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## Method Article

# *In Vitro* primary human airway epithelial whole exhaust exposure



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## A B S T R A C T

The method outlined in this article is a customization of the whole exhaust exposure method generated by Mullins et al. (2016) using reprogrammed primary human airway epithelial cells as described by Martinovich et al. (2017). It has been used successfully to generate recently published data (Landwehr et al. 2021). The goal was to generate an exhaust exposure model where exhaust is collected from a modern engine, real-world exhaust concentrations are used and relevant tissues exposed to assess the effects of multiple biodiesel exposures. Exhaust was generated, gently vacuumed into a dilution chamber where it was diluted 1/15 with air and then vacuumed into an incubator containing the primary cell cultures for exposure. Exhaust physico-chemical properties including combustion gas concentrations and particle spectra were then analyzed using a combustion

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gas analyzer and a Universal Scanning Mobility Particle Sizer. 24 h after exposure, cellular viability and mediator release were measured using Annexin-V/PI staining and mediator multiplexing kits respectively. This method was generated to test biodiesel exhaust exposures but can be easily adapted for any type of engine exhaust exposure or even potentially other respirable environmental exposures such as woodsmoke.

The main customization points for this method are:

- Exhaust generated by a diesel engine equipped with EURO VI exhaust after treatment devices including diesel particulate filter and diesel oxidation catalyst.
- The generated exhaust was diluted 1/15 with air to replicate real world exposure concentrations.
- Used primary human airway epithelial cells obtained from bronchoscope brushings from multiple volunteers and reprogrammed to allow multiple, comparative exposures from the same individual.

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## ARTICLE INFO

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*Keywords:* Biodiesel, Exhaust exposure, Environmental exposure, Airway epithelial cells, Exposure protocol

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## Specifications table

Subject Area	Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area	Environmental Exposure Toxicology
Method name	<i>In vitro</i> Primary Human Airway Epithelial Cell Whole Exhaust Exposure Protocol
Name and reference of original method	<b>Airway epithelial cell collection and reprogramming:</b> K.M. Martinovich, T. Iosifidis, A.G. Buckley, K. Looi, K.-M. Ling, E.N. Sutanto, E. Kicic-Starcevic, L.W. Garratt, N.C. Shaw, S. Montgomery, F.J. Lannigan, D.A. Knight, A. Kicic, S.M. Stick, Conditionally reprogrammed primary airway epithelial cells maintain morphology, lineage and disease specific functional characteristics, <i>Scientific Reports</i> , 7 (2017) 17971. <b>Whole exhaust exposure method:</b> B.J. Mullins, A. Kicic, K.-M. Ling, R. Mead-Hunter, A.N. Larcombe, Biodiesel Exhaust-Induced Cytotoxicity and Proinflammatory Mediator Production in Human Airway Epithelial Cells, <i>Environmental Toxicology</i> , 31 (2016) 44-57.
Resource availability	R package "lme4": <a href="https://cran.r-project.org/package=lme4">https://cran.r-project.org/package=lme4</a> R package "mgcv": <a href="https://cran.r-project.org/package=mgcv">https://cran.r-project.org/package=mgcv</a> FlowJo: <a href="https://www.flowjo.com/">https://www.flowjo.com/</a>

## Method details

### Sample collection

With informed parent/guardian permission, airway epithelial cells were derived from trans-laryngeal, non-bronchoscopic brushings of the tracheal mucosa of children through an endotracheal tube as previously described [2,3]. Volunteers were undergoing elective surgery at St John of God Hospital (Perth, Australia) for non-respiratory related conditions. Atopy was determined using a radio-allergo-sorbent test for a panel of common childhood allergens and positive results were excluded, alongside clinical diagnosis of bacterial or viral chest infections or any underlying chronic respiratory disease such as asthma. This study was approved by the St John of God Hospital Human Ethics Committee (901) and proof of approval is available on request.

### Conditional reprogramming

Conditional reprogramming of the brushings was performed as previously described [4]. Briefly, 125,000 *ex vivo* epithelial cells obtained from the brushings were grown in fibronectin pretreated T25 flasks (Falcon, Corning) and the F-medium containing ROCK inhibitor [4]. These flasks were

pre seeded with 125 000 cells from the murine embryonic fibroblast cell line NIH-3T3, which were irradiated with 3000 cGy  $\gamma$ -radiation (Gammacell® 3000 Elan; MDS Nordion) prior to being added to the flasks in order to establish an irradiated feeder cell layer. Epithelial cells were grown to ~90% confluence before being trypsinized with LONZA subculture reagent packs (LONZA, Switzerland) and cryogenically frozen at 500,000 cells per vial in 1 mL of freezing media (90% fetal calf serum, 10% dimethyl sulfoxide), labelled as passage zero (P0) cells. Cells were cryogenically frozen in Mr. Frosty freezing containers (Nalgene, ThermoFisher) at -80 °C before being moved to liquid nitrogen for longer term storage.

#### *Expansion for exposure stock vials*

Primary airway epithelial cell cultures were established and grown at 37°C in an atmosphere of 5%CO<sub>2</sub>/95% air under aseptic conditions. A vial of P0 cells for each volunteer was thawed, all cells seeded into a fibronectin pre-coated T75 flask (Falcon, ThermoFisher) with 375,000 irradiated NIH-3T3 feeder cells and fed 3 times weekly with the F-medium containing ROCK inhibitor [4]. At 80% confluence, epithelial cells were passaged with LONZA subculture packs, with two fibronectin pre-coated T75 flasks seeded at 375,000 cells with a 1:1 ratio with irradiated NIH-3T3. Remaining cells were divided into cryogenic vials at 500,000 cells per vial in 1 mL of freezing media. Cells were cryogenically frozen in Mr. Frosty freezing containers (Nalgene, ThermoFisher) and stored at -80 °C. This was repeated until cells reached passage 3 whereupon cells were discarded.

#### *Expansion for exposures*

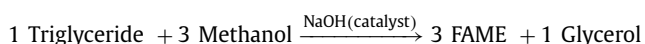
Once the stock of cryogenic vials was sufficient for the whole experiment, culturing for exposure began. Cryogenic vials were thawed as above into fibronectin pre-coated T75 flasks with 375,000 irradiated NIH-3T3 cells. Cells were passaged as above using LONZA sub-culture reagent packs. Additional flasks were made for each individual sample for the next passage with cells seeded at 375 000 cells with a 1:1 ratio with irradiated NIH-3T3. Remaining cells were collected into single cell suspension for set up in the next step. Cells were passaged weekly until they reached passage 6, whereupon they were discarded. This is due to later passages of cells (> 7) deviating from epithelial morphology.

#### *Set up for exposure*

Cells were resuspended in Basal Epithelial Basal Media supplemented with growth additives (BEGM®; LONZA, Switzerland) and seeded at 500 000 cells per dish in 35 mm x 10 mm Eppendorf cell culture dishes. Five dishes were seeded per ID per exposure. Four of these dishes were used in the exposure, with a randomized allocation throughout the baffleplate in order to help negate any effects caused by exhaust deposition differences within the exposure chamber. Empty spaces on the baffleplate were filled using blank dishes. The remaining dish containing cells was unexposed and used to collect protein for normalization of the immune mediator measurements, in order to account for slight differences in cell numbers. Cells were initially seeded in BEGM and then 24 h before exposure, the media was changed to a starvation media (BEGM without the addition of epithelial growth factor).

#### *Biodiesel creation*

Biodiesel, or fatty acid methyl ester (FAME), was created for each feedstock type using transesterification as previously described [5]:



Briefly, 4 kg of oil feedstock was poured into a large mixing bowl. Solid fats/oils were warmed on a heating plate until fully liquid before being poured. A methoxide solution was created by dissolving NaOH into methanol (> 99% purity) with exact amounts of each solution being adjusted for individual

**Table 1**

Exact amounts of methanol and NaOH needed for transesterification of 4 kg of oil for each feedstock tested. Amounts were adjusted using FAME profiles to calculate the molecular weight of each feedstock type.

Feedstock	Oil Mass (kg)	Volume of Methanol (mL)	NaOH (g)
Canola	4	575	40
Waste Cooking Oil	4	575	40
Soy	4	580	40
Tallow	4	575	50
Palm	4	635	50.4
Cottonseed	4	580	42.4

oils (Table 1). The methanol adjustment was based on FAME profile within the literature and later the FAME profile results of the mass spectrometry testing. The NaOH adjustments were based on the stoichiometric amount required for the amount of methanol needed for biodiesel generation, and the amount needed to neutralize the acidic pH of the various oils. Once NaOH had fully dissolved, methoxide was poured into the mixing bowl and the mixture stirred vigorously until clarification occurred. The resulting mixture was then poured into beakers containing water at a 1:1 ratio, sprinkled with salt (NaCl) to help separation and left to settle for 24 h. After settling, the top layer of FAME was removed from the beakers and the remaining solution discarded. Samples of the FAME were assessed via mass spectrometry (see below) and the remainder used to fuel the engine.

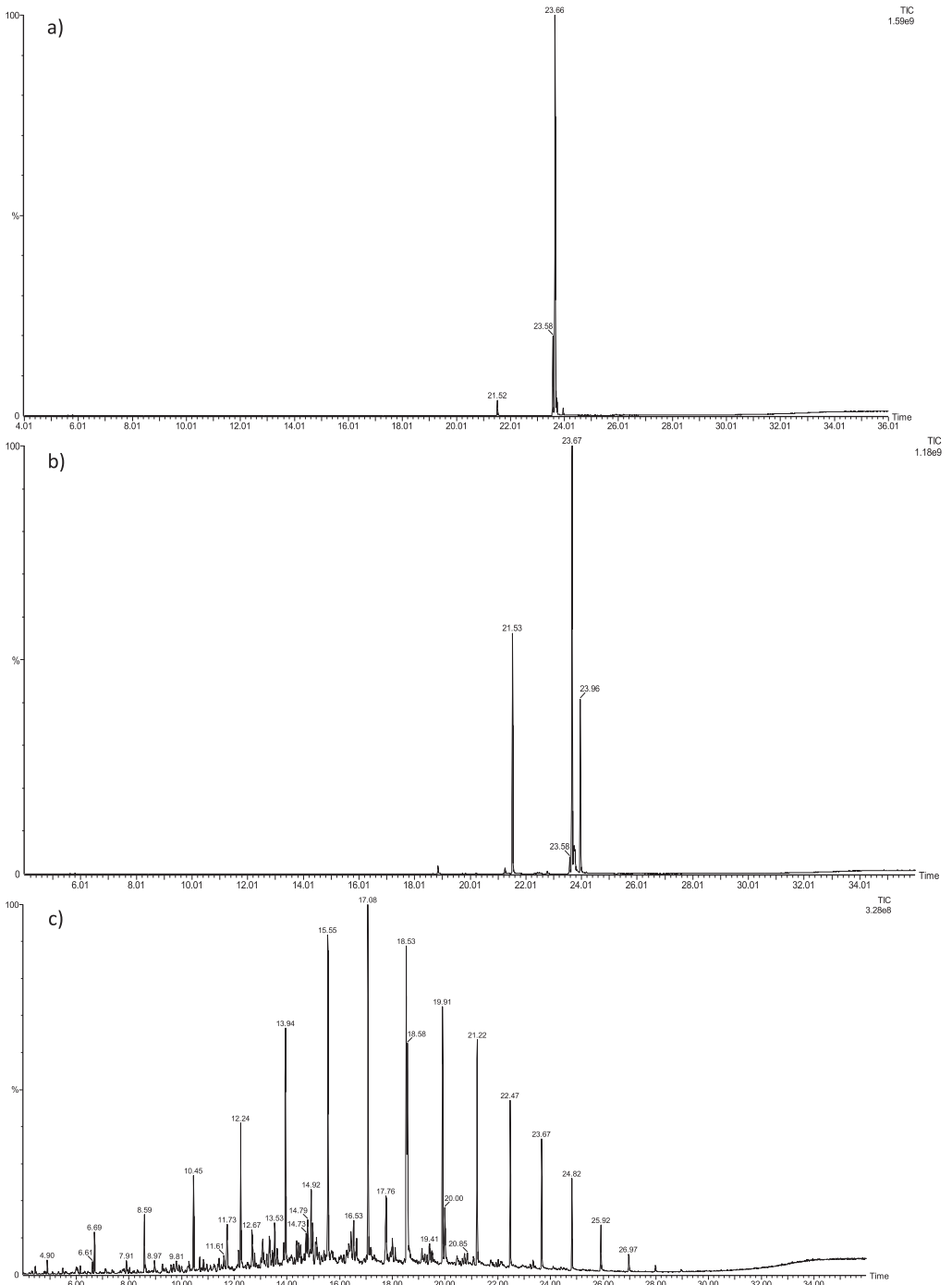
#### Mass spectrometry testing

Biodiesels were analyzed for their FAME content using gas chromatography mass spectrometry (GC-MS) analysis using a PerkinElmer Clarus 580 GC coupled to PerkinElmer Clarus SQ8S MS and an Elite-5MS column (30 m × 0.25 mm × 0.25 μm). Biodiesels were diluted 5 μL into 1.0 mL of hexane. The helium carrier gas had a constant flow of 1.0 mL/min. The injection port was 310 °C with a split ratio of 30:1. The temperature program was operated from 50 °C for 0.5 min, ramping at 8 °C/min until 310 °C and holding for 3.0 min. The mass spectrometer analyzed a mass range from 40 to 400 (m/z), from 4.0 to 36.0 min at 70 eV. Compounds were identified by comparison of mass spectra against National Institute of Standards and Technology (NIST) (08) MS library match and calculated retention index. Quantitation was via integration of the Total Ion Current chromatogram (Fig. 1). All biodiesels were found to contain > 99% FAME. Note we did two rounds of testing to optimize the transesterification process.

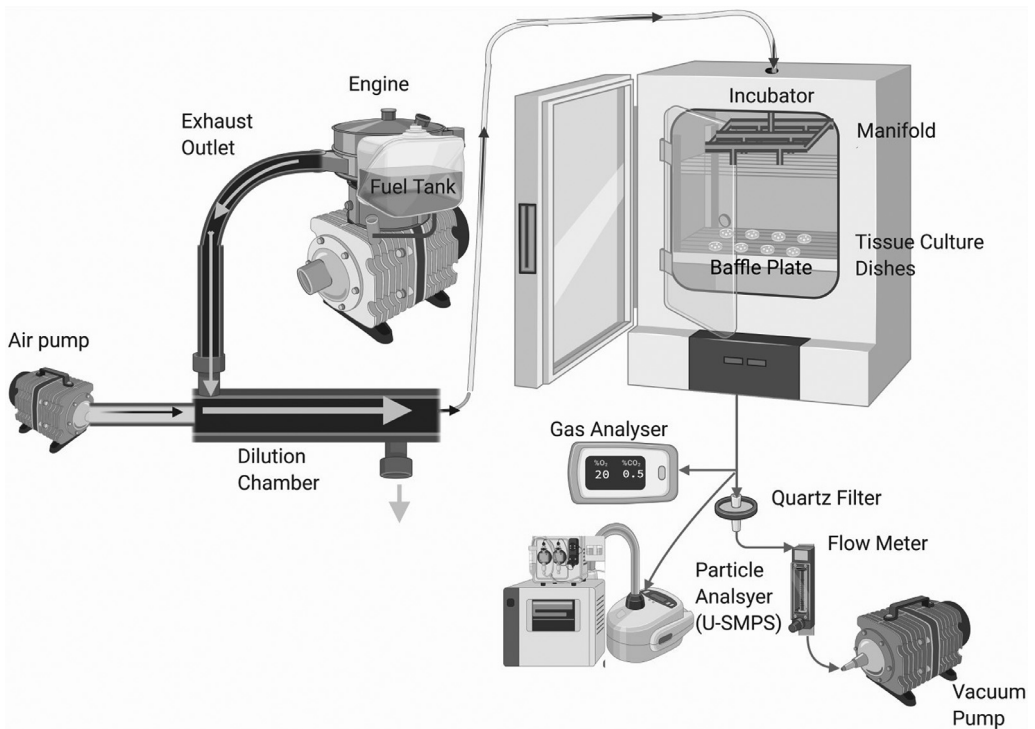
#### Exposure method

All exposures used exhaust generated from a diesel engine (Fig. 2) consisting of a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) coupled with a dynamometer. The engine was fitted with Euro V/VI after-treatment equipment consisting of an oxidation catalyst and diesel particulate filter (Daimler, Germany). The engine was run from cold start at a constant load of 40% and speed of 2000 rpm. It took approximately 2 h for the engine to cool back down to cold start levels after an exposure and so up to two exposures could be performed daily, one early morning and one early afternoon.

Using an air pump, exhaust was diluted 1:15 with air inside a dilution/mixing chamber attached to the exhaust piping and vacuumed through an isokinetic sampling point into a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) set at 36–37 °C for one hour. Exhaust was then vacuumed through a manifold spreader in order to minimize uneven exhaust dispersion before reaching the cell cultures inside the incubator. The cell culture dishes were set within a custom designed baffle plate, sealed around the edges where it attached to the incubator so that air/exhaust could only pass over the designated exit points (Fig. 3). Note, culture lids were removed prior to exposure start and the layout of culture dishes was randomized in order to minimize any effect caused by uneven exhaust dispersion. Culture dish gaps that did not contain a culture dish were filled with blank dishes. This



**Fig. 1.** Example chromatograms for (a) Soy biodiesel, (b) Tallow biodiesel and (c) ultra-low sulfur diesel for comparison.



**Fig. 2.** A diagram of our exposure set-up, adapted from [1]. This diagram was generated using [Biorender.com](https://biorender.com).

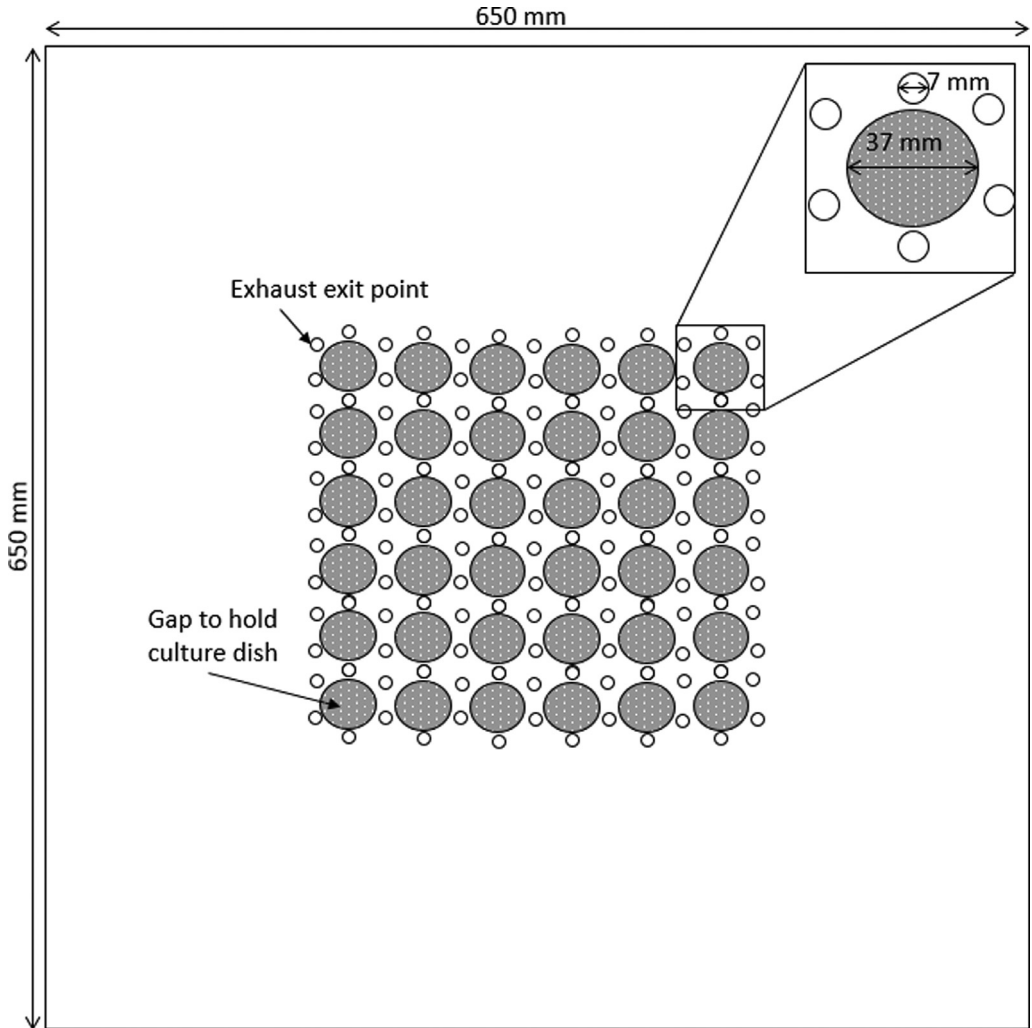
custom design was created using laser cut plastic and the exact size of the plastic sheet can be altered to fit other incubator models, although the size of the culture dish gaps and exhaust exit points should remain the same. The flow rate of the exhaust over the cells (10 L/min) was controlled by a flow meter situated just before the vacuum pump (Fig. 2). This flow rate, combined with a 1:15 dilution with air was sufficient to dilute exhaust down to real-world relevant exhaust concentrations but also high enough that we could expect to find toxic health impacts without needing to increase exposure time. Prolonged exposure time with high air dilutions risks the cells dehydrating due to the continuous flow of exhaust/air slowly drying out the media, thus biasing results.

Once cultures were exposed, the exhaust was vacuumed out to be analyzed for physico-chemical properties. This included the levels of combustion gas products using a combustion gas analyzer (TESTO 350, Testo, Lenzkirch, Germany) and particle concentrations between the sizes of 3–340 nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Measurements were taken every 10 min to monitor the cold start effect of the engine but the exact timing can be adjusted.

Exposure to Australian ultra low sulfur mineral diesel (ULSD, < 10 ppm sulfur) was used as a positive control and air exposure was used as a negative control. Due to diesel exhaust being highly humid as water vapor is a product of the combustion process, a portable air humidifier was set up within the incubator during all air control testing.

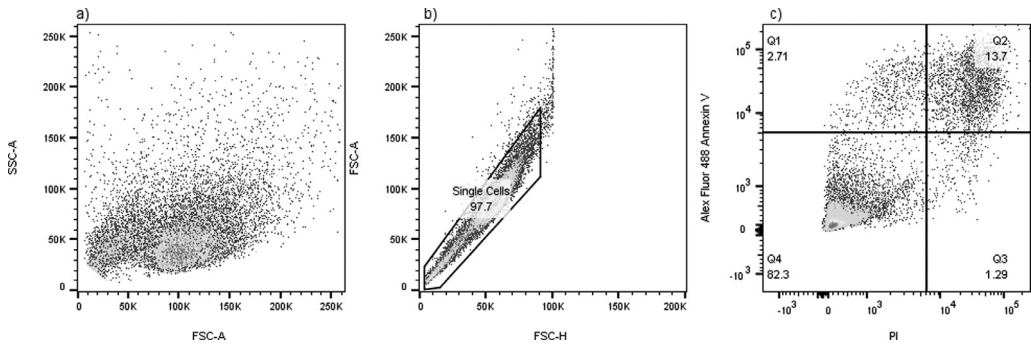
#### *Post exposure flow analysis and sample collection*

After exposure, cultures were left for 24 h to rest based on our previous data showing that 24 h was the optimum time to measure mediator response [6]. Post exposure analysis was performed separately for each exposure. After 24 h, protein was collected from the control dish by



**Fig. 3.** A diagram of our customized baffle plate. Cell culture dishes (35 mm wide) fit inside the bigger holes and exhaust is suctioned through the smaller holes.

adding lysis buffer directly to the dish with protease inhibitor added to 1x concentration (ROWE Scientific, Australia). The 4 exposure dishes had their supernatants pooled and collected for later analysis and were trypsinized using LONZA subculture reagents. A cell count was performed using a hemocytometer and 400,000 cells were aliquoted for flow cytometry analysis. The remaining cells were divided into two; one set having protein collected using lysis buffer with protease inhibitor added to 1x concentration, the other having RLT lysis buffer with  $\beta$ -mercaptoethanol added for later RNA extraction (RNeasy kits, Qiagen). The remaining aliquoted cells were analyzed using an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific, MA, USA). Briefly, cells were suspended in 1xAnnexin staining buffer, split into 4 groups and incubated for 15 min with either no stain, a single stain of a 1/40 dilution of Annexin V, Alexa Fluor™ 488 conjugate solution or 1  $\mu$ g/mL propidium iodide or both stains before undergoing flow cytometry analysis on a LSR Fortessa flow cytometer (BD Biosciences). The Annexin V staining was measured using the FITC channel and the



**Fig. 4.** The gating strategy used to analyze the flow cytometry data. This figure was taken from [1] supplementary. First cells were selected using forward and side scatter area (S2a). Next single cells populations were selected using forward scatter area and forward scatter height (S2b). Finally, cells were separated into Viable, Early Apoptotic, Late Apoptotic and Necrotic populations using the FIT-C channel to measure Alexa Fluor 488 Annexin V staining and the TEXAS Red channel to measure (S2c).

propidium iodide staining measured using the Texas Red channel. Analysis was performed using the FlowJo software (V10, BD Biosciences). The no stain and single stain samples were used to assess the correct position of the final gating step, helping to separate what counts as positive and negative staining for PI and Annexin V. This allowed gating to be performed without the need for compensation (Fig. 4). Annexin V -ve/PI -ve populations were counted as viable cells, Annexin V +ve/PI -ve as early apoptotic, Annexin V as late apoptotic and Annexin V -ve/PI +ve as necrotic [7].

### Protein analysis

Protein amounts in the control dishes were analyzed using Pierce BCA protein assays (ThermoFisher), as per the kit protocol. All samples were analyzed in duplicate at a 1/5 and 1/10 dilution and results averaged across dilutions. These data were used to normalize cytokine data in case of any discrepancies in cell number.

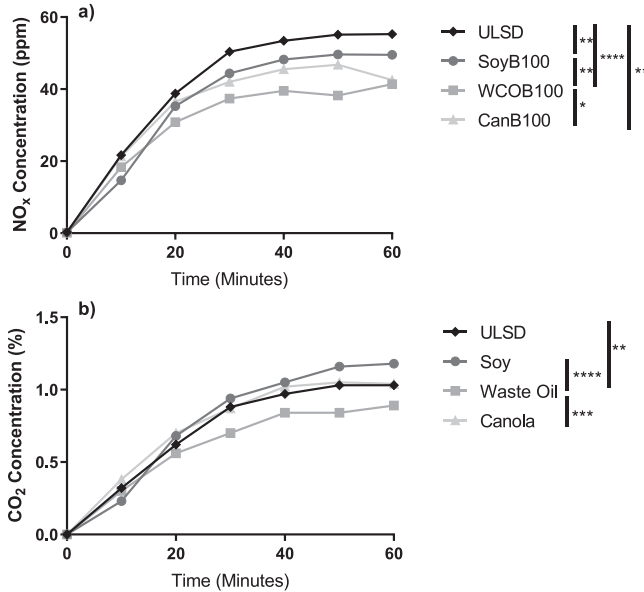
### Mediator analysis

Mediator analysis was performed Bio-Rad 27plx human cytokine kit (Bio-Rad, CA, USA) and accompanying software (Bio-Plex Manager, v6.1.1, Bio-Rad, Tokyo, Japan) as per the kit protocol. All samples were run undiluted in duplicate and then normalized using BCA results.

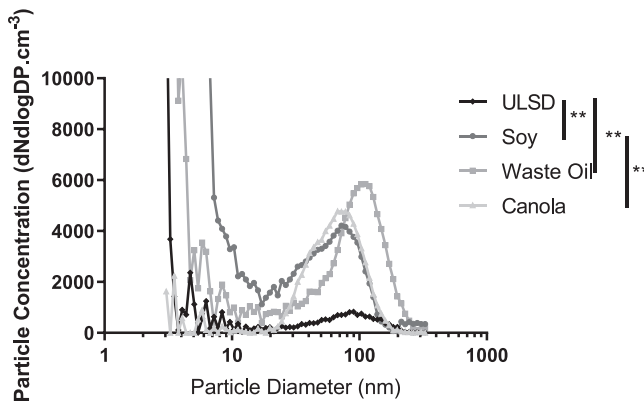
### Statistical analysis

All statistical analyses were completed using R statistical software (V3.4.3) [8] using the packages “mgcv” and “lme4” and the “gam” and “glm” functions respectively. *P*-values less than 0.05 were considered significant. A separate General Additive Model (GAM) file was fitted to each gas measurement with concentration as the response variable and time as the predictor which allowed for non-parametric fits caused by the cold start effect. All other statistical analyses, including total particle number and biological outcomes, were completed using multivariate general linear modelling (GLM) methodologies with the families “gaussian(log)” and “Gamma(inverse/log)” as best fit the data, applying a backwards elimination approach to remove insignificant predictive variables. Sample ID was used as a “factor” in all biological analyses to help account for variation between individual volunteers.





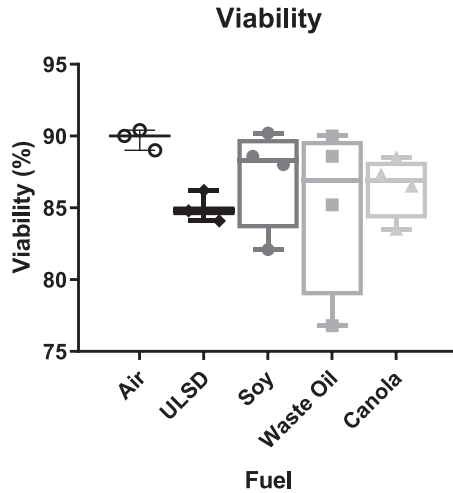
**Fig. 5.** Exhaust gas concentrations for the 1/10 diluted exhaust exposures. Results for a) nitrogen oxides (NO<sub>x</sub>) and b) carbon dioxide (CO<sub>2</sub>) are shown for the three tested biodiesel and commercial mineral diesel (ULSD). Significant results were obtained using gas concentrations and GAM methodologies (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ ).



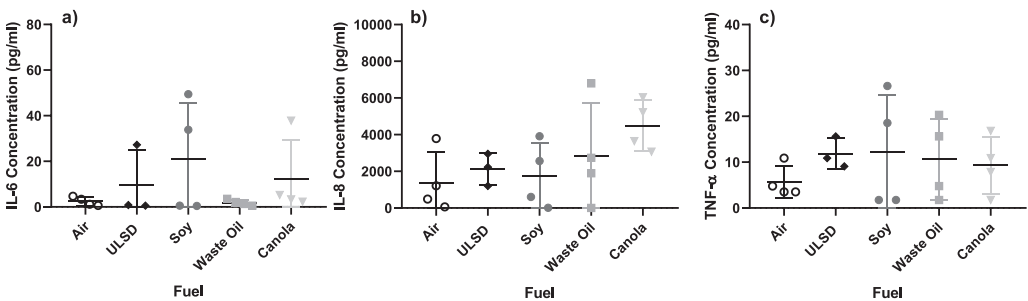
**Fig. 6.** Exhaust particle spectra for the concentrations for the 1/10 diluted exhaust exposures. The spectra for the three tested biodiesel and commercial mineral diesel (ULSD) are shown. Significant differences were obtained with total particle number using GLM methodologies (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ ).

## Method validation

We performed a pilot study using 4 different samples to assess the validity of our exposure methodology prior to starting the larger study. Exhaust was diluted 1/10 with Air for the pilot study (see below). We tested exhaust physico-chemistry and the toxicological results of exposure to the exhaust of a diesel engine running on commercial mineral diesel (ULSD) and three different types of biodiesels: Soy, Waste Cooking Oil and Canola. In this pilot study we tested our above methodology for the ability to analyse combustion gas concentrations (Fig. 5), particle spectra (Fig. 6), cellular viability



**Fig. 7.** Raw viability data for 4 samples exposed to the 1/10 diluted exhaust of the three tested biodiesel and commercial mineral diesel (ULSD). No significant differences were obtained in comparison to Air controls, likely due to small sample size.



**Fig. 8.** Inflammatory mediator release for 4 samples exposed to the 1/10 diluted exhaust of the three tested biodiesel and commercial mineral diesel (ULSD). The results for a) IL-6, b) IL-8 and c) TNF- $\alpha$  are shown. No significant differences were obtained in comparison to Air controls, likely due to small sample size.

(Fig. 7) and mediator release (Fig. 8). We were successful in obtaining all data but ultimately decided that the combustion gas levels seen in the pilot study were too high for real-world applicability and subsequently increased the dilution factor to 1/15. This increased dilution factor was used to obtain data which has now been published [1].

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgments

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Curtin University's Schools of Engineering and Curtin University's Graduate Research School for their support and funding. We would like to thank the West Australian Epithelial Research Program for supplying the patient samples. Thank you to all participants and families for contributing to the study.

## Additional information

One of the things that must be decided prior to beginning an exhaust exposure experiment is whether to include the cold start [9]. This occurs when the engine is started cold, as opposed to a hot start when the engine is run long enough to heat up, briefly turned off and then turned on again while still "hot". Cold starts result in higher emission levels which can impact results and thus it must be consistent whether exposures do or do not include the cold start effect.

We decided on the exposure time of one hour based upon our previous study where we tested only one type of biodiesel using a 1/10 exhaust dilution and found a one hour exposure to have the greatest toxicological effect [10]. A 1:15 dilution with air made exposures more relevant to real-world exhaust concentrations, however the higher air dilution also risked dehydrating the cells if we had also increased exposure time. Fuel combustion generates water and thus exhaust is highly humid, whereas without a humidifier, air is not. Prolonged exposure time with high air dilutions risks the continuous flow of exhaust/air slowly drying out the media, thus biasing results. Attempting to humidify the air prior to dilution risked collecting exhaust particles within the water droplets, thus artificially altering the exhaust deposition. That said, a lower exhaust dilution at 1:5 or 1:10 would increase the exhaust concentration and subsequent toxic effects at the risk of making the experiment irrelevant to real world exposures. This may still be a suitable alternative for studies looking into more subtle toxic mechanisms than viability or mediator release, where such an increase can easily be justified. Alternatively, further increasing the dilution to 1:20 or even 1:50, so long as the effect of dehydrating the cells can be negated, would make the experiment even more real world relevant at the risk of losing any of the resulting toxic effects to the background noise caused by high patient variability.

The 24 h rest period was chosen based on data obtained in [6], where the greatest immune response was seen after epithelial cells rested for 24 h. It is thus a possibility that the cells underwent some sort of recovery process during this rest period, and potentially impacted viability results if the cells either recovered or fully completed apoptosis to become apoptotic/necrotic bodies that would be difficult to be analysed by flow cytometry due to their small sizes. We chose the 24 h point because we believed it best answered the question "Can the effects of exposure still be observed a day later when people are likely being re-exposed, thus suggesting toxic effects can accumulate?". Thus, studies for which viability impacts are the priority, over that of immune mediator release, may want to include a shorter rest period.

In this study we also used linear regression to analyze the effect of the different components and found that exhaust gases were significantly correlated with various toxicological results, suggesting that the exhaust gases are crucial for analyzing the toxicity of exhaust exposures. This is absolutely something that must be kept in mind for future studies as the majority of diesel and biodiesel exhaust toxicity literature tends to focus on particulate matter alone [11–13].

In addition, we found that the maximum number of cell IDs one person can process at the post exposure stage without negatively affecting viability results was four, so each exposure will have to be done either multiple times in order to obtain a large enough sample size (as we did) or be processed by more people simultaneously. Stained cells could sit on ice for approximately 2 h before viability was negatively impacted.

Although this method was designed for exhaust exposure, it can easily be altered for other aerosol exposure types including, but not limited to, cigarette, wood fire smoke and household sprays. Exposure timing and dilution would have to be adjusted based on real world concentrations and humidity of the exposure being tested. Additionally, the cell type used can be easily replaced so that non-respiratory exposures can be tested. This could include skin or gut epithelium, which would also likely be exposed to air diluted concentrations of potentially toxic chemicals.

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