytokines for all exhausts. In addition, mechanisms of cell death skew towards necrosis after exhaust exposure in comparison to air, suggesting a high level of cell injury and trauma[50]. Previous studies using adult human airway epithelial cell lines have found similar results, with exposure to diesel exhaust inducing necrotic cell death[51]. The cytokine release after exhaust exposure is strongly indicative of an increased inflammatory response with biodiesel having the most pronounced effect, shown by an increase in the release of 6 cytokines in comparison to the controls; IL-1RA, IL-6, IL-8, G-CSF, GM-CSF and MIP-1β. The next highest inflammatory response is induced by ULSD exhaust exposure, causing increased release of 5 cytokines in comparison to the controls; IL-1RA, IL-1β, IL-6, VEGF and

GM-CSF. Finally, the B20 exhaust exposure significantly increased the release of 1 cytokine, IL-6, indicating a smaller inflammatory response.

Very few studies have focused on the effect of biodiesel exposure on human airway epithelial cell mediator production, particularly in less commonly studied cytokines such as IL-1RA, G-CSF and VEGF. The majority of studies that do test the immunogenic effect of biodiesel exposure focus on the release of IL-6 and IL-8, with sporadic attention paid to few other cytokines[7, 8, 52, 53]. The difference in the release of the inflammatory cytokine panel tested in this study suggests that this may be an oversight, with different exhausts inducing the production of different cytokines in comparison to air exposed controls. For example exposure to ULSD exhaust caused significant release of IL-1β, which is associated with the acute inflammatory response[54, 55], while exposure to soy biodiesel exhaust caused significant release of IL-8 and G-CSF, which are associated with neutrophilic inflammation[55, 56]. This suggests that exposure to the exhaust of either petroleum diesel or pure soy biodiesel induces different immune reactions, suggesting variations in the nexus points between the adaptive and innate immune responses[19, 54-58].

Interestingly, the release of cytokines also shows a pattern in response to exposure time. The 4 hour exposures show significantly increased production of 4 cytokines in comparison to the other two timepoints; IL-1RA, VEGF, TNF-α and RANTES. The 1 hour exposures show increased production of 5 cytokines in comparison to the other two timepoints; IL-1β, IL-6, G-CSF, GM-CSF and MIP-1β. The 2 hour exposures show no significant increase in immune mediators in comparison to the other two timepoints. This suggests that the 1 hour exposure time induces a slightly greater immune response in comparison to the 4 hour exposures and both the 1 and 4 hour exposures induce a greater immune response than the 2 hour exposures. Combined with the

significantly increased cell death in the 1 hour exposures, with some cell death and significantly increased necrotic cell death also observed in the 2 hour exposure, the earlier timepoints are more toxic than the later, with the 1 hour timepoint showing the most toxic effects followed by the 2 hour timepoint.

As the health effects of carbon monoxide are attributed to the binding of hemoglobin and thus low blood oxygen levels instead of a direct toxic effects on cells[59, 60], and the presence of carbon monoxide is eliminated in the exhaust in all fuels by the second hour via the catalytic converter and is thus unlikely to explain the presence of increased necrotic cell death at that timepoint, the cause of the more toxic effects in the 1 hour are likely to be attributed to the particulate matter which has a greater mass and number concentration and a smaller median size in the 1 hour when compared to both the 2 and 4 hour timepoints.

This is not the first study to find that exposure to a lower dosage of particles results in the most toxic effects[61, 62]. Previous studies have attributed this effect to particle agglomeration and we propose a similar effect occurred in this study. Smaller particles can more easily penetrate the cell membrane and cause injury to the cells. As exposure time increases, more and larger particles are added to the media. As diesel particulate matter is known to agglomerate readily in liquid[19], we propose that the addition of the larger particles causes particle concentration to reach a point that agglomeration occurs with the initial smaller, more damaging particles, preventing them from penetrating the cell membrane. This explains why the most cell death is observed in the 1 hour exposure where particle dosage is lowest and median particle size is smallest. A smaller amount of cell death is observed in the 2 hour, which has the second lowest particle dosage, and no death observed by the 4 hour timepoint. Indications of cell injury, including inflammatory cytokine release and necrotic cell death also decrease over time.

In conclusion, in our study, soy-based biodiesel exhaust contained more and smaller particulate matter, more gaseous respiratory irritants and exposure to the exhaust resulted in a higher percentage of cell death and a wider release of cytokines for a more varied immune reaction in comparison to mineral diesel exposure. As biodiesel usage becomes more widespread, environmental NO_x levels and ultrafine particulate pollution are likely to increase, leading to worrying concerns on health impacts in the wider community, particularly the impacts on childhood asthma severity.

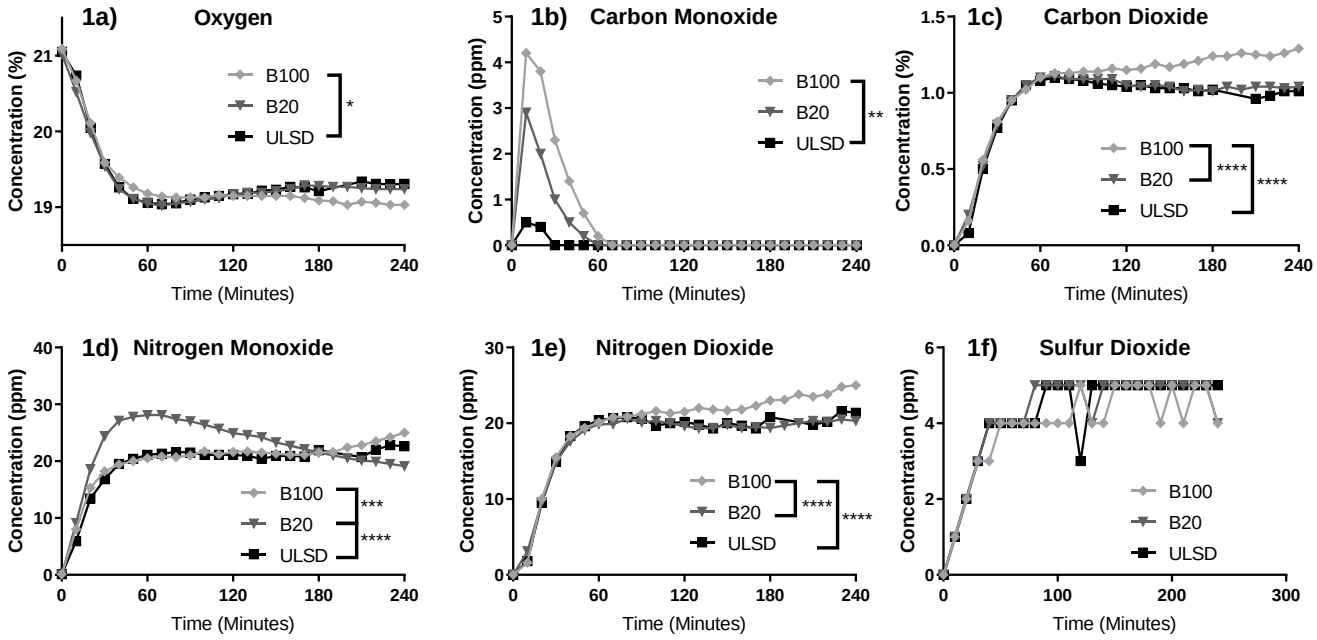


Figure 1: Combustion gas analysis from the diluted exhaust of the three different fuels types. Measurements were taken every 10 minutes for 4 hours (*=p value<0.5, **=p value<0.01, ***=p value<0.01, ****=p value<0.001). Figure 1a) and 1c) concentration measurements as a percentage, all other figures show concentration in parts per million (ppm).

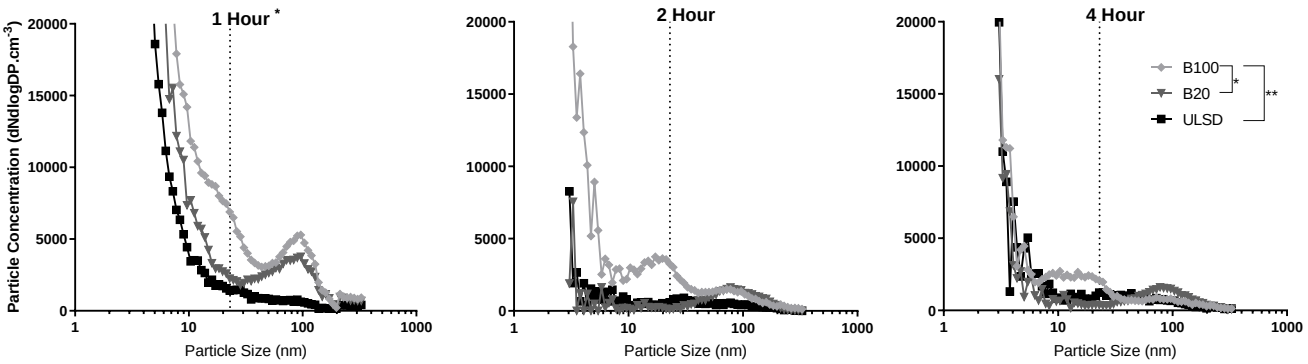


Figure 2: Particle size spectra for all three fuels (*=p value<0.05, **=p value<0.01, ****=p value<0.001). The dotted line indicates the particle size of 23 nm. Within fuels, particle size spectra are significantly different between the 1 hour and the 2 and 4 hour timepoints (p<0.001). Both B100 and B20 show peaks around the ultrafine particle size of 100 nm which is absent in the mineral diesel exhaust.

Table 1: Particle characteristics between the sizes of 3-340 nm for all fuels and timepoints.

Fuel	ULSD			B20			B100		
Time (Hours)	1	2	4	1	2	4	1	2	4
Particle Concentration (µg/m³)	34	10	19	84 (2.76)[a]	19 (1.90)	27 (1.42)	105 (3.09)	29 (2.9)	24 (1.26)
Median Particle Size (nm)	20	20	10	10	10	20	<10	20	10
Total Particle Number (particles/cm³)	521539	47765	113216	881298 (1.69)	45690 (0.96)	94510 (0.83)	1301691 (2.50)	224339 (4.70)	148467 (1.31)
Particle Number >23 nm (particles/cm³)	24117 [4.62%][b]	15044 [31.50%]	23298 [20.58%]	75980 (3.15) [8.62%]	26894 (1.79) [58.86%]	28641 (1.23) [30.30%]	113029 (4.69) [8.68%]	40377 (2.68) [18.00%]	24595 (1.06) [16.57%]
Particle Number <23 nm (particles/cm³)	497422 [95.38%]	32721 [68.50%]	89918 [79.42%]	805319 (1.62) [91.38%]	18797 (0.57) [41.14%]	65869 (0.73) [69.70%]	1188662 (2.39) [91.32%]	183962 (5.62) [82.00%]	123872 (1.38) [83.43%]

[a] Values in round brackets represent proportional increases in comparison to ULSD values.

[b] Values in square brackets represent the percentage of the total particle number concentration.

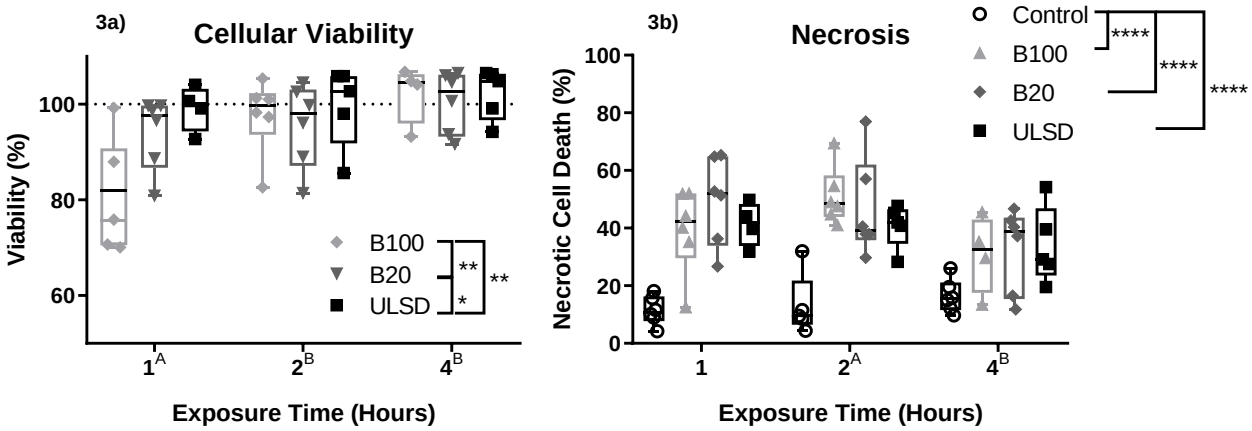
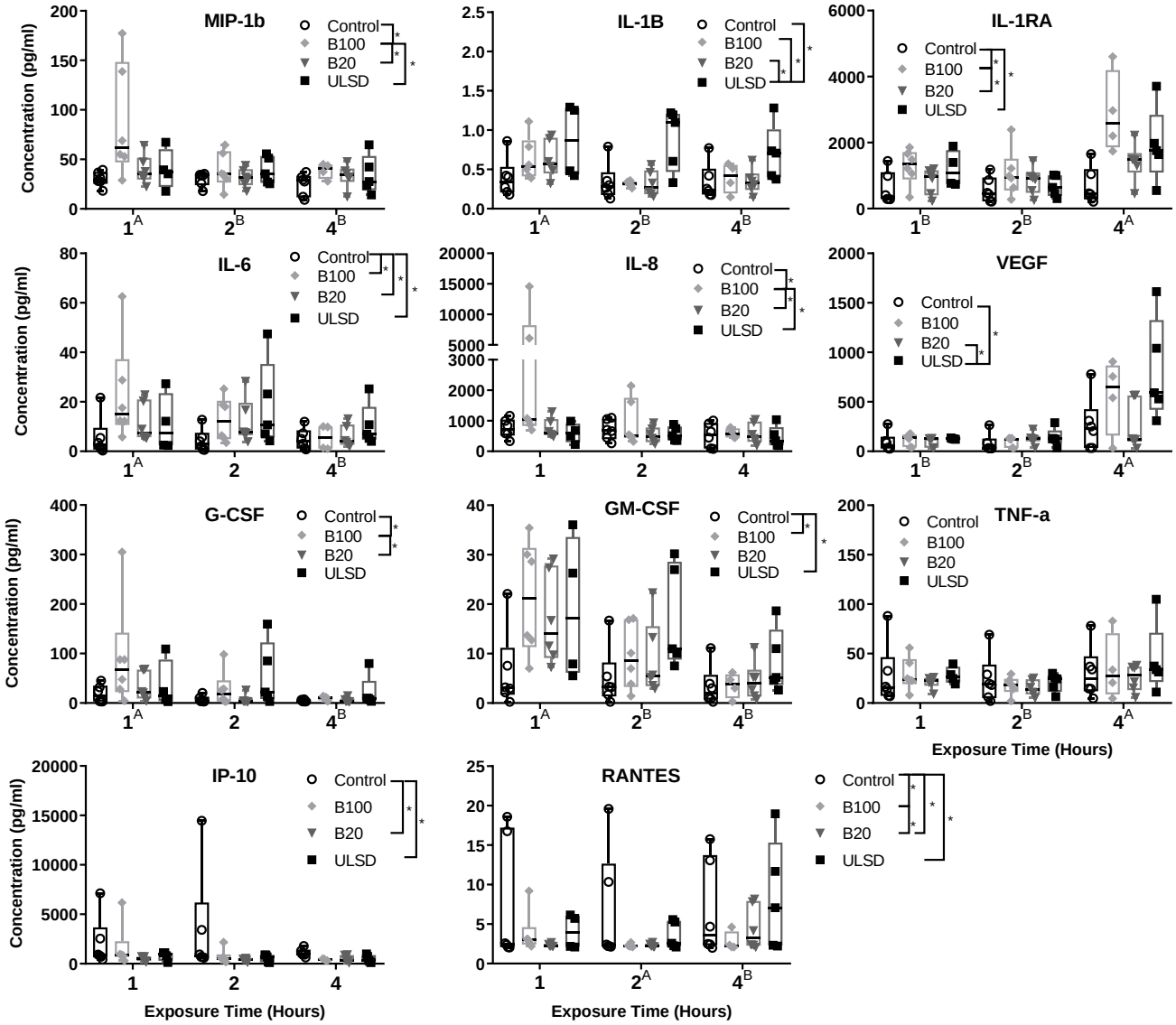


Figure 3: a) Cell viability measurements 24 hours after exposure using Annexin V staining. All results are normalized to control measurements (dotted line). 3b) Percentage of cell death via necrotic mechanisms 24 hours after exposure. Asterisk symbols on legend indicate significance between fuels (*=p value<0.05, **=p value<0.01, ****=p value<0.0001). Superscripts on x-axis indicate significant differences across time. A superscript of "A" indicates significant increase to a superscript of "B" (3a) $p<0.001$ and $p<0.0001$ for 1 vs 2 and 4 hours respectively, 3b) $p<0.05$). Bars indicate spread of data and median.



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380 **Figure 4:** Measured cytokine release for all fuels and times for 9 of the 11 cytokines released
381 above limit of detection (*=p value<0.05). A superscript of A indicates significant increase to a
382 superscript of B between timepoints (p<0.05). Boxplots indicate spread of data and median values.

383 **ASSOCIATED CONTENT**

384 Supporting information (PDF) including a diagram of the experimental setup and flow cytometry

385 gating is available free of charge at:

386 **DISCLOSURE:** The authors of this paper have no conflicts of interest to declare.

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Author Contributions

KRL, JH, AK, BJM and ANL performed the exposures. BJM and RM-H created the fuels with input from GK. KRL, JH and AK grew the cells for exposure. KRL performed analysis assays with input from JH and flow cytometry data analysis was performed by JH. RO'L adised on statistical analyses. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

B100, Pure soy biodiesel; B20, A 20% blend of soy biodiesel in mineral diesel; ULSD, commercial mineral diesel, O_2, oxygen; CO, carbon monoxide, CO_2, carbon dioxide; NO_x,

nitrogen oxides; NO, nitrogen monoxide; NO_2, nitrogen dioxide; SO_2, sulfur dioxide; IL-1β, interleukin 1 beta; IL-1RA, interleukin 1 receptor antagonist; IL-6, interleukin 6; IL-8, interleukin 8; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IP-10, interferon gamma inducible protein 10; MIP-β, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell expressed and secreted; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

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