Population Health

Development of a Screening Tool to Identify Safer Biodiesels

KATHERINE LANDWEHR
0000-0002-1543-6304

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

September 2021

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the St John of God Human Research Ethics Committee, approval number #901 and reciprocal human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number # HRE2018-0227.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number # ARE2020-16.

Katherine Landwehr 4th September 2021

Abstract:

Background and Aims: Biodiesel is a term which encompasses a range of fuels made from a wide variety of natural oils via the process of transesterification. It is often promoted as a sustainable replacement for commercial mineral diesel, and usage is increasing rapidly worldwide. As biodiesel fuel properties, and thus exhaust components, change depending on the type of oil it is made from, the series of studies described in this thesis had the over-arching aim of comparing the toxic/health effects of exposure to exhaust generated by the combustion of mineral diesel and biodiesel made from different, commonly used oils. The health effects of exposure to biodiesel blended (mixed) with mineral diesel at an appropriate ratio were also investigated. The secondary aim was to use the extensive dataset obtained by these comparative studies to develop a mathematical screening model which would allow new biodiesel fuels to be assessed for toxicity *in silico* without the need for further time-consuming and expensive biological testing.

Methods: The literature review (Chapter 1) conducted on diesel exhaust exposure studies advised on the typical levels of exhaust observed in occupational settings, and the exhaust concentrations used in all exposure studies was diluted to real-world levels. A series of *in vitro* and *in vivo* exposure models were used to assess the effects of biodiesel exhaust exposure. Initially (Chapter 2), a study was conducted whereby submerged human airway epithelial cell cultures were exposed to exhaust from an engine running on soy biodiesel, a 20% blend of soy biodiesel in diesel or mineral diesel (or air as a control) for 1, 2 or 4 hours with the goal of establishing an appropriate exposure protocol/duration for following "high-throughput" experiments. Exhaust physicochemical properties including exhaust gas concentrations and particulate matter concentration, number and size were measured. After exposure, key toxicity/health outcomes including cellular viability and mediator production were assessed. This study led to that described in Chapter 3, whereby submerged cultures were exposed to one of six different biodiesel exhausts (Canola, Waste Cooking Oil, Soy, Tallow, Palm or Cottonseed oils plus mineral diesel exhaust and air controls) for 1 hour and a similar set of outcomes assessed. Concurrently, the effects of exposure to relevant 20% biodiesel blends were assessed (Chapter 4) using the same protocols and outcome measures. The data gleaned from Chapters 2, 3 and 4 allowed for a targeted study using human airway epithelial cells grown at the air liquid interface to generate a 3D lung model (Chapter 5), and a targeted *in vivo* study (Chapter 6). In both of these studies, the "best" and "worst" biodiesels (Canola and Tallow) were selected for further detailed investigation, again in comparison with mineral diesel and air controls. In the air-liquid interface *in vitro* study, cells were exposed for 1 hour and outcome measures included permeability, protein release and mediator response in the apical and basal compartments. In the *in vivo* study, adult male BALB/c mice were exposed to Canola or Tallow biodiesel exhaust (or mineral diesel exhaust or air control) for 2 hours once, or 2 hours daily for 8 consecutive days. Twenty-four hours after the final exposure a wide range of outcomes, focussing on respiratory health were assessed. These included lung volume, lung function and responsiveness to methacholine, cellular inflammation, mediators, protein and phospholipid levels in bronchoalveolar lavage, systemic inflammation and parameters of airway and lung morphometry. A wide range of biological samples were bio-banked for future analyses. Finally (Chapter 7) key data from chapter 3, plus fuel fatty acid methyl ester (FAME) profiles (a key chemical property of biodiesel fuel) were used to generate and validate a linear regression based mathematical screening model allowing the toxicity of other biodiesels to be estimated.

Results: In all chapters, exhaust physiochemistry varied significantly between not only diesel and biodiesel but also between the different biodiesel types, with the higher exhaust concentrations showing all biodiesels to contain significantly more particles at smaller sizes, particularly Canola, Soy and Waste Cooking Oil biodiesels (Chapters 2, 3 and 4). The particle spectra of the higher dilutions showed that all biodiesel exhausts had an additional peak in particle concertation around the 23 nm size that wasn't present in mineral diesel exhaust. The exhaust gas concentrations were highly variable, with higher concentrations of exhaust showing biodiesel to have more, or similar levels of nitrogen oxides to diesel (Chapters 2 and 3) and the lower exhaust concentrations showing similar or lower levels (Chapters 5 and 6). Carbon dioxide concentrations were generally significantly higher in biodiesel than diesel in most experiments, especially for Tallow, Palm and Soy biodiesels (Chapter 2, 3, 6).

The toxicity study described in Chapter 2 showed than the lowest exhaust dosage, a 1-hour exposure, was more toxic with significantly decreased viability and increased mediator release. The high throughput 1-hour exposures for the 6 different biodiesels fuels (Chapter 3) found that the biodiesel FAME profile greatly impacted toxicological outcomes with the fuels with the highest number of unsaturated FAME molecules or the highest number of FAME molecules with two double bonds being the most toxic- Tallow then Soy and Palm in order of toxicity (more toxic than mineral diesel). In contrast, biodiesels with high levels of unsaturated FAME molecules (only one double bond) were the least toxic with equal or less toxicity than mineral diesel- Cottonseed, Waste Cooking Oil then Canola in order of most to least. This pattern was repeated in the blended fuels with the three most toxic and the three least toxic biodiesels being the same as seen for pure biodiesels (Chapter 4). The targeted *in vitro* air liquid interface and *in vivo* animal model studies (Chapters 5 and 6) were conducted using the most and least toxic biodiesel fuels (Tallow and Canola). They showed that Tallow biodiesel exhaust and Tallow biodiesel blend exhaust were once again the most toxic with increased permeability, increased protein in the supernatant and bronchoalveolar lavage (BAL) (indicating epithelial cell damage), the largest disruption in mediator release, increased inflammation in the BAL, increased responsiveness to methacholine and increased chord length indicating potential damage to airways. In contrast, Canola biodiesel was less toxic and caused some increased protein release in the supernatant, a smaller disruption to mediator release, impacts to lung volume and function, smaller inflammation in the BAL and a reduced responsiveness to methacholine compared even to air exposed controls. Exposure to diesel exhaust was in the middle in terms of health impacts. Of concern, single Tallow biodiesel and mineral diesel exposures in the *in vivo* study resulted in similar health impacts to the consecutive 8-day exposures, suggesting that only low dosages/short durations of exhaust exposure are needed induce toxicity. Finally, the screening model was successfully generated (Chapter 7) and tested, with biodiesels whose fuel properties were obtained from other studies being successfully inputted and the health effects predicted being within biological possibility, although the biodiesels with similar FAME profiles used to generate the model provided more reliable predictions. The model ultimately needs strengthening with data from biodiesels with more extreme FAME properties however it is the first of its kind to date.

Conclusions: The source oil used to make biodiesel significantly impacts the toxic effects of biodiesel exhaust exposure. Despite using high dilutions with air, exposure to more than half the biodiesels resulted in significant health effects equal to or more severe than those of mineral diesel. This has concerning implications for populations who have already been exposed to biodiesel exhaust. The screening model was successfully generated however it is

most effective when used on biodiesels with fatty acid methyl ester profiles similar to the ones used in this thesis. Future studies using a wider range of fatty acid methyl ester profiles could further strengthen the screening model.

In summary, this work has:

- Advised on acceptable occupational exposure limits of diesel exhaust particles.
- Performed a time course exposure showing that smaller dosages of exhaust are capable of inducing toxic effects.
- Conducted the most comprehensive toxicological analysis on the widest range of biodiesel to date in a single study.
- Conducted the most comprehensive toxicological analysis on the widest range of biodiesel blends to date in a single study.
- Extensively explored toxic capabilities of different biodiesel exhausts in a wide range of exposure models to show that the source oil used to make the biodiesel undoubtedly effects resulting exhaust toxicity.
- Developed a model that allows for the screening and comparison of health effects for new biodiesels *in silico*.

Therefore, there can be no doubt that this work has provided a significant and novel contribution to the fields of biodiesel exhaust toxicity.

Acknowledgements

I would like to acknowledge and thank my supervisors, A/Prof Alexander Larcombe, A/Prof Anthony Kicic and Professor Benjamin Mullins, as without their time and support I would not have made it this far. They helped to pave the path to make this work possible and their encouragement was invaluable. I would also like to thank Dr Rebecca O'Leary, a life saver who made the statistics work almost easy to understand, which I would not have thought possible before I started this PhD.

I would like to thank my family for starting me on this path, especially Pop for inundating me with David Attenborough documentaries and my sister for making me watch them over and over again until they had replaced the typical morning cartoons. Thank you all for supporting me and putting up with me even when it must have seemed like I had lost my mind in the midst of the PhD scramble. You joked that I was taking forever and that I needed to get a job already but four years is a reasonable time period I swear. I got there in the end! I would also like to thank my cat for keeping me sane(ish) and driving me batty in turn, especially when he sat on the backspace bar and deleted 20+ pages of my thesis. Cat, you are very lucky I had just saved my work or you would have made a lovely rug.

And finally, for all the obstacles that did their best to stop me; temperamental particle analysers, temperamental engines and exhaust equipment, weird settings on the flow cytometer that refused to change, faulty incubators, faulty air blowers, faulty humidifiers, freezers breaking, fridges breaking, several laboratory moves in a row, engines refusing to work because people kept removing the battery, people breaking the engine gasket, people with weird animal phobias, global pandemics, faulty software, terrible website designs that make no logical sense and finally unhelpful colleagues and lab managers who seemed to delight in making everything harder. Despite your best efforts, I succeeded.

Funding Source Acknowledgement:

The funding for this project was obtained through the Australian Research Council Discovery Project Grant DP170104346. I would like to acknowledge and thank Curtin University, the Australian Government, the Telethon Kids Institute and the Wal-yan Respiratory Centre for funding and support throughout and the Australian Research Council for providing the grant that made it possible. I would also like to thank Stan and Jean Perron for their funding and support for my work through the Stan and Jean Perron Excellence Award.

List of Publications:

Chapter 1: Landwehr, K. R., Larcombe, A. N., Reid, A., & Mullins, B. J. (2020). Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants? *Exposure and Health*. doi:10.1007/s12403-020-00379-0.

Chapter 2: Landwehr, K. R., Hillas, J., Mead-Hunter, R., O'Leary, R. A., Kicic, A., Mullins, B. J., & Larcombe, A. N. (2019). Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells. *Environmental Science & Technology*, 53(19), 11437-11446. doi:10.1021/acs.est.9b01671.

Chapter 3: Landwehr, K. R., Hillas, J., Mead-Hunter, R., Brooks, P., King, A., O'Leary, R. Kicic, A., Mullins, B. J., Larcombe, A. N. (2021). Fuel feedstock determines biodiesel exhaust toxicity in a human airway epithelial cell exposure model. *Journal of Hazardous Materials*, 420, 126637. doi: https://doi.org/10.1016/j.jhazmat.2021.126637

Chapter 5: Submitted to Particle and Fibre Toxicology September 2021.

List of Conference Abstracts:

1. Landwehr KR, Hillas K, Mead-Hunter R, King A, O'Leary R, Kicic A, Mullins B, Larcombe AN (2020) Toxicity of various biodiesel exhausts in an in-vitro human airway epithelial cell model. *Respirology* 25 Suppl 1:90.

2. Landwehr KR, Hillas J, Mead-Hunter R, King A, O'Leary RA. Kicic A, Mullins BJ, Larcombe AN (2019) Comparative toxicity of various biodiesel exhaust exposures compared with diesel. European Respiratory Society: 2019.

3. Landwehr KR, Hillas J, Mead-Hunter R, O'Leary RA, Knothe G, Kicic A, Mullins BJ, Larcombe AN (2019) Comparative toxicity of various biodiesel exhausts compared with mineral diesel in an in vitro human airway epithelial cell model. *Respirology* 24 Suppl 1:117.

4. Landwehr KR, Hillas J, O'Leary RA, Knothe G, Kicic A, Mullins BJ, Larcombe AN (2018) in vitro exposure of airway epithelial cells to soy biodiesel exhaust is less toxic compared with mineral diesel exhaust. *Respirology* 23 Suppl 1:112.

List of Conference Presentations:

State:

1. Wal-Yan Respiratory Centre, Annual Respiratory Meeting, 2020, "*Comparative toxicity of various biodiesel exhausts compared with commercial mineral diesel in in vitro and in vivo models."*
2. Institute for Respiratory Health: 20th Anniversary Symposium, 2019, "*Comparative toxicity of various biodiesel exhausts compared with commercial mineral diesel in an in vitro human airway epithelial cell model."*
3. Australian Society for Medical Research: WA Annual Scientific Meeting, 2018, "*Is in vitro exposure of airway epithelial cells to soy biodiesel exhaust less toxic compared with mineral diesel exhaust?"*

National:

1. Thoracic Society of Australia and New Zealand: Annual Scientific Meeting, Virtual Event 2021, *"Toxicity of various biodiesel exhausts in human airway epithelial cells."*
2. Australian Society for Medical Research: National Annual Conference, Online 2020, *"Comparative toxicity of commercial mineral diesel and tallow and canola biodiesel exhausts in an in vitro air-liquid interface human airway epithelial cell model"*.
3. Australian Society for Medical Research: National Annual Conference, Perth 2019, *"Comparative toxicity of various biodiesel exhausts compared with commercial mineral diesel in an in vitro human airway epithelial cell model."* My abstract was chosen for media release.
4. Thoracic Society of Australia and New Zealand: Annual Scientific Meeting, The Gold Coast 2019, *"Comparative toxicity of various biodiesel exhausts compared with commercial mineral diesel in an in vitro human airway epithelial cell model."*
5. Australian College of Toxicology and Risk Assessment: Annual Scientific Meeting, Perth 2018, *"Comparative toxicity of biodiesel and mineral diesel exhaust in an in vitro airway epithelial cell model."*
6. Thoracic Society of Australia and New Zealand: Annual Scientific Meeting, Adelaide 2018, ""*Is in vitro exposure of airway epithelial cells to soy biodiesel exhaust less toxic compared with mineral diesel exhaust?"*

International:

1. Accepted for poster presentation at the annual Asia Pacific Conference Online, November 2021, *"Toxicity of two different biodiesel exhausts in primary human airway epithelial cells grown at air-liquid interface."*
2. Accepted for poster presentation at the annual European Toxicology Conference Online, September 2021, *"Toxicity of two different biodiesel exhausts in a murine exposure model."*
3. European Respiratory Society Annual Congress, Madrid, Spain, 2019, "*"Comparative toxicity of various biodiesel exhausts compared with commercial mineral diesel in an in vitro human airway epithelial cell model."*
4. Australia – China Centre for Air Quality Science and Management Annual Meeting, Perth 2018, "*Comparative toxicity of biodiesel and mineral diesel exhaust in an in vitro airway epithelial cell model."*

Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by myself (Katherine Rae Landwehr). All published chapters have me as the first author. Contributions by colleagues are described and signed in the Appendix where the Copyright Permissions can also be found. I also declare that there is some similarity of my thesis with my previously published papers, specifically within the methodology and results sections which as stated, were primarily designed, experimentally, executed, interpreted, and written by myself.

Abbreviations:

A1; Air 1-day exposure
A8; Air 8-days exposure
ALI; Air liquid interface
B100; 100% biodiesel fuel
B20; Blended mineral diesel fuel with 20% biodiesel
BAL; Bronchoalveolar lavage
BE; Biodiesel exhaust
C1; Canola 1-day exposure
C8; Canola 8-days exposure
CO; Carbon Monoxide
CO_2; Carbon Dioxide
DOC; Diesel Oxidation Catalyst
DPF; Diesel particulate filter
FAME; Fatty acid methyl esters
FRC; Functional residual capacity
G; Tissue damping
G-CSF; Granulocyte colony-stimulating factor
GM-CSF; Granulocyte-macrophage colony-stimulating factor
H; Tissue elastance
HVO; Hydro-treated vegetable oil
IFN-γ; Interferon gamma
IL-10; Interleukin 10
IL-12(p40); Interleukin 12(p40)
IL-12(p70); Interleukin 12(p70)
IL-13; Interleukin 13
IL-17; Interleukin 17
IL1-RA; Interleukin 1 receptor antagonist
IL-1α; Interleukin 1-alpha
IL-1β; Interleukin 1-beta
IL-2; Interleukin 2
IL-4; Interleukin 4
IL-5; Interleukin 5
IL-6; Interleukin 6
IL-7; Interleukin 7
IL-8; Interleukin 8
IL-9; Interleukin 9
IP-10; Interferon gamma-induced protein 10
KC; Keratinocyte chemoattractant
MCh; Acetyl β-methacholine chloride
MCP-1: Monocyte chemoattractant protein 1
MIP-1α; Macrophage inflammatory protein 1-alpha
MIP-1β; Macrophage inflammatory protein 1-beta
NO; Nitrogen Monoxide
NO_2; Nitrogen Dioxide
NO_x; Nitrogen oxides
O_2; Oxygen
PAHs; Polycyclic aromatic hydrocarbons
PDGF-bb; Platelet derived growth factor BB
PM; Particulate matter

PN; Particle Number
P_{rs}; Transrespiratory pressure
RANTES; Regulated on activation, normal T cell expressed and secreted
R_{aw}; Airway resistance
RME; Rapeseed methyl esters
SME; Soy methyl ester
SO_2; Sulfur Dioxide
T1; Tallow 1-day exposure
T8; Tallow 8-days exposure
TGV; Thoracic gas volume
TNF-α; Tumour necrosis factor-alpha
U1; ULSD 1-day exposure
U8; ULSD 8-days exposure
ULSD; Ultra-low sulfur diesel
VEGF; Vascular endothelial growth factor
WCO; Waste Cooking Oil
Z_{rs}; Respiratory system impedance
η; Hysteresivity

Table of Contents

Table of Contents for Figures and Tables

Chapter 1.0: Critical review of diesel exhaust exposure health impact research relevant to occupational settings: are we controlling the wrong pollutants?

*Katherine R. Landwehr[1,2], Alexander N. Larcombe[1,2], Alison Reid[1], Benjamin J. Mullins[1]

[1]School of Public Health, Curtin University, PO Box U1987, Perth, WA, 6845

[2]Respiratory Environmental Health, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, WA, 6009

Corresponding Author*:

Email: katherine.landwehr@telethonkids.org.au

Post: Northern Entrance, Perth Children's Hospital, 15 Hospital Avenue, Nedlands, Western Australia, 6009

Keywords: Diesel Exhaust Exposure, Occupational Exhaust Exposure, Occupational Exposure Limit, Occupational Diesel Exhaust Limit

Reprinted by permission from Exposure and Health: Springer Exposure and Health, Landwehr, K. R., Larcombe, A. N., Reid, A., & Mullins, B. J. (2020). Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants? *Exposure and Health*. doi:10.1007/s12403-020-00379-0[COPYRIGHT] (2020)

Abstract

Introduction: Diesel exhaust emissions and exposure of workers in occupational settings are topics which have attracted increased attention after IARC classification as a group 1 carcinogen (IARC, 2018). There is ongoing debate over appropriate exposure limits for occupationally exposed workers. This review consolidates recent research findings relevant to setting appropriate exposure limits, with a specific focus on newer engine and after-treatment technologies.

Method: Appropriate online databases were searched for studies published since 2005 focussing on the health effects of whole diesel exhaust exposure. Engines that used exhaust after-treatment devices including both a diesel oxidation catalyst and a diesel particulate filter were classified as new technology engines. All other studies were classified as using older technology engines.

Results: Exposure to diesel exhaust from both engine classifications resulted in negative health impacts on the lungs, heart and brain. Study participants with asthma, allergy or respiratory disease were more at risk of negative effects caused by diesel exhaust exposure than healthy subjects.

Conclusion: Based on the published literature, an occupational limit of an average diesel exhaust concentration below 50 µg/m^3 of diesel exhaust particles, 35 µg/m^3 of elemental carbon, is appropriate to limit health effects. To meet this limit, many diesel engines will need to be equipped with after-treatment technology such as a DPF. However, the use of a DPF had little to no impact on measured health effects despite the removal of over 90% by weight of particles. This negates the feasibility of using particle mass based limits.

1.0.1 Diesel Exhaust

Diesel exhaust was classified as a class 2a; probable human carcinogen by the International Agency for Research on Cancer (IARC) in 1989. This classification changed to class 1; definitely carcinogenic to humans in 2012 based primarily on a series of studies conducted on 12315 occupationally exposed hardrock miners. The risk was greatest in surface workers with a standard mortality ratio (SMR) of 1.33 (1.06-1.66, 95% C.I.) compared with underground workers 1.21 (1.01-1.45, 95% C.I.), despite the underground workers having an average respirable elemental carbon (EC) exhaust exposure level that was over 75 times higher than the surface workers. This may be attributed to background exposures unrelated to DE in the study population or the effect of DE aging and being exposed to sunlight, ozone and other environmental factors which can cause DE components to become more toxic (Attfield et al., 2012; Silverman et al., 2012).

Diesel exhaust can be separated into two main components; the gaseous phase and the particulate matter (PM) phase. Gaseous components include carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (NO_x) and sulfur dioxide (SO_2) as well as additional gas phase chemical species such as polycyclic aromatic hydrocarbons (PAH) and volatile organic compounds (VOC). The PM is composed of mostly solid EC particles with potentially toxic chemicals such PAH, VOC, aldehydes, ketones and heavy metals ad/ab-sorbed to the particles (Carrara & Niessner, 2011; Fontaras et al., 2009; Hu et al., 2013; Prokopowicz, Zaciera, Sobczak, Bielaczyc, & Woodburn, 2015; Riley et al., 2018). Diesel exhaust can contain hundreds of different chemical species and concentrations can change significantly depending on engine type, speed, load, whether accelerating or decelerating, starting temperature and the usage of exhaust after-treatment devices (J. Bünger et al., 2000; Fontaras et al., 2009; Hemmingsen, Møller, Nøjgaard, Roursgaard, & Loft, 2011; Hesterberg et al., 2011; George Karavalakis, Stournas, & Bakeas, 2009; Khalek, Bougher, Merritt, & Zielinska, 2011; Kisin, Shi, Keane, Bugarski, & Shvedova, 2013).

Of most concern are the ultrafine particles found within DE. These particles, at less than 100 nm in size, comprise the majority of DE PM with particles smaller than 30 nm comprising over 90% of the total number of particles but only accounting for 10% of the total PM mass (D. Kittelson, Watts, & Johnson, 2002; Ris, 2007). Ultrafine particles are capable of penetrating deeper into the lungs than larger sized particles, dispersing over a greater percentage of lung volume and thus causing a greater respiratory irritant effect (Oberdörster, Celein, Ferin, & Weiss, 1995; Seaton, Godden, MacNee, & Donaldson, 1995). Smaller particles have a greater surface area to volume ratio, meaning that a greater amount of potentially toxic substances can adhere to the surface for a given mass of PM (Mullins, Kicic, Ling, Mead-Hunter, & Larcombe, 2016; Yoza, Matsumoto, & Matsunaga, 2002) and thus a greater amount of toxic chemicals are deposited in the lungs. Exposure to ultrafine particles is associated with pulmonary inflammation (Oberdörster et al., 1995) and exacerbation of existing lung diseases including asthma (Evans, Halterman, Hopke, Fagnano, & Rich, 2014; Seaton et al., 1995). Ultrafine particles are also capable of penetrating into the cardiovascular system and cause a range of adverse health effects including increased blood pressure and heart failure (Brook et al., 2010).

Alone, each individual component of the exhaust can cause its own unique health effects and combined they can interact to cause more complicated health impacts such as cancer as well as impacting the cardiovascular, respiratory and neurological systems (Benbrahim-Tallaa et al., 2012; Kristen E. Cosselman et al., 2012; Heidari Nejad et al., 2015; Larcombe et al., 2014; Levesque, Surace, McDonald, & Block, 2011; Mills et al., 2007; Zhu et al., 2012). This makes studying the effects of whole exhaust preferable to those of isolated components, such as PM alone, where the effects of the gas components and their interaction with PM is lost (Abe, Takizawa, Sugawara, & Kudoh, 2000; Larcombe, Kicic, Mullins, & Knothe, 2015).

1.0.2 Changes in Engine Technology, Exhaust After-Treatment Devices and Emission Limits

Using diesel particulate filters (DPF), diesel oxidation catalysts (DOC) and other exhaust after-treatment devices, the components of DE change dramatically. A DPF is capable of removing approximately 90% of PM by mass. Elemental carbon is preferentially removed and ratios of EC to organic carbon reduce from ~3 to 0.5 (Khalek et al., 2011). In exhaust without a DPF, EC makes up approximately 75% of PM by weight (US EPA, 2002), which reduces to approximately 13% after the use of a DPF. In the ultrafine particle range, larger sized particles closer to 100 nm in size are removed from the exhaust more successfully than smaller sizes (Khalek et al., 2011).

The EURO, US EPA and the US TIER classification systems have been developed as emission standards for light-heavy vehicles on road, heavy duty vehicles on road and off road engine emissions respectively. Most engines classified as EURO IV, US EPA 2007 or US TIER 4 and above require exhaust after-treatment devices for compliance and engines classified as EURO IV and above generally require the latest high-pressure common-rail electronic fuel injection systems (Dallmann & Menon, 2016). The aim of this review is to consolidate recent DE exposure and health effects research findings relevant to setting appropriate DE exposure limits, with a specific focus on newer engine and after-treatment technologies.

1.0.3 Methods

PubMed was searched using “Diesel Exhaust” combined with the individual search term “Exposure Health Effect”, limiting the search to results published after 2005 and finding over 600 studies that matched the search criteria. In addition, the databases Embase and Cinahl Plus were searched using the term “Diesel Exhaust Exposure Health Effect”, limiting the search to results not included as part of the PubMed/Medline database and studies published after 2005. Over 200 studies matched the search criteria. Only articles from the search which matched the review criteria, as well as relevant cited references therein, were reviewed. Studies were excluded if they were not in English, if the results were based on data obtained before 2005, if the diesel fuel used was not classified as ultra-low-sulfur diesel (<15ppm sulfur), or if it exceeded 10% biodiesel concentration, if whole exhaust was not used and finally if the concentration of the exhaust used, or the health outcomes measured were not relevant to occupational exposure settings.

The cut-off date of 2005 was selected based on diesel fuel legislation to limit sulfur levels in commercial diesel fuel. The legislation was introduced in multiple countries in the mid

2000's with several years taken to complete the change over (Kavanagh, 2014). If studies did not specify the amount of sulfur within the diesel fuel used, assumptions were made based on the country the study was performed in and the date that the legislation for ultra-low-sulfur diesel was introduced. If the date of publication fell outside of that range, the study was excluded (Figure 1.0.1).

Relevant studies were separated into occupational exposure studies, acute human exposure studies, *in vivo* exposure studies and *in vitro* exposure studies. Acute human exposure, *in vivo* and *in vitro* studies were further separated into the use of new or older technology engines. Studies that used exhaust from an engine either classified as EURO IV, US EPA 2007 or TIER 4 and above, or as being paired with a DPF and DOC, were classified as using new technology engines. Studies that did not specify engine type, used exhaust from an engine without both after-treatment devices or used an engine at a lower EURO or TIER classification were defined as using older technology.

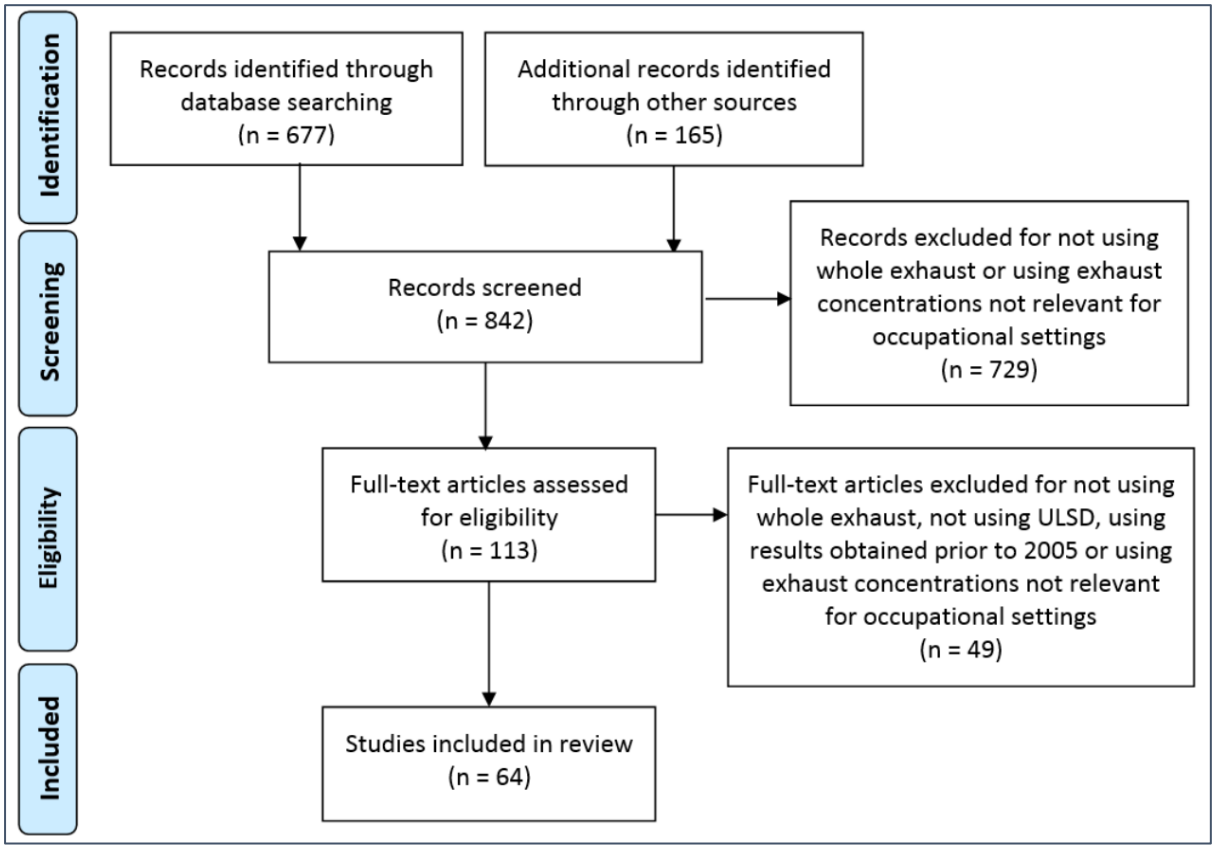


Figure 1.0.1: An overview of the methodology used to select appropriate articles for review.

1.0.4 Occupational Exposure Studies

A common method of completing occupational exposure studies is to focus on population data in order to collect potential health consequences of DE exposure. As a consequence, the majority of DE exposure data are obtained before 2000, for example (Attfield et al., 2012; Kachuri et al., 2016; Olsson et al., 2011; Silverman et al., 2012; Vermeulen et al., 2014), when sulfur levels in fuel were high (>500 ppm and in some cases >5000 ppm) (Kavanagh, 2014) and diesel engines were not equipped with exhaust after-treatment devices. In order to

measure the consequences of a lifetime of occupational exposure to a substance, a lifetime has to have passed, making such studies difficult when the substance being measured (i.e. new technology diesel engine exhaust) is still newly introduced into the workplace. Thus few studies have looked at new technology exhaust and fewer still have looked at the health consequences of exposure. Since the occupational studies reviewed did not specify the type of engine technology used, all are classified as old technology studies.

That said, studies focussing on populations that have been more recently exposed to DE at occupational concentrations have been published (Table 1.0.1), including a series of studies on a cohort of diesel engine testers in China (Bassig et al., 2017; Dai et al., 2018; Niu et al., 2018; H. Wang et al., 2017). These studies found that the greater the concentrations of DE exposure and the longer the time period the workers had been exposed for, the more the immune response was dysregulated (Dai et al., 2018; H. Wang et al., 2017), with the highest exposure levels (greater than 397 $\mu g/m^3$) resulting in significantly lower inflammatory cytokine levels in blood serum (Dai et al., 2018). This was theorised to be a mechanism for increased lung cancer risk as the immune system has an important role in eliminating cancerous cells. Exposure to ~282 $\mu g/m^3$ resulted in decreased lung function, compared to control subjects with an exposure level of ~92 $\mu g/m^3$ (H. Wang et al., 2017). At DE exposure concentrations of ~268 $\mu g/m^3$, increased DNA damage was found in peripheral blood lymphocytes, as well as DNA hypomethylation changes associated with DNA damage in blood samples of exposed subjects, compared to control subjects exposed to DE concentrations of ~92 $\mu g/m^3$ (Xiao Zhang et al., 2015; Xiao Zhang et al., 2016). Occupational DE exposures that resulted in above 1.08 µg/g urinary creatinine, a marker of PAH exposure and corresponding to an exposure of ~170 $\mu g/m^3$ of fine PM (~110 $\mu g/m^3$ total carbon) were associated with higher levels of cancer biomarkers (Niu et al., 2018). An exposure level of approximately 100 $\mu g/m^3$ found immune alterations similar to published lung cancer risk studies, suggesting a significantly higher risk of lung cancer (Bassig et al., 2017).

Studies outside of the diesel engine tester cohort found that mechanics occupationally exposed to DE, at an estimated level of 250 $\mu g/m^3$, exhibited cytotoxic and genotoxic damage to buccal epithelial cells, and peripheral blood lymphocytes. In addition, damage to DNA in both cell types was correlated with years of service, suggesting that longer periods of exposure to DE resulted in a greater amount of DNA damage (León-Mejía et al., 2019), which has implications for lung cancer risk (Bonassi, El-Zein, Bolognesi, & Fenech, 2011; León-Mejía et al., 2019). A lifetime exposure (approximately 45 years) to 14 $\mu g/m^3$ of EC in Western Australian miners was estimated to result in an increase of 5.5 (2.7-9.2, 95% confidence interval) lung cancer deaths per 1000 male workers. An exposure of 44 $\mu g/m^3$ of EC was estimated to result in an increase of 38 (19-97, 95% confidence interval) lung cancer deaths per 1000 male workers (Peters et al., 2017). Exposure was measured between 2003 and 2011 when new technology engines were still being introduced, such that the total PM exposure levels are estimated to be 19 and 59 $\mu g/m^3$ respectively. Norwegian tunnel finishing workers occupationally exposed to DE at approximately 37.8 $\mu g/m^3$ of EC, (~50 $\mu g/m^3$ PM assuming that the majority of exposure was from old technology engines) found that in comparison to non-exposed control subjects, the tunnel workers had more DNA damage in their peripheral blood mononuclear cells, altered blood plasma profiles and dysregulated expression of several micro RNAs, including some related to carcinogenesis, cell death and oxidative stress (Rynning et al., 2019).

Occupational DE exposure studies reported effects on lung function and biomarkers that correlated with increased cancer risk (Bassig et al., 2017; Dai et al., 2018; León-Mejía et al., 2019; Niu et al., 2018; Rynning et al., 2019; H. Wang et al., 2017). All studies reported increased risks of lung cancer in workers occupationally exposed to DE. Occupational exposures below 100 $\mu g/m^3$ of PM increased DNA damage, immune alterations in a pattern related to increased lung cancer incidence and an estimated risk of 38 lung cancers per 1000 workers exposed to approximately 44 $\mu g/m^3$ EC (approximately 59 $\mu g/m^3$ PM).

1.0.5 Acute Human Exposure Studies

We found no studies that examined the effects of new engine technology exhaust exposure on humans and only one study that focussed on the health effects of acute exposure to DE with and without a DPF on humans. Thus, all studies that involve acute exposure of humans to high levels of DE have used old technology diesel engines. All but two studies used exposure chambers and participants were exposed to either diluted whole DE at a variety of concentrations and/or air as a control. The measured end points focussed primarily on the cardiovascular system with fewer studies focusing on the respiratory system. No study exposed participants to DE for more than three hours. Further information on the exposure methodology can be found in Table 1.0.2.

Studies using DE exposure concentrations between 350-300 µg/m3 found minor and major cardiovascular effects with increased arterial stiffness (Lundbäck et al., 2009), increased endothelial dysfunction in patients at risk for heart failure (Vieira et al., 2016), reduced vasodilation (Lucking et al., 2011; Mills et al., 2011), increased thrombus formation (Lucking et al., 2011) and increased blood pressure after two hours of exposure (Mills et al., 2011; Tong et al., 2014). In addition, altered blood plasma profiles were found in healthy individuals and altered blood plasma profiles and altered micro RNA expression in peripheral blood were found in individuals with an allergy or asthma (Giles, Tebbutt, Carlsten, & Koehle, 2018; Yamamoto et al., 2013; Xin Zhang, Hirota, Yang, & Carlsten, 2016). DNA hypomethylation was found in genes associated with oxidative stress and inflammation in asthmatics (Jiang, Jones, Sava, Kobor, & Carlsten, 2014). Respiratory effects have also been reported, with altered micro RNA and transcription profiles and DNA hypomethylation associated with increased oxidative stress in epithelial cell brushings (Clifford et al., 2017; Rider et al., 2016) and increased airway hyperactivity and obstruction in individuals with asthma or allergies (Hussain et al., 2012; Xin Zhang et al., 2016). Healthy individuals exposed for 30 minutes to 300 µg/m3 of DE reported significant irritation of the nose, throat and chest after exposure (Giles, Carlsten, & Koehle, 2018). In comparison, heart rate was not affected (Lucking et al., 2011; Vieira et al., 2016). Some studies reported no changes in blood pressure after 30-60 minutes of exposure (Giles, Carlsten, et al., 2018; Giles, Tebbutt, et al., 2018; Lucking et al., 2011) and no changes in markers of inflammation and platelet activation (Giles, Carlsten, et al., 2018; Lucking et al., 2011), balance was not affected and no changes were found in central nervous system biomarkers (Cliff et al., 2016; Curran, Cliff, Sinnen, Koehle, & Carlsten, 2018).

Exposures to DE at concentrations between 300 and 200 $\mu g/m^3$ resulted in similar health effects. Eye irritation was reported by 11 out of 18 healthy subjects exposed for up to three hours, and nose and throat irritation was diagnosed by a medical professional (Wierzbicka et al., 2014). Healthy subjects had decreased induced vasodilation (Barath et al., 2010), increased vasoconstriction (Peretz, Sullivan, et al., 2008) and increased blood pressure and inflammation after two hours of exposure (Kristen E. Cosselman et al., 2012; Tong et al.,

2014) as well as altered gene expression in peripheral blood mononuclear cells (Peretz et al., 2007). In contrast, 60 minutes of exposure did not affect heart rate variability, systemic inflammation or blood pressure in 18 healthy male volunteers (Barath et al., 2010). No indications of oxidative stress was found in individuals with metabolic syndrome (J. Allen et al., 2009) and no changes in heart rate variability were found after two hours of exposure (Peretz, Kaufman, et al., 2008).

Studies examining concentrations of DE below 100 µg/m^3 report half the amount of vasoconstriction in comparison 200 µg/m^3 exposures completed in the same study, suggesting a linear dose response although these levels were not significantly different to air exposures (Peretz, Sullivan, et al., 2008), increased airway inflammation in healthy subjects (Annelie F Behndig et al., 2011), allergic inflammation and viral-induced immune responses in allergic rhinitics (Pawlak et al., 2016) and decreased lung function, increased airway acidification and increased respiratory inflammation in asthmatics exposed for two hours at exhaust concentrations up to 75 µg/m^3 (J. J. Zhang et al., 2009). No thrombotic effect was found in subjects with metabolic syndrome (C. Carlsten et al., 2008), no impact on heartrate was observed (Lucking et al., 2011; Peretz, Kaufman, et al., 2008; Tong et al., 2014), no impact on vasoconstriction was found (Lucking et al., 2011) and there was no evidence of respiratory epithelial cell damage in healthy, allergic or asthmatic individuals following exhaust exposure at 100 µg/m^3 for two hours (Annelie F. Behndig et al., 2015).

One study compared the health impact of exposure to DE with and without a DPF on 19 healthy volunteers. The use of a DPF decreased PM concentration from 320 to 7.2 µg/m^3. Study participants who were exposed to whole, unfiltered exhaust for one hour had increased thrombotic formation and reduced vasodilation. The use of a DPF negated the effects of exposure on vasodilation and decreased the thrombotic effect as well (Lucking et al., 2011).

Approximately 60% of acute human exposure studies used exhaust exposure concentrations between 250-350 µg/m^3, suggesting that this level of exposure is the concentration where an observable response is likely to occur using short exposure periods (Tong et al., 2014). At this level of exposure, health effects are noticeable by the participants themselves with reports of irritation to mucosal surfaces such as the nose, throat and eyes. Most studies examined the cardiovascular system (Steiner, Bisig, Petri-Fink, & Rothen-Rutishauser, 2016). As exposure levels decreased to 100 µg/m^3, reported health effects lowered in severity and more studies began reporting negative outcomes. Those that reported positive results mostly involved individuals with asthma or allergy, suggesting that they may be an at risk population that requires closer monitoring.

In addition, combining the results of both the occupational and acute human exposure studies shows potential to explore genetic alterations and DNA damage as potential biomarkers for disease and lung cancer risk after DE exposure. Rynning et al found DNA damage in peripheral blood mononuclear cells associated with DE exposure (Rynning et al. 2019) and León-Mejía et al found DNA damage of the buccal cheek cells and lymphocytes after continued DE exposure, with the amount of DNA damage correlating with years of service and thus years of exposure (León-Mejía et al., 2019). Thus, with more testing, there is potential to use either a cheek swap or a blood test to quantify DNA damage as a marker of increased cancer risk after DE exposure. Hypomethylation is another potential marker of DE exposure (Clifford et al. 2017), particularly hypomethylation and other changes to genes

related to DNA damage responses such as p16, RASSF1A, and MGMT which are frequently found to be dysregulated in cancer (Zhang et al. 2016). Alternatively, the plasma miRNA profile could be another marker as studies have found it to be significantly altered after DE exposure (Rynning et al. 2019; Rider et al. 2016), with several of the altered miRNA expressions such as miR-31–5 p, miR-20b-5p, miR-196b-5p, miR-4500 and miR-340 being associated with cancer (Rynning et al. 2019).

1.0.6 *In Vivo* (Animal Model) Exposure Studies

Approximately 74% of studies involving animal models focus on the respiratory and central nervous systems. A strength of animal studies is that long-term exposure can be compressed into the relatively short life span of experimental animals, meaning that lifetime exposures can be completed in a much shorter period of time than in comparative human occupational studies. In addition, animal models can also be exposed to much higher concentrations than used in either occupational or acute studies involving human subjects. Thus the majority of *in vivo* studies reviewed expose animals for a large range of exhaust concentrations over longer periods of time, including months or even years, which also represent greater proportions of their life expectancy, than human studies. Six *in vivo* studies examined the effects of acute exposure. Information on animal type and exposure methodology can be found in Tables 1.0.3 and 1.0.4.

1.0.6.1 *Older Engine Technology*: The majority of *in vivo* exposure studies use old technology diesel engines and exposure concentrations vary greatly (between 50 and 3000 $\mu g/m^3$).

Studies that exposed animals to DE concentrations between 3000 and 2000 $\mu g/m^3$ for one to twelve weeks resulted in negative respiratory and neurological effects. A 3.2 fold greater DNA mutation frequency was found in the lungs of mice exposed for twelve weeks compared with air exposed controls suggesting greater cancer risk (Hashimoto et al., 2007), large increases in neuroinflammation were found in the brains of mice exposed for 4 weeks (Shannon Levesque et al., 2011) and increased lung inflammation was found in mice exposed for less than a week, with allergic mice exhibiting greater symptoms (Stevens, Krantz, Linak, Hester, & Gilmour, 2008). In contrast, more recent studies exposing rats for one or four weeks to ~2000 $\mu g/m^3$ found only minor histopathological changes and inflammatory effects in the lungs (Magnusson et al., 2019) and minor oxidative stress in the brain (Valand et al., 2018).

Exposing mice to DE between the concentrations of 1000 and 2000 $\mu g/m^3$ has effects on the transcription of stress related genes in the brain (Lung, Cassee, Gosens, & Campbell, 2014). Similar to an exposure concentration of 3000 $\mu g/m^3$, a 3.1 fold increase of DNA mutations was found in the lungs of mice exposed to 1000 $\mu g/m^3$ for twelve weeks, suggesting greater cancer risk (Hashimoto et al., 2007).

Studies exposing mice and rats to DE concentrations between 500 and 1000 $\mu g/m^3$ found oxidative stress and increased inflammation in the lungs of rats exposed to 950 $\mu g/m^3$ for < one week (Tsukue, Kato, Ito, Sugiyama, & Nakajima, 2010), increased neuroinflammation in mice exposed to 650 $\mu g/m^3$ for 4 weeks (similar to that found in 2000 $\mu g/m^3$ exposure concentrations) (Shannon Levesque et al., 2011), increased flu severity in mice exposed to 500 $\mu g/m^3$ for < two weeks (Gowdy et al., 2010) and an increased effect of chemically

induced arrhythmia in hypertensive rats exposed to 500 µg/m^3 for < one week (Hazari et al., 2015). Increased respiratory inflammation was found in allergic mice, but not healthy mice, exposed for < one week and the effects were lower than that found in mice exposed to 2000 µg/m^3, suggesting dose-response relationships in these particular outcomes (Stevens et al., 2008).

In vivo exposure to DE concentrations between 300 µg/m^3 and 100 µg/m^3 results in neurological effects including impaired neurogenesis in male mice exposed to 250 µg/m^3 for < one day (Coburn, Cole, Dao, & Costa, 2018), increased neuroinflammation in mice exposed for four weeks to 173 and 149 µg/m^3 (Gerlofs-Nijland et al., 2010; Win-Shwe, Yamamoto, Fujitani, Hirano, & Fujimaki, 2008) and impact on object recognition ability in mice exposed to 129 µg/m^3 for twelve weeks (Win-Shwe, Fujimaki, Fujitani, & Hirano, 2012). No impact was found on spatial learning abilities in mice exposed to 149 µg/m^3 for four weeks (Win-Shwe et al., 2008). Health impacts on other systems included increased respiratory inflammation in normal mice and increased respiratory inflammation and oxidative stress in asthmatic mice exposed to 200 µg/m^3 for seven weeks or 169 µg/m^3 for eight weeks respectively (Bai et al., 2011; Tanaka, Aoki, et al., 2013; Tanaka, Takano, et al., 2013), unfavourable changes in atherosclerotic plaques in mice exposed to 200 µg/m^3 for seven weeks (Bai et al., 2011), changes in steroidogenesis in male rats exposed for four weeks to 149 µg/m^3 (Yamagishi et al., 2012), an increased effect of chemically induced arrhythmia in hypertensive rats exposed to 150 µg/m^3 for less than a week (Hazari et al., 2015), increased allergic symptoms in asthmatic mice exposed to 100 µg/m^3 for twelve weeks (Matsumoto et al., 2006) and increased oxidative stress in the lungs of rats exposed for 3 days to 100 µg/m^3, although no impact on respiratory inflammation was found (Tsukue et al., 2010).

Exposure studies that used DE concentrations below 100 µg/m^3 found mild increases in the effect of chemically induced arrhythmia in hypertensive rats exposed to 50 µg/m^3 for < one week (in comparison to exposure to 150 and 500 µg/m^3) (Hazari et al., 2015), increased oxidative stress in the lungs and minor impact on respiratory inflammation of rats exposed to 60 µg/m^3 for < one week (in comparison to exposure to 950 µg/m^3) (Tsukue et al., 2010), minor increases in respiratory inflammation in asthmatic mice exposed for eight weeks to 39 µg/m^3 (in comparison to exposures to 169 µg/m^3) (Tanaka, Aoki, et al., 2013), some impact on steroidogenesis in male rats exposed to 38 µg/m^3 for eight weeks (Yamagishi et al., 2012) and no impact on object recognition in mice exposed to 47 µg/m^3 for twelve weeks or oxidative stress in mice exposed to 36 µg/m^3 for eight weeks (Tanaka, Takano, et al., 2013; Win-Shwe et al., 2012).

1.0.6.2 ***New Engine Technology:*** Seven *in vivo* studies exposed animals to the exhaust generated from new technology diesel engines. Exhaust concentrations never exceeded 200 µg/m^3 and all studies were published in the past five years. Rats exposed to 182 µg/m^3 for one and four weeks displayed changes in gene expression of the brain which suggests minor oxidative stress, although no histopathological effects were found and the differences compared to rats exposed to old technology exhaust at a concentration of 2000 µg/m^3 were minor (Valand et al., 2018). Magnusson et al. found minor respiratory inflammation and oxidative stress in the lungs of rats exposed to approximately 170 µg/m^3 for one and four weeks. No differences were found when compared to rats exposed to old technology exhaust at a concentration of 2000 µg/m^3 (Magnusson et al., 2019). Douki et al. found minor

indications of accumulated lung DNA damage in rats exposed to less than 100 µg/m^3 for three weeks, however effects were found to be worse with new technology exhaust when compared to old, suggesting that toxicity was associated with the ultrafine particulates and the gas phase of the exhaust (Douki et al., 2018). A series of studies exposed rats to 12 µg/m^3 of exhaust for 28-30 months and found only limited effects, including minor histopathological changes and mild increases in inflammatory and thrombotic markers however no damage to DNA was recorded and no increases in tumour development were found (Bemis, Torous, & Dertinger, 2015; Conklin & Kong, 2015; Hallberg, Ward, Hernandez, Ameredes, & Wickliffe, 2015; McDonald et al., 2015).

In *in vivo* exposure studies using old technology exhaust, exposure concentrations varied greatly with the highest exposures resulting in a range of health impacts to the respiratory, cardiovascular and neurological systems of mice and rats. The severity of these effects decreased as exposure decreased. Results were similar for new technology studies, however the few studies available limit the conclusions that can be drawn. Once again, animals with conditions simulating asthma or allergy displayed worse symptoms and the study with the lowest exhaust exposure concentration that still reported exposure health impacts used asthmatic mice as subjects, highlighting potentially susceptible populations. Some studies also reported increased influenza severity in mice exposed to DE, which may highlight another susceptible population that wasn't found in the human exposure studies.

1.0.7 *In Vitro* (Cell Model) Exposure Studies

Most *in vitro* studies into the effects of DE exposure use DE particles collected on quartz filters and added directly to the media the cells are grown within (Maria C. Zarcone et al., 2016). Using this approach to estimate the potential health consequences of exhaust exposure is limited as it ignores the health consequences of the exhaust gases entirely. In addition, the particles collected on the filter agglomerate, sticking together to generate an artificial particle spectrum made of larger particles, often removing the ultrafine particles from the sample and thus from the subsequent analysis of exposure health effects (Morin et al., 2008). This approach often underestimates health consequences of exposure and over 16 times higher concentrations of particles are needed to generate the same health consequences as exposure to whole exhaust (Lichtveld et al., 2012). All *in vitro* studies included in this review use whole exhaust instead of pre-collected particles and focus on the damage caused to the respiratory epithelium, either using primary human epithelial cells or the alveolar carcinoma cell line A549. All cells were grown at an air-liquid interface, exposing one side of the cell model directly to the diluted DE (Tables 1.0.5 and 1.0.6).

1.0.7.1 *Older Engine Technology*: Studies exposing cells to old technology diesel engine exhaust have mostly focussed on cell damage, oxidative stress and inflammatory responses. A549 cells exposed to 1600 µg/m^{3}at air liquid interface displayed inhibited proliferation and increased oxidative stress (Okubo, Hosaka, & Nakae, 2015). The same cells exposed to exhaust after the use of a DPF, at a concentration of 470 µg/m^3, exhibited suppressed immune reactivity in comparison to air exposed controls. Oxidative stress was decreased in comparison to the DE exposure concentration of 1600 µg/m^3, however the decreased immune response after exposure was only found in those cells exposed to the DPF equipped exhaust (Okubo et al., 2015). A549 cells exposed to 1300 µg/m^3 at air-liquid interface displayed increased cell death and increased oxidative stress (Kooter et al., 2013). While differentiated primary human bronchial airway epithelium grown at air-liquid interface and exposed to DE at a concentration of 850 µg/m^3, displayed increased oxidative stress and increased PAH

adduct formation but no loss of viability (Hawley, L'Orange, Olsen, Marchese, & Volckens, 2014).

Three studies exposed differentiated primary human airway epithelial cells collected from healthy volunteers and volunteers with COPD to a range of exhaust concentrations and types (M. C. Zarcone et al., 2018; Maria C. Zarcone et al., 2016; Maria C. Zarcone, van Schadewijk, Duistermaat, Hiemstra, & Kooter, 2017). In a study that used old technology exhaust, Zarcone et al. (2016) found that exposing the cells to ~1200 $\mu g/m^3$ induced the production of inflammatory markers, oxidative stress, cellular death and increased permeability after 150 minutes of exposure. At 430 $\mu g/m^3$ they found increased oxidative stress after 150 minutes and increased permeability after 375 minutes. At 140 $\mu g/m^3$ only decreased permeability was recorded (Maria C. Zarcone et al., 2016).

1.0.7.2 ***New Engine Technology:*** Only three *in vitro* exposure studies were found that examined new technology DE exposure. Exposure to 1500 $\mu g/m^3$ for 60 minutes induced oxidative stress and decreased the defence response to infection, although no cellular death occurred (Maria C. Zarcone et al., 2017). Primary human airway epithelial cells exposed to three different, much lower, exhaust concentrations found that the lowest concentration (34 $\mu g/m^3$) had no impact on healthy cells, the second lowest concentration (82 $\mu g/m^3$) increased oxidative stress in healthy cells and the highest concentration (206 $\mu g/m^3$) increased oxidative stress in healthy cells and decreased host defence in COPD derived cells (M. C. Zarcone et al., 2018). Differentiated primary human airway epithelial cells exposed to a DE concentration of 35.3 $\mu g/m^3$ found increased oxidative stress and increased PAH adduct formation. No difference in health effects was observed between new technology exhaust and old technology exhaust at a concentration of 800 $\mu g/m^3$ (Hawley et al., 2014).

Table 1.0.1: Key data from selected occupational human exposure studies using old technology DE. Studies use average PM/EC readings to assess levels in the work place and thus assume that workers are exposed to the measured level of diesel exhaust for the entirety of their shifts. All EC measurements are assumed to be from old technology engines (~75% of the total PM measurement).

Average Concentration of Diesel Exhaust PM (µg/m3, mean (±SD))	Source	Cohort Demographic	Health Outcomes
19*	Peters, de Klerk et al. 2017	Personal EC exposure for 8614 Australian Miners collected between 2003 and 2015	Increased lung cancer risk: estimated 5.5 (2.7-9.2, 95% C.I.) extra lung cancer deaths per 1000 workers
50*	Rynning, Arlt et al. 2019	69 Norwegian tunnel finishing workers and 69 unexposed control subjects working at similar construction sites	Increased DNA damage in peripheral blood mononuclear cells in never smoking, former smoking and daily smoking subjects ((~mean; 5^{th}-95^{th} percentile DNA adducts per 10^8 nucleotides) control vs exposed for never smoking, former smoking and daily smoking subjects: 0.87; 0.64-1.12 vs 1.03, 0.74-1.33, 0.91; 0.69-1.22 vs 1.24; 0.79-1.90, 1.10; 0.90-1.26 vs 1.48; 0.97-2.01 respectively). Micro RNA dysregulation, including several related to carcinogenesis, cell death and oxidative stress
59*	Peters, de Klerk et al. 2017	Personal EC exposure for 8614 Australian Miners collected between 2003 and 2015	Increased lung cancer risk: estimated 38 (19-97, 95% C.I.) extra lung cancer deaths per 1000 workers
100	Bassig, Dai et al. 2017	54 male workers employed at a diesel engine testing facility and 55 unexposed male control workers	Levels of nine inflammatory markers altered in directions associated with lung cancer risk. The largest differences between control and exposed subjects were found in CRP (42.7% decrease), IL-21 (23.5 % decrease) and CCL15 (21.2% increase) (mean±SD (pg/ml) in control vs exposed: 1.6x107±2.3x107 vs 9.2x106±1.4x107, 3.4±8.6 vs 2.6±4.9 and 2260.4±997.2 vs 2740.5±1098.4 respectively
~170	Niu, Zhang et al. 2018	137 male exposed diesel engine tester and 127 male non-exposed workers	Exceeding 1.08 µg/g urinary creatinine, approximately 110 µg/m3 total carbon exposure, was associated with increased cancer biomarkers such as micronucleus, and thus increased risk of cancer
250	León-Mejía, Luna-Rodríguez et al. 2019	120 diesel exhaust exposed Columbian mechanics and 100 unexposed control subjects	Cytotoxic and genotoxic damage to buccal epithelial cells and peripheral blood lymphocytes ((mean±SD) frequency of micronucleation in buccal epithelial cells control vs exposed: 6.13±2.49 vs 16.89±10.16 ($p<0.001$). Comet assay damage index, % tail DNA and frequency of micronucleation in blood lymphocytes, control vs exposed: 107.05±27.88 vs 131.22±48.15 ($p<0.05$), 23.39±9.18 vs 30.91±17.52 ($p<0.05$) and 4.02±2.54 vs 10.36±6.56 ($p<0.001$)). Micronucleation of lymphocytes correlated with years of service ($r=0.370$, $p < 0.0001$)
268	Zhang, Duan et al. 2015	117 male exposed diesel engine tester and 112 male non-exposed control workers	Increased DNA damage in peripheral blood lymphocytes, in comparison to exposures at 92 µg/m3. Exposed workers exhibited a 2, 7.8, and 4.3 fold increase in the means of the micronucleus, nucleoplasmic bridge and nuclear bud frequencies (mean±SD of control vs exposed subjects: 3.54%±2.64% vs 7.04%±3.32, 0.22%±0.46% vs 1.71%±1.28%, 1.18%±1.37% vs 5.11%±3.63% respectively)
268	Zhang, Li et al. 2016	117 male exposed diesel engine tester and 112 male non-exposed control workers	DNA hypomethylation of three DNA damage response genes (p16, RASSFIA and MGMT) and slight immune dysregulation in comparison to exposures at 92 µg/m3. Methylation in p16, RASSF1A, and MGMT decreased by 0.36% (0.11-0.60%, 95% C.I), 0.46% (0.14-0.79%, 95% C.I.) and 0.55% (0.15-0.95%, 95% C.I.) respectively and monocyte levels were lower in exposed workers (5.01%±1.72% vs 4.40%±1.12%, $p = 0.014$)
282	Wang, Cui et al. 2017	117 male exposed diesel engine tester and 112 male non-exposed control workers	Lower lung function, decreased serum markers of local inflammation and increased serum markers systemic inflammation in comparison to exposures at 92 µg/m3. The longer the exposed workers had worked at the facility the greater the immune dysregulation displayed. Measures of FEV1/FVC decreased from 88.5% (80.5-98.1%, 90% C.I.) to 86.0% (76.5-94.0%, 90% C.I.). Local inflammation was measured using serum CC16 (17.1 ng/ml (9.3–29.1 ng/ml, 90%

			C.I.) vs 13.9 ng/ml (7.5–25.6 ng/ml, 90% C.I.) in healthy vs exposed subjects), systemic inflammation was measured using serum CRP levels (0.47 ng/ml (0.06–6.36 ng/ml, 90% C.I.) vs 0.91 ng/ml (0.30–4.90 ng/ml, 90% C.I.)
>397	Dai, Ren et al. 2018	41 male exposed diesel engine testers and 46 male unexposed controls	Reduced inflammatory cytokine response in blood serum. Cytokines IL-8 and Mip-1β had significantly decreased release in the highest exposed subjects (median (pg/ml) (10-90th percentile) in healthy vs exposed subjects= 11.9 (8.5-18.1) vs 9.4 (8.4-11.9) and 71.1 (31.4-130.8) vs 30.8 (8.9-59.4) respectively). Cytokine MCP-1 also displayed a significant inverse relationship with exposure levels

- SEM = standard error of the mean, SD= standard deviation, C.I.= confidence interval,

~ Data obtained from graphical forms and thus is an approximation only

* Exposure levels measured in EC, adjusted to PM.

Table 1.0.2: Key experimental data from selected acute human exposure studies using old technology DE. All studies diesel exhaust exposure levels below 400 µg/m^3 and no exposure occurred for more than three hours.

Concentration of Diesel Exhaust (µg/m3)	Source	8 Hour TWA (µg/m^3)	Exposure Time (hours)	Times Exposed to Diesel Exhaust	Cohort Demographic	Exposure Method	Engine Classification	Health Impacts in Acute Exposures
7.2	Lucking, Lundback et al. 2011	0.9	1	2	19 non-smoking healthy males (mean age, 25±3 years)	Exposure chamber	NS*	Exhaust paired with a DPF had no impact on vasoconstriction and mild increased thrombotic effects in comparison to more severe effects at 320 µg/m^3 unfiltered exhaust (increase of approximately 4% in stenosed coronary artery simulation, not significant compared to either air or unfiltered exhaust. Not significantly different to air in patent coronary arteries but significantly decreased in comparison to the unfiltered exhaust)
<75	Zhang, McCreanor et al. 2009	<18.75	2	1	60 non-smoking asthmatics (18-55 years old)	Controlled roadside exposure	Mix	Decreased lung function, increased airway acidification and increased respiratory inflammation in asthmatics, more severe asthmatics displayed greater symptoms ((mean±SE) FEV1 and FVC decreased by 3.23%±1.04% and 3.06%±0.29% respectively two hours after DE exposure (p=0.004), exhaled breath condensate pH decreased by 1.99%±0.05 three hours after exposure (p=0.002), 22 hours after exposure measurements of sputum myeloperoxidase increased by 521%±12.58% (p=0.014))
100	Pawlak, Noah et al. 2016	25	2	1	22 allergic rhinitics (11 exposed to air, 27.5±8.7 years, 11 exposed to exhaust, 25.6±4.7 years)	Exposure chamber	NS	Increased inflammation (eosinophil cationic protein levels (mean±SD) = 92.78±111.3 and 112.1±97.54 for air and DE exposure respectively (p=0.04)) and prolonged viral induced eosinophil activation effects in subjects with allergic rhinitis
100	Behndig, Shanmuganathan et al. 2015	25	2	2	32 asthmatics, 13 rhinitics and 21 healthy controls (18-41 years old)	Exposure chamber	NS[a]	No evidence of epithelial cell damage following exposure
100	Tong, Rappold et al. 2014	25	2	3	6 healthy glutathione-S-transferase-Mu 1 null adults (50-71 years old)	Exposure chamber	NS	No cardiovascular effects, increased inflammatory effects (18 hour pose exposure fold change (95% C.I.) for venous blood monocyte counts of 1.22 (1.00, 1.44) (p<0.05))
100	Peretz, Kaufman et al. 2008	25	2	2	16 healthy adults (18-49 years old)	Exposure chamber	NS[b]	No consistent cardiovascular effects
100	Peretz, Sullivan et al. 2008	25	2	2	10 healthy adults and 17 adults with metabolic syndrome (18-49 years old)	Exposure chamber	NS[b]	Half the amount of vasoconstriction (0.05mm decrease in brachial artery diameter) in comparison to 200 µg/m3 exposures, suggesting a linear dose response. Not significantly different to air
100	Carlsten, Kaufman et al. 2008	25	2	2	16 adults with metabolic syndrome (18-49 years old)	Exposure chamber	NS[b]	No cardiovascular effects in metabolic syndrome patients

100	Behndig, Larsson et al. 2011	25	2	2	32 non-smoking asthmatics and 23 non-smoking healthy controls (18-45 years old)	Exposure chamber	NS[a]	Increased airway inflammation in healthy subjects but not asthmatics (submucosal neutrophil counts (~median; IQR, cells/mm^2) and bronchial wash IL-6 release (~median; IQR, pg/ml) in healthy subjects, control vs exposed: 57.3; 25.2-75.6 vs 71.1; 48.1-153.5 ($p<0.01$), 3.1; 1.6-4.9 vs 4.9; 2.7-7.1 ($p<0.05$))
200	Peretz, Peck et al. 2007	50	2	3	5 non-smoking healthy adults (20-31 years old)	Exposure chamber	NS[b]	Altered genetic profile in peripheral blood mononuclear cells (2.4% or 1240 out of 54675 probe sets significantly changed in response to DE exposure)
200	Cosselman, Krishnan et al. 2012	50	2	1	45 healthy non-smokers (18-49 years old)	Exposure chamber	NS[b]	Increased blood pressure (an increase of 3.8 mmHg (95% CI: −0.4, 8.0) and 5.1 mmHg [95% CI: 0.7, 9.5] for 30 minutes and 60 minutes of exposure respectively), no impact on heart rate
200	Allen, Trenga et al. 2009	50	2	1	10 adults with metabolic syndrome (18-49 years old)	Exposure chamber	NS[b]	No effect on patients with metabolic syndrome
200	Peretz, Kaufman et al. 2008	50	2	2	16 healthy adults (18-49 years old)	Exposure chamber	NS[b]	No consistent cardiovascular effects
200	Tong, Rappold et al. 2014	50	2	3	6 healthy glutathione-S-transferase-Mu 1 null adults (50-71 years old)	Exposure chamber	NS	Increased inflammation in venous blood samples (18 hour post exposure fold change (95% C.I.) of 1.07 (0.96, 1.19) ($p<0.05$) and 1.02 (0.97, 1.07) ($p<0.1$) for neutrophil and platelet count respectively), no cardiovascular effects
200	Peretz, Sullivan et al. 2008	50	2	2	10 healthy adults and 17 adults with metabolic syndrome (18-49 years old)	Exposure chamber	NS[b]	Increased vasoconstriction (brachial artery diameter decrease of 0.11 mm (95% C.I., 0.02–0.18))
250	Barath, Mills et al. 2010	31.25	1	1	18 non-smoking healthy males (21-30 years old)	Exposure chamber	NS	Decreased chemically induced vasodilation (e.g. (~mean±SEM, ml/100ml tissue/min) forearm blood flow vasodilation after intra-brachial infusion of 1000 pmol/min bradykin control vs exposed: 20.6±1.7 vs 18.2±1.7), no effect on heart rate variability, inflammation or blood pressure
280	Wierzbicka, Nilsson et al. 2014	105	3	2	Healthy non-smoking adults (40-66 years old)	Exposure chamber	NS	Irritant effects- chest, throat and nose symptoms (clinically diagnosed and reported by participants using post exposure questionnaires). No reported symptoms at 15 minutes of exposure. Symptoms reported after 75 minutes and symptoms worsened after 135 minutes of exposure
300	Giles, Carlsten et al. 2018	18.75	0.5	3	18 non-smoking recreationally active males (24.5±6.2 years)	Exposure chamber	TIER-3c	Irritant effects- chest, throat and nose symptoms (reported by participants using post exposure questionnaires), no changes in blood pressure
300	Giles, Tebbutt et al. 2018	18.75	0.5	3	18 non-smoking recreationally active males (24.5±6.2 years)	Exposure chamber	TIER-3c	Altered blood plasma profiles (two hours post exposure, endothelin-1 significantly decreased in comparison to air (mean±SD (pg/ml) = 1.48±0.28 and 1.36±0.37 for air and DE exposures respectively ($p=0.037$). High intensity exercise during exposure increased plasma NO_x levels the DE exposed group in comparison to controls: (~mean±SD (µmol/L) = 19.7±7.7 vs 13.8±5.9 respectively. No changes in blood pressure or markers of inflammation

300	Hussain, Laumbach et al. 2012	37.5	1	1	16 non-smoking asthmatics (20-42 years old)	Exposure chamber	NS	Decreased lung function, increased airway hyperactivity and obstruction in individuals with asthma (FEV1% decreased by 3.3% 24 hours after exposure (p=0.043), 20% reduction in forced expiratory volume in one second (PC20) decreased by 4.8 mg/ml (C.I. 95% 1.23–8.35, p=0.012))
300	Rider, Yamamoto et al. 2016	75	2	2	15 non-smoking healthy volunteers with atopy to house dust mite, birch or Pacific grass (19-49 years old)	NS	NS	Altered micro RNA and transcription profiles (expression of six miRNA and ten mRNA were significantly altered after exposure to DE alone)
300	Clifford, Jones et al. 2017	75	2	1	17 non-smoking healthy adults (20-46 years old)	Exposure chamber	TIER-3c	DNA hypomethylation in airway epithelial cells. Exposure to DE or allergen primes response to second exposure
300	Zhang, Hirota et al. 2016	75	2	1	17 non-smoking atopic adults (17-49 years old)	Exposure chamber	TIER-3c	Altered genetic and plasma profile, decreased lung function and increased airway hyperactivity in subjects with allergies (coexposure to DE and allergen resulted in 10.23±42.0 mg/ml reduction in PC20 (p=0.15) and a 5.2% mean reduction in FEV1 after DE exposure in comparison to control)
300	Jiang, Jones et al. 2014	75	2	1	16 non-smoking asthmatics (19-35 years old)	Exposure chamber	TIER-3c	In asthmatics, changes to DNA methylation occurred at 2827 CpG sites after exposure to diesel exhaust but not filtered air. The majority of changes were hypomethylation. Methylation changes occurred in genes associated with oxidative stress and inflammation in asthmatics
300	Tong, Rappold et al. 2014	75	2	3	6 healthy glutathione-S-transferase-Mu 1 null adults (50-71 years old)	Exposure chamber	NS	Diastolic blood pressure increased by 5mmHg ((mean±SEM) before and after DE exposure= 78.3±3.7 and 83.3±4.0 respectively)
300	Cliff, Curran et al. 2016	75	2	1	27 non-smoking healthy adults (19-49 years old)	Exposure chamber	TIER-3c	No effect on blood Central Nervous System biomarkes
300	Curran, Cliff et al. 2018	75	2	1	28 non-smoking healthy adults (19-49 years old)	Exposure chamber	TIER-3c	No effect on balance after exposure
301	Yamamoto, Singh et al. 2013	75	2	2	13 non-smoking asthmatics (19-35 years old)	Exposure chamber	TIER-3c	In asthmatics, the expression of 81 micro RNA's in blood were found to change after DE exposure. Changes associated with increased oxidative stress
320	Lucking, Lundback et al. 2011	40	1	2	19 healthy males (mean age, 25±3 years)	Exposure chamber	NS	Reduced vasodilation and increased thrombus formation (forearm blood flow vasodilation (~mean±SEM, ml/100ml tissue/ min) decreased after intra-brachial infusion of 1000 pmol/min bradykin control vs exposed: 19.0±1.5 vs 20.5±2.1. Thrombus formation increased by 21.8% ($p<0.001$) and 14.8% ($p<0.05$) in simulations of patent and stenosed coronary arteries respectively after exposure to DE). No changes in blood pressure, heart rate, markers of inflammation and platelet activation
325	Vieira, Guimaraes et al. 2016	14	0.35	2	26 adults at risk of heart failure (51±9 years) and 15 healthy controls (45±10 years)	NS	NS	Increased endothelial dysfunction in patients at risk for heart failure (decrease in the reactive hyperemia index from 2.17 (IQR: 1.8 to 2.5) to 1.72 (IQR: 1.5 to 2.2; p=0.002) after exposure to DE. Values under 2 associated with increased endothelial dysfunction. Increased B-type natriuretic peptide in peripheral blood from 47.0 pg/ml (IQR: 17.3 to 118.0 pg/ml) to 66.5 pg/ml (IQR: 26.5 to 155.5 pg/ml; p=0.004) after exposure to DE). No changes in heart rate variability

348	Mills, Miller et al. 2011	75	2	2	16 non-smoking healthy males (18-32 years old)	Exposure chamber	NS	Reduced vasodilation and increased blood pressure after DE exposure (mean±SD: systolic blood pressure increased from 133±3 to 145±4 mmHg. Forearm blood flow vasodilation (~mean±SEM, ml/100ml tissue/ min) decreased after intra-brachial infusion of 1000 pmol/min bradykin control vs exposed: 16.6±2.2 vs 19.1±2.6)). No changes to resting heartrate
350	Lundbäck, Mills et al. 2009	43.75	1	1	12 non-smoking healthy males (21-30 years old)	Exposure chamber	NS[a]	Increased arterial stiffness (30 minutes after exposure, augmentation pressure and augmentation index increased: (mean±SEM, air vs DE exposure) -2.5±0.7 vs -1.8±0.8 (p=0.01) and -7.9±2.2 vs -5.8±2.7 (p=0.02) respectively). No effect on heartrate or blood pressure

- SEM = standard error of the mean, SD= standard deviation, C.I.= confidence interval, IQR = interquartile range

~ Data obtained from graphical forms and thus is an approximation only
* = NS - Not Specified
a= Volvo TD45, 4.5L four cylinder 1991 engine model.
b= Turbocharged direct-injection 5.9-L Cummins 2002 B-series diesel engine (model 6BT5.9G6) and a 100-kW generator.
c= EPA Tier 3-compliant, 6.0 kW Coliseum GY6000 generator, with 406 cc Yanmar L 100 EE 4-stroke diesel generator

Table 1.0.3: Key experimental data from selected *in vivo* animal exposure studies using old technology DE.

Concentration of Diesel Exhaust (µg/m^3)	Source	8 Hour TWA (µg/m^3)	Exposure Period	Animal	Engine Classification	Health Impacts in Older Technology Exhaust Exposures
38	Yamagishi, Ito et al. 2012	23.8	5 h/day, 5 days/week, 1, 2 or 3 months	Rat	NS*	Some effects on steroidogenesis in male rats (increased plasma testosterone after 2 months exposure ($p<0.05$), decreased plasma luteinizing hormone which regulates testosterone biosynthesis after 3 months ($p<0.05$), no effect on hippocampus)
36	Tanaka, Takano et al. 2013	22.5	5 h/day, 5 day/week, 8 weeks	Mouse	NS	No effects of oxidative stress in lungs (8-OHdG expression used as marker) of healthy or asthmatic mice
39	Tanaka, Aoki et al. 2013	24.4	5 h/day, 5 day/week, 8 weeks	Mouse	NS	Minor increases in respiratory inflammation in asthmatic mice, some indications of oxidative stress in the lungs (increased eosinophil number in bronchoalveolar lavage fluid (BALF), increased IFN-γ and IL-5 release ($p<0.05$), increased myeloperoxidase levels in BALF ($p=<0.05$))
47	Win-Shwe, Fujimaki et al. 2012	29.4	5 h/day, 5 day/week, 3 months	Mouse	NS	No impact on object recognition
50	Hazari, Haykal-Coates et al. 2015	25	4 h/day, 1 or 5 days	Rat	NS	Mild increased effect of chemically induced arrhythmia (in comparison to 150 and 500 µg/m^3) (decreased dose of aconite needed to induce arrhythmia ($p<0.05$))
60	Tsukue, Kato et al. 2010	30	6 h/day, 1-7 days	Rat	NS	Impact on respiratory inflammation and increased oxidative stress (decreased macrophage levels ($p<0.05$), increased release of 8-OHdG ($p<0.001$) after 3 days of exposure)

82	Karthikeyan, Thomson et al. 2013	41	4 h/day, 1 and 3 days.	Rat	NS	Inflammation and increased oxidative stress in lungs and negative cardiovascular effects (increased number of BALF neutrophils, increased BALF levels of KC, MIP-1α and MCP-1 after single exposure, increased number of BALF macrophages after multiple exposures. Increased lung mRNA levels of *IL-6*, *TNF-α*, *HO-1* and *SOD2* after single exposure, increased *HO-1* and *iNOS* and decreased *SOD2* after multiple exposures. Increased levels of plasma endothelins). Greater effects than higher exhaust concentration without DPF usage
100	Matsumoto, Hiramatsu et al. 2006	87.5	7h/day, 5 days/week, 12 weeks	Mouse	NS	Increased allergic symptoms in asthmatic mice (increased bronchoconstriction after methacholine challenge ($p<0.001$), increased expression of *IL-4*, *IL-5*, *IL-13*, *MDC* and *RANTES* mRNA in lung tissue, increased release of IL-4 and RANTES in BALF ($p<0.05$)), effects not prolonged with continuous exposure
100	Tsukue, Kato et al. 2010	50	6 h/day, 1-7 days	Rat	NS	Increased respiratory oxidative stress (increased release of 8-OHdG after 3 days of exposure ($p<0.001$)), no impact on respiratory inflammation
129	Win-Shwe, Fujimaki et al. 2012	80.6	5 h/day, 5 day/week, 3 months	Mouse	NS	Impact on object recognition (increased exploration time using novel object recognition test, increased inability to recognise familiar objects, decreased *CaMKIV* and *EAAT4* mRNA expression in hippocampus ($p<0.05$))
149	Win-Shwe, Yamamoto et al. 2008	93.1	5 h/day, 5 days/week, 4 weeks	Mouse	NS	Increased neuroinflammation but no impact on spatial learning (increased expression of IL-1β and TNF-α mRNA in the hippocampus, increased expression of *NR1*, *NR2A* and *NR2B* mRNA in hippocampus ($p<0.05$), no change in results for the Morris Water Maze Behaviour Test)
149	Yamagishi, Ito et al. 2012	93.1	5 h/day, 5 days/week, 1, 2 or 3 months	Rat	NS	Effects on steroidogenesis in male rats (increased concentrations of plasma and testicular testosterone after 1 month or exposure and increased androstenedione concentrations in hippocampus after 1 month of exposure ($p<0.05$))
150	Hazari, Haykal-Coates et al. 2015	75	4 h/day, 1 or 5 days	Rat	NS	Increased effect of chemically induced arrhythmia (decreased dose of aconite needed to induce arrhythmia ($p<0.05$))
169	Tanaka, Takano et al. 2013	105.6	5 h/day, 5 day/week, 8 weeks	Mouse	NS	Increased oxidative stress in asthmatic mice compared to both air exposed and healthy control (increased levels of 8-OHdG in BALF)
169	Tanaka, Aoki et al. 2013	105.6	5 h/day, 5 day/week, 8 weeks	Mouse	NS	Increased respiratory inflammation and markers of oxidative stress in asthmatic mice (increased neoutrophil, eosinophil and lymphocyte cell number in BALF, increased release of IL-5, IL-6, IL-13, MCP-1, TARC, MDC, Eotaxin and KC in BALF, increased levels of myeloperoxidase in BALF ($p<0.05$))
173	Gerlofs-Nijland, van Berlo et al. 2010	129.7	6 h/day, 5 days/week, 4 weeks	Mouse	NS	Increased neuroinflammation (increased expression of TNF-α and IL-1α in different regions of the brain ($p<0.05$))
200	Bai, Kido et al. 2011	150	6 h/day, 5 days/week, 7 weeks	Mouse	NS	Unfavourable changes in atherosclerotic plaques and increased respiratory inflammation (increased plaque lipid content, cellularity, foam cell content and smooth muscle content ($p<0.05$), increased expression of oxidative stress markers iNOS, CD36 and nitrotyrosine and enhanced systemic lipid and DNA oxidation in plaques ($p<0.05$). Increased levels of alveolar macrophages and increased number of alveolar macrophages positive for the presence of particles ($p<0.01$))
250	Coburn, Cole et al. 2018	187.5	6 hour	Mouse	NS	Impaired neurogenesis and increased neuroinflammation in male mice (decreased cell proliferation in hippocampus and increased mRNA expression of TNFα and MDA in cerebral cortex and hippocampus ($p<0.05$))
277	Karthikeyan, Thomson et al. 2013	138.5	4 h/day, 1 and 3 days.	Rat	NS	Inflammation and increased oxidative stress in lungs and negative cardiovascular effects (increased number of BALF neutrophils and increased BALF levels of KC after single exposure, increased number of BALF macrophages after multiple exposures. Increased lung mRNA levels of *IL-6*, *TNF-α*, *HO-1* and *SOD2* after single exposure, decreased *HO-1* and *SOD2* after multiple exposures. Increased levels of plasma endothelins)

500	Gowdy, Krantz et al. 2010	250	4 h/day, 1-14 days	Mouse	NS	Increased flu severity, (increased viral titres of influenza A/HongKong/8/68 and increased BALF neutrophils at days 4 and 8 post infection and increased responsiveness to methacholine ($p<0.05$). Increased mRNA expression of *INF-β* and *TNF-α* and decreased mRNA expression of *INF-γ* and *IL-12p40* in lungs ($p<0.05$). Increased expression of TNF-α in BALF ($p<0.05$))
500	Hazari, Haykal-Coates et al. 2015	250	4 h/day, 1 or 5 days	Rat	NS	Increased effect of chemically induced arrhythmia (decreased dose of aconite needed to induce arrhythmia ($p<0.05$))
500	Stevens, Krantz et al. 2008	250	4 h/day, 4 days	Mouse	NS	Increased respiratory inflammation in allergic mice but not in healthy mice (increased number of neutrophils, eosinophils and lymphocytes in BALF ($p<0.05$))
650	Levesque, Taetzsch et al. 2011	325	4 h/day, 5 days/week, 4 weeks	Mouse	NS	Increased neuroinflammation (similar to that found in 2000 μg/m^3 exposures) (increased mRNA expression of *TNF-α*, *MIP-1α* and increased nitrotyrosine in whole brain homogenate, increased expression of IL-1β, IL-6, TNF-α, MIP-1, RAGE and IBA-1 after exposure in different regions of the brain ($p<0.05$))
950	Tsukue, Kato et al. 2010	712.5	6 h/day, 1-7 days	Rat	NS	Impact on respiratory inflammation (increased number of cells, increased levels of lymphocytes and decreased levels of macrophages in BALF ($p<0.05$)
1000	Hashimoto, Amanuma et al. 2007	1000	12 h/day, 7 days/week, 4, 12 and 24 weeks	Mouse	NS	A 3.1 fold increase in DNA mutation burden in the lungs of *gpt* delta transgenic mice (G:C→A:T transitions were the predominant *gpt* transgene mutation, no difference in mutation burden to the 3000 μg/m^3 exposures)
1700	Lung, Cassee et al. 2014	637.5	3 h/day, 5 days/week, 4 weeks	Mouse	NS	Impact on transcription of stress related genes in the brain (AP-1 levels significantly decreased in the brain after DE exposure)
2000	Magnusson, Dziendzikowska et al. 2019	1500	6 h/day, 7 days or 6 h/day, 5 day/week, 4 weeks	Rat	EURO V (-DPF)	Minor histopathological changes and oxidative stress in lungs (focal mild emphysema and mild mononuclear infiltrate in the lungs, disrupted redox signalling pathways)
2000	Valand, Magnusson et al. 2018	1500	6 h/day, 7 days or 6 h/day, 5 day/week, 4 weeks	Rat	EURO V (-DPF)	Minor oxidative stress in brain (genes associated with oxidative stress and inflammation differentially expressed after DE exposure). No histopathological changes in frontal cortex or hippocampus. No differences in comparison to exposures with a DPF.
2000	Stevens, Krantz et al. 2008	1000	4 h/day, 4 days	Mouse	NS	Increased respiratory inflammation, allergic mice display greater symptoms (increased neutrophil numbers in BALF of normal mice ($p<0.05$), increased neutrophil, eosinophil and lymphocyte numbers in BALF of allergic mice, lIL-6 release in BALF of allergic mice ($p<0.01$), 49 enriched gene sets with 619 core genes were differentially expressed in normal mice after exposure to DE in comparison to air, 23 enriched gene sets with 412 core genes were differentially expressed in asthmatic mice after exposure to DE in comparison to air)
2000	Levesque, Taetzsch et al. 2011	1000	4 h/day, 5 days/week, 4 weeks	Mouse	NS	Increase in neuroinflammation (increased mRNA expression of *TNF-α*, *MIP-1α* and increased nitrotyrosine in whole brain homogenate, increased expression of IL-1β, IL-6, TNF-α, MIP-1, RAGE and IBA-1 after exposure in different regions of the brain ($p<0.05$))
3000	Hashimoto, Amanuma et al. 2007	3000	12 h/day, 7 days/week, 4, 12 and 24 weeks	Mouse	NS	A 3.2 fold increase in DNA mutation burden in the lungs of *gpt* delta transgenic mice (G:C→A:T transitions were the predominant *gpt* transgene mutation, no difference in mutation burden to the 1000 μg/m^3 exposures)

*= NS - Not Specified.

Table 1.0.4: Key experimental data from selected *in vivo* animal exposure studies using new technology exhaust.

Concentration of Diesel Exhaust (µg/m3)	Source	8 Hour TWA (µg/m^3)	Exposure Period	Animal	Engine Classification	Health Impacts in New Technology Exhaust Exposures
12	Bemis, Torous et al. 2015	12	16 h/day, 5 days/week, 2 years.	Rat	US EPA 2007	No increase in micronucleation in blood samples
12	Hallberg, Ward et al. 2015	12	16 h/day, 5 days/week, 2 years.	Rat	US EPA 2007	No DNA damage in either serum or lung tissue, no increase in serum oxidative stress markers
12	McDonald, Doyle-Eisele et al. 2015	12	16 h/day, 5 days/week, 2 years.	Rat	US EPA 2007	No tumour development and mild negative histopathological change in the lungs (periacinar epithelial hyperplasia, bronchiolization, accumulation of macrophages, and periacinar interstitial fibrosis. Associated with the gas components of the exhaust)
12	Conklin and Kong 2015	12	16 h/day, 5 days/week, 2 years.	Rat	US EPA 2007	Mild inflammatory and cardiovascular effects in female rats (increased serum levels of IL-6 and sICAM-1 and decreased total non-high-density-lipoprotein cholesterol)
<100	Douki, Corbière et al. 2018	<37.5	3 Hours, 5 days/week, 3 weeks	Rat	EURO IV	Limited accumulation of lung DNA damage and effects of gene expression (limited induction of *γ-H2AX* and acrolein adducts and 171 genes dysregulated in comparison to air controls. Greater effects in exposures that used a DPF in comparison to exposures that did not)
170	Magnusson, Dziendzikowska et al. 2019	127.5	6 h/day, 7 days or 6 h/day, 5 day/week, 4 weeks	Rat	EURO V	Minor histopathological changes and oxidative stress in lungs (focal mild emphysema and mild mononuclear infiltrate in the lungs, disrupted redox signalling pathways, redox pathways more disrupted with the use of a DPF than without)
182	Valand, Magnusson et al. 2018	136.5	6 h/day, 7 days or 6 h/day, 5 day/week, 4 weeks	Rat	EURO V	Minor oxidative stress in brain (genes associated with oxidative stress and inflammation differentially expressed after DE exposure). No histopathological changes in frontal cortex or hippocampus. No differences in comparison to exposures without a DPF.

*= NS - Not Specified.

Table 1.0.5: Key experimental data from selected *in vitro* exposure studies using old technology exhaust. All studies human airway epithelial cells and use air-liquid interface cultures.

Concentration of Diesel Exhaust µg/m^3)	Source	8 Hour TWA (µg/m^3)	Exposure Period (Minutes)	Cohort Demographic	Engine Classification	Old Technology Exhaust
140	Zarcone, Duistermaat et al. 2016	17.5-109.38	60-375	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS	Decreased permeability (higher TEER measurement compared to air controls ($p<0.01$))
430	Zarcone, Duistermaat et al. 2016	53.75-335.93	60-375	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS	Increased oxidative stress and permeability (increased NQO1 mRNA expression and lower TEER measurement compared to air controls ($p<0.05$))
470	Okubo, Hosaka et al. 2015	19.58-117.5	20-120	Alveolar basal epithelial cell line A549	NS	Suppressed immune response and increased oxidative stress (decreased release of IL-8, *HO-1* mRNA expression levels increased ($p<0.001$))
850	Hawley, L'Orange et al. 2014	8.86-106.29	5-60	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS	Increased oxidative stress and increased cellular responses to diesel pollutants (PAHs) (increased mRNA expression of *HO-1* and *CYP1A1* ($p<0.01$)). No loss of viability
1200	Zarcone, Duistermaat et al. 2016	150-937.5	60-375	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS	Increased inflammation, cell death, permeability and oxidative stress (increased *IL-8*, *CHOP*, *GADD34*, *HMOX1* and *NQO1* mRNA expression, increased LDH release, decreased TEER measurement ($p<0.05$))
1300	Kooter, Alblas et al. 2013	975	90	Alveolar basal epithelial cell line A549	EURO III	Increased cell death and increased oxidative stress (decreased Alamar Blue concentration, decreased GSH/GSSG ratio and increased HO-1 levels)
1600	Okubo, Hosaka et al. 2015	66.7-400	20-120	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS	Inhibited cell proliferation and increased oxidative stress (*HO1* mRNA and protein expression levels increased ($p<0.001$))

*= NS - Not Specified.

Table 1.0.6: Key experimental data from selected *in vitro* exposure studies using new technology exhaust. All studies human airway epithelial cells and use air-liquid interface cultures.

Concentration of Diesel Exhaust ($\mu g/m^3$)	Source	8 Hour TWA ($\mu g/m^3$)	Exposure Period (minutes)	Cohort Demographic	Engine Classification	New Technology Exhaust
34	Zarcone, Duistermaat et al. 2018	25.5	360	Mucociliary differentiated primary bronchial epithelial cells obtained from both normal and COPD patients	EURO V	No effect on oxidative stress levels
35	Hawley, L'Orange et al. 2014	0.37-4.41	5-60	Mucociliary differentiated primary bronchial epithelial cells obtained from normal volunteers	NS*	Increased oxidative stress and increased cellular responses to diesel pollutants (PAHs) (increased mRNA expression of *HO-1* and *CYP1A1* ($p<0.01$)). No loss of viability
82	Zarcone, Duistermaat et al. 2018	61.5	360	Mucociliary differentiated primary bronchial epithelial cells obtained from both normal and COPD patients	EURO V	Increased oxidative stress (increased mRNA expression of *HMOX1* and *NQO1* 90 minutes post exposure ($p<0.01$))
206	Zarcone, Duistermaat et al. 2018	64.37-154.5	150- 360	Mucociliary differentiated primary bronchial epithelial cells obtained from both normal and COPD patients	EURO V	Increased oxidative stress and decreased defence response to infection in COPD derived cells (increased mRNA expression of *HMOX1* and *NQO1* 90 minutes post exposure ($p<0.01$), increased IL-8 mRNA expression and decreased BiP mRNA expression in NTHI infected COPD derived cells after DE exposure in comparison to air exposed controls)
1500	Zarcone, van Schadewijk et al. 2017	187.5	60	Mucociliary differentiated primary bronchial epithelial cells obtained from both normal and COPD patients	TIER 4	Increased oxidative stress and decreased defence response to infection (increased mRNA expression of *HMOX-1*, *CHOP*, *GADD34* and *IL-8* and decreased NTHI-induced mRNA expression of *BiP* and *S100A7* ($p<0.05$))

*= NS - Not Specified.

1.0.8 Occupational Exposure Limits and their Applicability

The Australian Institute of Occupational Hygienists recommends a DE occupational exposure limit of 100 µg/m^3 as a time weighted average over 8 hours, measured as elemental carbon (AIOH, 2017). In America the DE exposure limit in a non-coal mining setting was set in 2008 at 160 µg/m^3 total carbon (MSHA 2016). However, there is no particle mass exposure limit set for non-mining settings (OSHA 2013). As of 2019, the European Union have introduced occupational DE exposure limits of 50 µg/m^3 EC, to be put into effect in 2023 in non-mining settings and 2026 in a mining setting (EU 2004). Previous DE exposure health effect reviews have recommended an occupational exposure limit of 100 µg/m^3 of diesel PM in total, which is equivalent to approximately 75 µg/m^3 EC (Taxell & Santonen, 2017). Using the acute human studies reviewed in this report as the basis for the cross comparison, this limit is accurate for reducing the health effects of short term exposure in healthy workers. However, this limit fails to take the safety and comfort of workers with asthma or allergy into account and is far above occupational exhaust concentrations where studies found significantly increased lung cancer risk. Previously published reviews recommended the lower occupational exposure threshold of 50 µg/m^3 of respirable EC (approximately 67 µg/m^3 of PM) in order to reduce lung cancer risk (Möhner & Wendt, 2017). This current review, based on the acute human exposure studies and the occupational exposure studies, suggests a limit below 50 µg/m^3 of PM, approximately 35 µg/m^3 EC, would be more suitable. This level is below the exposure concentrations where effects were observed among asthmatics and below the concentrations that found the higher lung cancer risks (an increase of 38 (19-97, 95% confidence interval) lung cancer deaths per 1000 male workers (Peters et al., 2017) and a significant increase in DNA damage and dysregulation of micro RNA's, some of which were associated with carcinogenesis (Rynning et al. 2019)). In addition, this limit is supported by *in vivo* exposure studies, where exposure concentrations at 50 µg/m^3 only resulted in mild health effects.

However, it should be noted that exposure limits based on both the mass of EC, as well as the mass of total PM, are limited in their long-term applicability. In order to meet the suggested 50 µg/m^3 PM occupational limit, most if not all diesel equipment must be fitted with exhaust after-treatment devices, including a DPF. Diesel particulate filters remove particles from the exhaust, however they preferentially select for EC above other particle types (Hawley et al., 2014; Khalek et al., 2011) skewing the exhaust output and eliminating EC as a predictive measure for overall exhaust exposure, making any occupational limits based on EC unreliable.

Occupational limits based on particle mass have their own drawbacks. Evidence is accumulating that it is particle size and particle number that contribute more towards health impact than total particle mass (Cauda, Ku, Miller, & Barone, 2012; Hawley et al., 2014; Ramachandran, Paulsen, Watts, & Kittelson, 2005), making occupational limits based on mass, without accounting for particle size and number, a questionable decision. The latest European Emission Standards take this into account and have set limits for both particle mass and particle number (EU-Commission, 2011).

In addition, multiple studies published in the last decade are reporting little to no change in health impacts after the use of a DPF. In *in vivo* and *in vitro* exhaust exposure studies that compare exposure health effects before and after the use of a DPF, few to no decreases in

health impact are found (Douki et al., 2018; Gioda et al., 2016; Hawley et al., 2014; Karthikeyan et al., 2013; Magnusson et al., 2019; Okubo et al., 2015; Steiner, Czerwinski, Comte, Müller, et al., 2013; Valand et al., 2018) with only a few adverse cardiovascular events, including thrombosis and vasoconstriction, being decreased or prevented in an acute human exposure study (Lucking et al., 2011). Diesel particulate filters remove more than 90% by mass of particles from the exhaust (Hawley et al., 2014; Lucking et al., 2011; Magnusson et al., 2019; Valand et al., 2018). However, they cannot be 100% efficient given pressure drop constraints of the system, therefore some particles (generally in the smaller size ranges) will pass through the DPF. Also at the operating temperatures of a DPF, many particles (such as PAHs) are liquid and can migrate through the filter and be resuspended (ChemSpider; Hawley et al., 2014; Khalek et al., 2011). Indeed PAH can melt as low as 80^{o}C and boil as low as 200^{o}C, both of which are well below typical exhaust temperatures (ChemSpider). The addition of after treatment devices, such as a DOC, may even generate additional nitro-PAH's (Carrara & Niessner, 2011; Inomata, Fushimi, Sato, Fujitani, & Yamada, 2015). This suggests that either the exhaust gases are having a greater effect on health than previously thought or that ultrafine particles, and the toxic chemicals potentially adsorbed to their surface, are responsible for the majority of health impacts caused by diesel PM (Douki et al., 2018; Hawley et al., 2014; Karthikeyan et al., 2013; Tanaka, Takano, et al., 2013). Thus, using occupational limits based on particle mass, an exhaust exposure that was over the limit where negative health consequences occur would read as under with the use of a DPF, and yet the DPF would have little to no impact on decreasing the health impacts on an exposed worker.

Limits on particle number should also be addressed. Studies have found NO_x to be a reliable indicator of DE exposure, as long as the majority of sources contributing to the NO_x concentrations are diesel engines (Hedmer et al., 2017; Taxell & Santonen, 2017). Equipment that measure NO_x concentrations are also less expensive than the equipment needed for EC measurement (Hedmer et al., 2017) and thus an additional occupational limit based on NO_x should not prove to be an expensive burden on industry. However, a more thorough review on the health effects of NO_x and its applicability as a DE exposure predictive measurement should be conducted before any sort of limit is put into effect. In future, more research needs to be conducted on the health effects of exposure to new technology diesel engine exhaust and further occupational studies need to be based on the possible health outcomes of the increasing application of new technology engines in industry.

1.0.9 Limitations: The majority of literature was sourced from PubMed using strict search criteria and thus it is possible that relevant studies were missed. Studies were only included if they were written in English and thus relevant studies in other languages were also excluded. This review focussed on studies relevant to occupational exposure settings and thus studies that used exhaust concentrations not relevant to occupational exposure conditions were not included.

The studies included in this review use a wide variety of engine types with varying emission classifications and after-treatment devices. Details of engine specifications and settings used during the exposures are limited, if they are reported at all. This, combined with the wide range of exposure outcomes measured, makes firm conclusions difficult for setting occupational DE exposure limits. Consistency in experimental designs and strict guidelines

for reporting engine specifications and settings in DE exposure research would help immensely in solving this issue.

Many of the occupational exposure and acute human exposure studies also use exclusively male subjects and more research needs to be done to verify that occupational DE exposure has similar health impacts in both men and women. The few animal studies that compare both sexes often show differences in response between males and females (Coburn et al. 2018; McDonald et al. 2015; Conklin et al. 2015). There is insufficient evidence to assess whether these differences also exist in humans. In addition, very few studies exist that exposed human, animal or tissue to "new technology" exhaust and thus further research is needed to confirm the findings of this review. Future studies in DE exposure effects should concentrate on using newer technology engines and after-treatment devices in order to consolidate the health effects of exposure to "new technology" engine exhaust before it becomes more widely used in an occupational setting.

1.0.10 Conclusion:

In conclusion, an occupational exposure limit of 100 $\mu g/m^3$ is too high as it does not take increased lung cancer risk caused by high levels of DE exposure into effect. A limit of 50 $\mu g/m^3$ is more appropriate if lung cancer risk and the effects of exposure on workers with asthma, allergy and respiratory disease are accounted for. An occupational exposure limit based on EC is not appropriate as after-treatment devices preferentially remove it from the exhaust, making it an unreliable indicator of exhaust exposure. After-treatment devices also make occupational limits based on particle mass unreliable at best and additional limits, such as ones based on particle number or NO_x concentrations, are needed in order for occupational exhaust exposures to be reliably monitored.

Declarations:

Funding: This review was supported by the Department of Mines, Industry Regulation and Safety (Western Australia), Curtin University and the Telethon Kids Institute.

Conflicts of interest: The authors of this paper have no conflicts of interest to declare.

Availability of data and material: All data and citations used within this review are available online.

Code availability: Not applicable.

Authors' contributions: KRL reviewed and wrote the majority of this manuscript with ANL, AR and BJM editing and providing expertise and advice on how to convey and interpret the results of the reviewed articles.

Chapter 1.1: Biodiesel Exposure Studies Summary

1.1.1 What is Biodiesel?

Biodiesel is a renewable fuel created through the transesterification of organic fats and oils (Gerhard Knothe, de Castro, & Razon, 2015). Transesterification is the process whereby the organic group of an ester (in this case triglycerides from organic fats or oils) is replaced with the organic group of an alcohol (usually methanol), using a base such as sodium hydroxide as a catalyst. This generates glycerol and Fatty Acid Methyl Esters (FAME), otherwise known as biodiesel (Figure 1.1.1). This mixture is then washed with water to remove the glycerol and any unreacted alcohol and left to separate. After separation, the top layer of FAME is extracted and ready to be used as a fuel whereas the bottom layers, a combination of glycerol, water and unreacted alcohol, are either thrown out or processed for glycerol extraction (Beer, Grant, & Campbell, 2007).

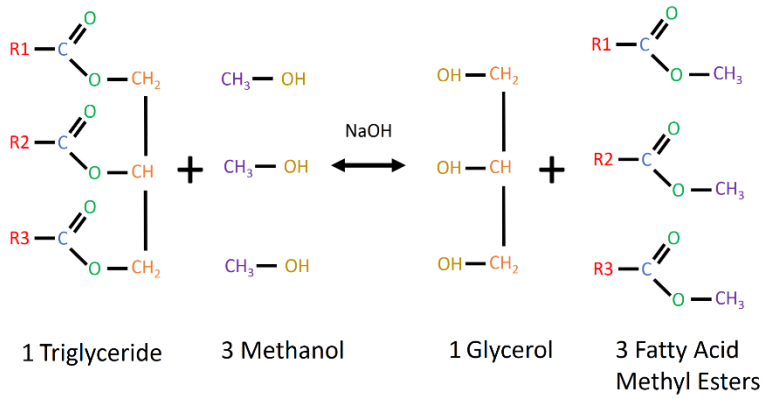


Figure 1.1.1: An example of a general biodiesel transesterification reaction. The fat/oil (triglyceride) from the base feedstock is mixed with methanol using a base such as sodium hydroxide as a catalyst to create glycerol and fatty acid methyl esters, otherwise known as biodiesel.

Biodiesel can be used to directly replace commercial mineral diesel within many engines, including those currently on-road (Fontaras et al., 2009). Usage has been increasing steadily worldwide and has increased 50 fold since 2000, from under 1000 million litres to over 46000 million litres (EIA, 2020a) (Figure 1.1.2). Biodiesel is generally added to mineral diesel as the sulfur extraction process required to turn crude mineral diesel into ultra-low-sulfur diesel greatly reduces the lubricity of the fuel, necessitating the use of additives to improve it back to usable standards. Biodiesel is one of those additives, with this being a key reason why many countries allow up to a certain percentage (usually between 5-7%) of biodiesel to be added to commercial mineral diesel without labelling (EU, 2016; ASTM, 2020a; Price, 2019). Additionally, due to climate change concerns and the push towards the use of “greener” fuel alternatives, some countries are now increasing the mandated blend up to 12% (Barros, 2020), and others allow 20% blends of biodiesel in commercial diesel to be sold with appropriate labelling (EERE, 2020; ASTM, 2020b). Public transport that uses pure biodiesel fuel is also on trial in various places worldwide, such as Norway (Biodiesel International, 2019).

One of the advantages of biodiesel is that it can be made from almost any fat and oil, hereby termed feedstocks. This includes common feedstocks such as those used for cooking (canola oil, soy oil, palm oil, etc.), feedstocks from non-edible oils (such as algal oil and jatropha oil) and feedstocks commonly generated as waste products including used cooking oils from vats and friers and waste animal fats (Beer et al., 2007; Hannon, Gimpel, Tran, Rasala, &

Mayfield, 2010; Møller, Scholten, Roursgaard, & Krais, 2020; Silitonga, Hassan, Ong, & Kusumo, 2017). The type of oil used to make the biodiesel changes from country to country. For example, rapeseed is amongst the most common in Europe whereas in the USA it is soybean (Møller et al., 2020). Worldwide the most common feedstocks are soy, rapeseed (canola), animal fats, waste cooking oil, palm, cottonseed and corn (Eea, 2013; EIA, 2020a, EIA, 2020b; OECD/FAO, 2020). In Australia, the most common feedstock types are canola, tallow, palm and waste cooking oil (ARENA, 2018; Beer et al., 2007).

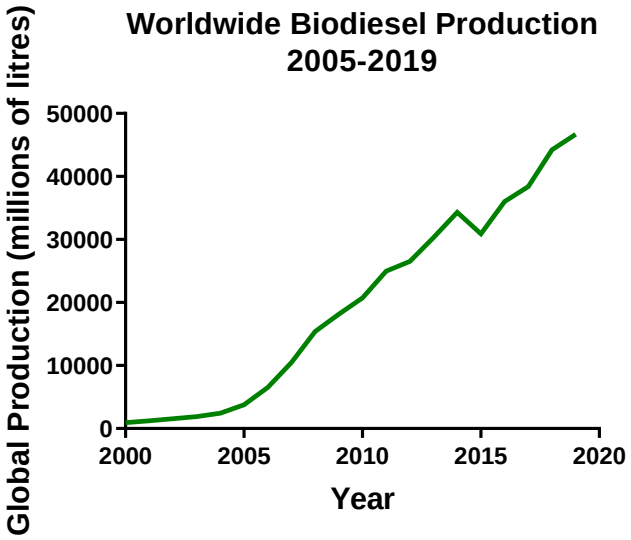


Figure 1.1.2: Global biodiesel production since 2000. Adapted from the US Energy Information Administration international energy statistics (EIA, 2020a): https://www.eia.gov/international/overview/world

1.1.2 Fuel Characteristics:

The fuel characteristics of biodiesel change significantly depending on the type and purity of oil used to create it (Graboski, McCormick, Alleman, & Herring, 2003; Omidvarborna, Kumar, & Kim, 2016). These include flash point (lowest temperature at which vapours ignite with an ignition source), viscosity, oxygen content, cetane number (a measure of the fuel ignition delay with higher values meaning a more complete combustion) and iodine number (a measure of fatty acid bond saturation with higher numbers indicating more unsaturated bonds). In general, biodiesels tend to have higher flashpoints, viscosity, cetane number and iodine numbers than commercial mineral diesel (Graboski et al., 2003). For example, soy biodiesel has a flash point of 167°C, a viscosity of 4.55mm^2/s at 40°C, oxygen content of 11.16% by weight, a cetane number of 59, and an iodine number of 133 (Graboski et al., 2003). In comparison, tallow biodiesel had a flashpoint of 173°C, a viscosity of 4.91mm^2/s at 40°C, oxygen content of 11.74% by weight, a cetane number of 64.8 and an iodine number of 64 (Graboski et al., 2003). Thus, soy biodiesel ignites at a lower temperature, is less viscous, has a lower oxygen content, a higher ignition delay and contains more unsaturated fatty acid bonds than tallow biodiesel. Australian ultra-low sulfur diesel has a flashpoint of 79°C, a viscosity of 3.05mm^2/s at 40°C, and a cetane number of ~49 (SHELL, 2018). Thus, it ignites at a much lower temperature, is less viscous and has a higher ignition delay than both soy and tallow biodiesel. These differences alter the combustion of the different fuels, ultimately altering the properties of the exhaust generated (Fontaras et al., 2009; Graboski et al., 2003;

G. Knothe & Steidley, 2005; McCormick, Graboski, Alleman, Herring, & Tyson, 2001; Omidvarborna et al., 2016).

Biodiesel and mineral diesel also differ in terms of contaminants present due to the different extraction processes involved in their production. The base feedstocks used to create biodiesel are also exposed to organic contaminants during oil extraction, such as protein, cell debris and chemicals such as pesticides. Mineral diesel extracted from underground has a high sulfur content and thus undergoes a series of refinement procedures to reduce the sufur levels, exposing the fuel to a variety of potential chemical contaminants (Gao, Guo, Xing, Zhao, & Liu, 2010; He, Van Gerpen, & Thompson, 2010; Sheehan, Camobreco, Duffield, Graboski, & Shapouri, 1998). This, combined with the different fuel characteristics, can greatly alter the concentrations of various exhaust components.

1.1.3 Exhaust Components:

Due to the diesel fuel combustion process,

Fuel + Oxygen → Carbon Dioxide + Water,

combusting diesel and biodiesel results in broadly similar exhaust physico-chemical outputs (Chapter 1.1). Since combustion is not always fully completed, and fuels contain contaminates, exhaust outputs can include thousands of different chemical species (J. Bünger et al., 2000; Fontaras et al., 2009; Hemmingsen et al., 2011; Hesterberg et al., 2011; George Karavalakis et al., 2009; Khalek et al., 2011; Kisin et al., 2013), although the more common types include carbon monoxide, nitrous oxides (NO_x), sulfur oxides, particulate matter (PM), volatile organic compounds, polycyclic aromatic hydrocarbons (PAHs) and carbonyl compounds (including aldehydes and ketones) (Fontaras et al., 2009). The PM is composed of mostly solid elemental carbon particles ranging in size from ultrafine particles (<100 nm in size) to PM10 (<10 µM in size), with many of the other potentially toxic compounds mentioned above adhered/adsorbed to the surface of those particles (Carrara & Niessner, 2011; Fontaras et al., 2009; Hu et al., 2013; Prokopowicz et al., 2015; Riley et al., 2018).

Previous studies have generally found biodiesel exhaust to contain more NOx, less PM (by weight) and more ultrafine particles (by number) than mineral diesel, as well as more PAHs which has concerning implications for increased exhaust toxicity (Fontaras et al., 2009; Gioda et al., 2016; Graver, Frey, & Hu, 2016; Mullins et al., 2016; Westphal et al., 2013). The increased NO_x and decreased PM is attributed as an effect of the increased oxygen content within biodiesel generating a more complete combustion. As PM is a product of incomplete combustion (i.e. the fuel is not fully combusted into CO_2 and H_2O) a more complete combustion caused by a greater availability of oxygen would instead mean more exhaust gases are generated. Greater availability of oxygen for reactions would also mean greater amounts of NO_x can be produced. This inverse relationship between NO_x and particulate matter emissions has also been observed outside of biodiesel, when oxygenates were added to mineral diesel fuel (Liotta & Montalvo, 1993; Nikanjam, 1993).

Conversely, unburned fuel is also of great concern when running an engine on biodiesel, especially since diesel engines have been greatly optimised for mineral diesel (J. Bünger et al., 2000). Despite combustion being more complete (due to biodiesels higher flashpoint temperature, and thus higher combustion temperature) a greater proportion of unburnt biodiesel fuel can pass through into the exhaust. This unburnt fuel undergoes pyrolysis in the high

exhaust temperatures generated by combustion, causing complex reactions and generating PAHs as well as other potentially toxic chemicals such as aldehydes and ketones (V. André et al., 2015; G. Karavalakis, Bakeas, Fontaras, & Stournas, 2011; Ravindra, Sokhi, & Van Grieken, 2008; Rhead & Hardy, 2003). This chemical profile is unique to the type of biodiesel combusted as the kinetics of pyrolysis change with fuel type (Chien, Lu, Chai, & Boreo, 2009; Omidvarborna et al., 2016).

1.1.4 Health Effects:

Despite the potentially more harmful health impacts of biodiesel exhaust exposure, biodiesel only has limited studies on health effects with the majority of studies in the field only looking into the impact of the fuel on the engine or improving the efficiency of transesterification (Ghogare, Chen, & Xiong, 2020; Larcombe et al., 2015; Madden, 2016; Møller et al., 2020; K. J. Swanson, Madden, & Ghio, 2007) and is generally used in blends with mineral diesel (EERE, 2020; F. Li, Liu, Ni, & Wang, 2019; Peng, 2017), the potential health impacts of biodiesel exhaust exposure are largely unknown. As both biodiesel and diesel share many of the same exhaust physico-chemical characteristics, it is likely that biodiesel exhaust exposure will result in many of the same general health effects as mineral diesel exhaust exposure, although their severity in comparison to mineral diesel is largely unknown.

Studies using exposure models to compare exhaust health impacts between mineral diesel and biodiesel suffer from methodological limitations that make firm conclusions difficult to make (Larcombe et al., 2015; Møller et al., 2020). They are often contradictory (Møller et al., 2020), with some stating biodiesel is more cytotoxic and immunogenic than diesel (Brito et al., 2010; Mullins et al., 2016), while others state that diesel is more cytotoxic and mutagenic than biodiesel (Hemmingsen et al., 2011; Mutlu et al., 2015). When they are comparatively assessed, studies generally show that blended fuels (i.e. biodiesel mixed with mineral diesel) are worse than pure fuels in terms of oxidative stress, cytotoxicity and mutagenic effects (Ackland, Zou, Freestone, Van De Waasenburg, & Michalczyk, 2007; Adenuga, Wright, & Atkinson, 2016) or that there are no differences between fuels in terms of genotoxic effects (Cervena et al., 2017). Previous studies have also found that extended exposure to diesel exhaust or 20% biodiesel blends caused no noticeable impacts on the health effects measured (Magnusson et al., 2017). Despite exhaust gases having a known impact on health (V. André et al., 2015), the majority of biodiesel exhaust exposure studies assess only the particle phase using bacterial mutagenicity assays, immortalised cell lines (some of which are not even human or derived from respiratory tissue (J. Bünger et al., 2000; Jalava et al., 2012; Mutlu et al., 2015; Westphal et al., 2013)) or animal models instilled/aspirated with exhaust particle extracts (Bendtsen et al., 2020; Yanamala et al., 2013). Bacterial mutagenicity studies potentially exaggerate mutagenic effects (Szikriszt et al., 2016), immortalised cell lines are limited in how accurately they can mimic human tissue and negate genetic variability (Kicic, Sutanto, Stevens, Knight, & Stick, 2006) and installation/aspiration use in mouse models does not mimic accurate exposure routes (Larcombe et al., 2014). In addition, the particles for these studies are often collected on filters despite particulate matter from both exhausts agglomerating to from larger sizes when collected this way (Morin et al., 2008). This generates an artificial particle spectrum, removing ultrafine particles from the exhaust and skewing the resulting health effects as ultrafine particles are epidemiologically linked to greater health impacts compared to other particle sizes (Breitner et al., 2011; Oberdörster et al., 1995). A more appropriate model of exposure would be to use respiratory tissue derived

from multiple volunteers or animal models and expose them to whole exhaust in order to more appropriately model the particle spectra of exhaust.

A clear understanding of the health effects of biodiesel exhaust exposure is also made difficult in that previous studies treat biodiesels made from different feedstocks as one overarching fuel, to the point that they do not always mention the exact type of feedstock used to make their biodiesel (Ackland et al., 2007; Hawley et al., 2014). The type of feedstock clearly impacts biodiesel fuel properties and hence exhaust characteristics (Graboski et al., 2003), which in turn will alter the resulting health impacts. Despite this, the majority of existing studies only test biodiesel made from one feedstock type (Larcombe et al., 2015; Møller et al., 2020). In addition, pure biodiesel fuel (i.e. biodiesel that is not blended with mineral diesel) is not always tested with some studies only testing the blended fuels (Barraud et al., 2017; Magnusson et al., 2019). This provides added "real-world" relevance as commercial biodiesels are typically sold as blends, but ignores the effects of exposure to unblended fuels, which are also commonly used, and potentially masks the differences between feedstocks as the biodiesel content in the tested blends are rarely greater than the mineral diesel content. This, combined with the wide variety of methods used for testing (Table 1.1.1), in which different engine types (Brito et al., 2010; Valand et al., 2018), engine settings (e.g. steady state *vs* drive cycle (V. André et al., 2015; Hawley et al., 2014)), presence/absence and type of exhaust after-treatment technology (Skuland et al., 2017; Unosson et al., 2021), diesel reference fuel (with sulfur levels varying between <500 ppm and <10 ppm (Brito et al., 2010; Mullins et al., 2016)) and exposure models (e.g. bacterial mutagenic assays, cell line exposures, animal model exposures and human exposures) for the health effects testing (Mullins et al., 2016; Mutlu et al., 2015; Unosson et al., 2021; Valand et al., 2018) are used, makes comparing outcomes from previously studies difficult.

Despite this, I have collated previous studies in this field by conducting a broad literature search in the PubMed database in late June 2021. I used the terms "biodiesel exhaust" and "health", as well as relevant citations within. From the 97 results obtained, titles and abstracts were examined for studies using diesel as a reference fuel, using whole exhaust (i.e. not just collected particulate matter) and directly assessing health impacts instead of just extrapolating from engine tests. All articles were written in English. The 15 studies that met these criteria were a mixture of cell line, animal and human exposure models, which were then analysed further for key methodological aspects (e.g. type of engine and engine settings, type of biodiesel, exhaust physico-chemical characteristics) and health effect outcomes (Table 1.1.1).

1.1.5 Literature Review Results:

Of the 15 studies that met the inclusion criteria, four were *in vitro* airway epithelial cell exposure studies, ten used animal models and one subjected human volunteers to biodiesel exhaust. PM concentrations varied greatly between studies, with the lowest being 10 $\mu g/m^3$ and the highest 24, 000 $\mu g/m^3$, which is greater than a 2000-fold difference. Engine configurations changed between different studies, however seven of the 15 studies used old technology engines (no exhaust after-treatment devices), seven compared results of exhaust exposure with and without a diesel particulate filter (DPF) and/or diesel oxidation catalyst (DOC) and one study used a new technology engine equipped with both a DPF and a DOC. Although not always overtly stated, I assumed that engines that met EURO 4 or above

compliance were fitted with both after-treatment devices. The majority of studies (13 of 15) stated the type of biodiesel used and three of the 15 used diesel reference fuels that contained more sulfur than ultra-low sulfur diesel levels (ULSD, <16 ppm). Of particular interest is that of all 11 studies that measured NO_x, only two found it to be higher in biodiesel and biodiesel blends compared with mineral diesel, despite previous studies on engine exhaust parameters showing that biodiesel generates more NO_x than diesel (Fontaras et al., 2009; Graver et al., 2016).

A total of nine studies used 100% biodiesel fuel for at least one of the exposures, the remainder using solely blends between 7% and 30%. For the studies using 100% biodiesel, four reported more harmful health effects after biodiesel exhaust exposure compared with after diesel exhaust exposure, four had the same and one was less harmful. Of note, the blend was found to be less harmful than both pure fuels in two of those studies. For the studies than only used blended fuel, three found biodiesel blends to have more harmful health outcomes than diesel, one found them to be the same and two found the blends to be less harmful. Overall, 47% of the studies found biodiesel to be more toxic than diesel, 33% found no significant differences in health impacts and 20% found biodiesel to be less toxic.

Almost three-quarters of the studies used biodiesel fuels made from either rapeseed (6/15) or soy (5/15) feedstocks, with 14 of the studies using methyl esters and one using ethyl esters. The remaining fuels were generated from hydro-treated vegetable oil (3/15), sewerage (1/15) and/or used unstated biodiesel types (3/15). Of the 7 studies that used rapeseed biodiesel, two reported more harmful health outcomes than diesel, three were the same as diesel and two reported less harmful outcomes. A similar variety of outcomes were reported for soy biodiesel exhaust exposure (three of five studies identified more harmful health outcomes for biodiesel, one found no significant differences between biodiesel and diesel exhaust exposure and one found soy biodiesel exhaust to be less harmful than diesel).

Overall, the literature suggests that feedstock plays a role in biodiesel exhaust toxicity. More than twice as many studies (60%) found that soy biodiesel exhaust was more toxic than mineral diesel exhaust, compared with 29% of studies on rapeseed biodiesel. However, this should be taken cautiously as the pool of studies is so small that definite conclusions cannot be drawn and there are enough methodological differences in both engine set-up and methods used to test for health effects that directly comparing between the different studies is inadvisable. It should also be noted that since rapeseed and soy dominated the biodiesel types in the studies and yet are only two of the many types of biodiesel used worldwide (Eea, 2013; EIA, 2020b; OECD/FAO, 2020), conclusions as to the possible toxicity of other biodiesel types cannot be made. Thus, a study that directly compares multiple biodiesel types beyond just soy and rapeseed in order to explore the role of feedstock type on exhaust toxicity and potentially to find the least toxic feedstock type, keeping methodologies and engine parameters as consistent as possible so direct comparisons can be made between fuels, is urgently needed.

Table 1.1.1: Results from a literature review using the PubMed database and the words "Biodiesel Exhaust" and "Health" as well as relevant citations within.

Study	Exposure Model Type	Engine Used	Engine Settings	Biodiesel Used	Highest Biodiesel Concentration PM	More NOx than Diesel?	More PM (mass) than Diesel?	Health effects tested	Biodiesel Worse than Diesel?
Hawley et al., 2014	Differentiated primary human airway epithelial cells grown at ALI	JD 4045H PowerTech Plus (with and without DPF)	Constant: 2400 rpm, 75% load	100% BD ULSD	+DPF=235.6 $\mu g/m^3$ -DPF=21.7 $\mu g/m^3$	No	No	Viability, Oxidative stress, PAH response	Same for all outcomes, DPF had no effect/slightly increased health effects
V. André et al., 2015	AMES Bacterial mutagenicity assay, cell lines exposed at ALI (human airway epithelial A549), rat lung slices	Euro 3 Standard (with and without DOC and DPF)	Artemis Cycle (Urban Section)	30% RME 7% RME ULSD	Not stated, dilution ratios used	Not stated	Not stated	Mutagenicity in bacterial assay, DNA damage in eukaryote cells	Worse, more mutagenic at higher blends but similar DNA damage observed, mutagenicity still observed after DPF
Mullins et al., 2016	Submerged Cell Line Culture (Human Airway Epithelial Cell Lines NuLi-1 and 10KT)	Light-medium duty diesel engine (Isuzu 4BD1-T, 3.9L) with DOC and DPF	Constant: 1800 rpm, 20% load	100% RME, 20% RME, ULSD	~10 $\mu g/m^3$	No	Yes	Inflammation (IL-6, IL-8 and RANTES), Cell viability	Worse for both blend and pure fuel
Barraud et al., 2017	Cell lines exposed at ALI (human airway epithelial A549)	Euro 3 Standard (with and without DOC and DPF)	Artemis Cycle (Urban Section)	30% RME 7% RME ULSD	Not stated, ratio dilutions used	Not stated	Not stated	Cell viability, Oxidative stress, DNA damage	Better, same for viability and oxidative stress, better in terms of DNA damage
Brito et al., 2010	Animal (mice)	BD-2500 CFE; Branco)	Not stated	100% SEE, 50% SEE, 500 ppm Sulfur diesel	550 $\mu g/m^3$	Not measured	Not applicable- fuels diluted to PM concentration	Changes to heart rate and blood pressure, Inflammation in BAL, serum and bone marrow	Worse, more inflammation and greater cardiac effects in both blend and pure fuel
Gavett et al., 2015	Animal (normal and sensitised mice)	0.32 L Yanmar engine driving a 3.8kW Pramac generator	Constant load of 3 kW RPM not stated	100% SME, 20% SME, ULSD	50 $\mu g/m^3$, 150 $\mu g/m^3$, 500 $\mu g/m^3$	No	Not applicable- fuels diluted to PM concentration	Methacholine response, Inflammation in BAL and serum	Better for both blend and pure fuel, less inflammatory effects and no changes to methacholine response for all fuels

de Brito et al., 2018	Animal (mice)	DE electrical generator (BD-2500 CFE; China)	Not stated	100% Sewage methyl esters, 50 ppm Sulfur diesel (5% biodiesel)	579 µg/m^3, 1139 µg/m^3	Yes	Not applicable- fuels diluted to PM concentration	Changes to heart rate and blood pressure, Inflammation in BAL, serum and bone marrow, Lung histology and immunohistochemistry	Same overall effect, some outcomes higher in diesel than biodiesel and vice versa. No consistent pattern.
Farraj et al., 2015	Animal (normal and hypertensive rats)	Yanmar L70 diesel engine and Pramac E3750 generator	Constant load 5.8 hp (4.3 kW), 3600 RPM	100% SME 20% SME ULSD	50 µg/m^3, 150 µg/m^3, 500 µg/m^3	No	Not applicable- fuels diluted to PM concentration	Heart rate variability and electrocardiogram, Inflammation in BAL and serum assessed	Blend is better than both pure fuels for cardiac impact and inflammation, pure biodiesel slightly worse than diesel in terms of cardiac effects, blood cholesterol alterations and systemic inflammation
Hazari et al., 2015	Animal (hypertensive rats)	Yanmar L70 diesel engine and Pramac E3750 generator	Constant load 5.8 hp (4.3 kW), 3600 RPM	100% SME 20% SME ULSD	50 µg/m^3, 150 µg/m^3, 500 µg/m^3	No	Not applicable- fuels diluted to PM concentration	Arrhythmia assessed	Blend is better than both pure fuels, pure biodiesel induced slightly greater effects than diesel
Bass et al., 2015	Animal (normal and hypertensive rats)	Yanmar L70 diesel engine and Pramac E3750 generator	Constant load 5.8 hp (4.3 kW), 3600 RPM	100% SME, 20% SME, ULSD	50 µg/m^3, 150 µg/m^3, 500 µg/m^3	No	Not applicable- fuels diluted to PM concentration	Glucose tolerance, Inflammation in BAL, Blood count, Lung histology, Thoracic aorta gene transcription	Same, better in terms of lung effects, worse in terms of cardiac with the blend middling for both
Douki et al., 2018	Animal (rats)	Euro4-compliant supercharged common rail direct injection diesel Engine (with and without DPF)	NEDC drive cycle	30% RME, ULSD	- DPF: 24 mg/m^3, + DPF: <100 µg/m^3	Yes	Yes	Lung genotoxicity, Telomerase activity in lungs, Gene expression analysis on genes associated with DNA repair and cell cycle regulation, Oxidative stress, DNA damage	Better, less genotoxic and less genes related do DNA damage and cell cycle regulation affected, exhaust after DPF more toxic than exhaust before
Valand et al., 2018	Animal (rats)	Fiat Panda 1.3 JDT (2014) with a Euro 5 engine (with and without DPF)	Chassis dynamometer used, settings not stated	7% RME, 20% RME, 13% HVO + 7% RME	RME: - DPF: 2.02 mg/m^3, + DPF: 182 µg/m^3 HVO: - DPF: 2.13 mg/m^3, + DPF: 194 µg/m^3	No	No	Brain histology, Gene expression changes in brain	Same, no differences observed between groups, DPF filtration only had a minor effect despite drastic drop in PM amounts

Dziendzikowska et al., 2018	Animal (rats)	Fiat Panda 1.3 JDT (2014) with a Euro 5 engine (with and without DPF)	Not stated	7% BD 7% BD + 13% HVO	- DPF: 2.13 mg/m^3, + DPF: 190 $\mu g/m^3$	No	No	Changes to blood profile and inflammation, DNA damage	Worse, more red blood cells, immune dysregulation and increased DNA damage, usage of DPF increased toxic health effects
Magnusson et al., 2019	Animal (rats)	Fiat Panda 1.3 JDT (2014) with a Euro 5 engine (with and without DPF)	Static conditions	7% BD 7% BD + 13% HVO	- DPF: 2 mg/m^3, + DPF: 190 $\mu g/m^3$	Not stated	No	Inflammation in BAL, Lung histology, Lung gene expression, Lung DNA damage	Slightly worse, greater oxidative stress, DPF caused greater gene dysregulation
Unosson et al., 2021	Human Volunteers (Exposure Chamber)	Volvo TD40 GJE, 4.0 L, 4 cylinders	Urban European Transient Cycle	100% RME, 30% RME, LSD	300 $\mu g/m^3$	Yes	Yes	Thrombus formation, Vascular endothelial function, Biomarkers of inflammation, platelet activation and fibrinolysis were measured in the blood	Same, no differences observed between exposures

BD= Biodiesel
DOC= Diesel oxidation Catalyst
DPF=Diesel particulate filter
HVO= Hydro-treated vegetable oil
PM= Particulate matter
RME= Rapeseed methyl esters
SME=Soy methyl ester
ULSD= Ultra-low sulfur diesel

Chapter 2: Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells.

*Katherine R. Landwehr[1,2], Jessica Hillas[2], Ryan Mead-Hunter[1], Rebecca A. O'Leary[3], Anthony Kicic[1,2,4,5,6], Benjamin J. Mullins[1], Alexander N. Larcombe[1,2], on behalf of AusREC[7,8,9], WAERP[1]

[1]Occupation, Environment and Safety, School of Public Health, Curtin University, PO Box U1987, Perth, WA, 6845

[2]Respiratory Environmental Health, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, WA, 6009

[3]Department of Primary Industries and Regional Development, Perth, WA, 6151.

[4]Department of Respiratory and Sleep Medicine, Perth Children's Hospital, Nedlands, Perth, WA, 6009.

[5]School of Biomedical Sciences, The University of Western Australia, Perth, WA, 6009.

[6]Centre for Cell Therapy and Regenerative Medicine, The University of Western Australia, Perth, WA, 6009.

[7]Telethon Kids Institute, Centre for Health Research, The University of Western Australia, Nedlands, Western Australia, Australia, 6009.

[8]Priority Research Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, Newcastle, New South Wales, Australia, 2305.

[9]Robinson Research Institute, University of Adelaide, North Adelaide, South Australia, Australia, 5006.

Key Words: Biodiesel, Soy Biodiesel, Diesel, Exhaust, Exhaust Exposure, Exhaust Gas Analysis, Exhaust Particle Analysis, Health, Airway Epithelial Cell, Cellular Viability, Inflammation, Cytokine Release,

Published in Environmental Science and Technology: Reprinted (adapted) with permission from Landwehr, K. R., Hillas, J., Mead-Hunter, R., O'Leary, R. A., Kicic, A., Mullins, B. J., & Larcombe, A. N. (2019). Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells. Environmental *Science & Technology, 53*(19), 11437-11446. doi:10.1021/acs.est.9b01671 Copyright 2019 American Chemical Society.

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.

2.1 INTRODUCTION:

Since limited battery storage capability decreases the feasibility of electrical engines in long distance transport and goods shipping (Amjad, Neelakrishnan, & Rudramoorthy, 2010), and the inefficiency of natural gas storage limits natural gas engine capabilities in long distance haulage (Camuzeaux, Alvarez, Brooks, Browne, & Sterner, 2015), 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 (J. Bünger et al., 2000), biodiesel is gaining popularity as a renewable, sustainable fuel due to its ability to directly replace diesel fuel in many engines (Fontaras et al., 2009). However, as biodiesel usage is predicted to increase worldwide (OECD/FAO, 2015; EIA, 2020a), concerns have been raised over the health impact of exposure to its exhaust emissions (Larcombe et al., 2015).

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 (J. Bünger et al., 2000; Fontaras et al., 2009; Graver et al., 2016; Mullins et al., 2016). 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 (Fontaras et al., 2009; Graver et al., 2016). Few compare the health effects to exhaust exposure (Larcombe et al., 2015; Madden, 2016; K. J. Swanson et al., 2007). Those that do primarily use the Ames mutagenic assay (Jürgen Bünger et al., 2000; Mutlu et al., 2015) or immortalized cell lines (Cervena et al., 2017; Mullins et al., 2016) 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 (V. André et al., 2015; Larcombe et al., 2015). Particle concentrations are also rarely relevant to real world exposure levels, often being far too concentrated to simulate a realistic dosage (V. André et al., 2015). In addition, in *in vitro* based studies, the cell lines used are not always human, or even derived from respiratory tissue (J. Bünger et al., 2000; Jalava et al., 2012). 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 (Kicic et al., 2006).

As exhaust is typically inhaled, health complications can occur in the respiratory (Benbrahim-Tallaa et al., 2012; Larcombe et al., 2014), circulatory (Mills et al., 2007) and immune systems (Nejad et al., 2015). Of concern, inhalation of ultrafine exhaust particles has

been correlated with exacerbation of childhood asthma (Evans et al., 2014), and associations between air pollution from major roads and decreased lung function in children have been identified (Gauderman et al., 2015; Gauderman et al., 2007). 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 (Ginsberg, Perkovich Foos, & Firestone, 2005), meaning that over the same period of time, they are exposed to a larger dosage of exhaust than adults (Ginsberg et al., 2005; Saadeh & Klaunig, 2014). 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 (Z. Chen, Salam, Eckel, Breton, & Gilliland, 2015; Gauderman et al., 2007; Svanes et al., 2004). 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.

2.2 MATERIALS AND METHODS:

2.2.1 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 (Gerhard Knothe et al., 2015), 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.

2.2.2 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.

2.2.3 Sampling & tissue culture: Airway epithelial cells were derived via trans-laryngeal, non bronchoscopic brushing of the tracheal mucosa of children through an endotracheal tube as previously described (Kicic et al., 2006; Lane, Burgess, Kicic, Knight, & Stick, 2005). Primary cell cultures were established as previously described (Martinovich et al., 2017), and grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. Cells were passaged weekly and used before passage 6 in all experiments. Prior to exposure, 35 mm diameter cell culture dishes (Eppendorf, Hamburg, Germany) were seeded with 500 000 cells, 4 dishes per patient per exposure group, and maintained as submerged cultures using Basal Epithelial Basal Media (BEBM® ; LONZA, Switzerland) supplemented with growth additives (SingleQuots™ Supplement Pack; LONZA, Switzerland) (now termed BEGM). Twenty four hours prior to experimentation, cells were placed in starvation media, consisting

of BEGM minus epithelial growth factor. Volunteers were age and gender matched and split into 2 groups (n=6) whereupon the cells were exposed to either the control or the exhausts. After exposure, supernatants were collected and stored at -80°C for cytokine analysis. Cells were collected and stained with Annexin-V (BD Biosciences, CA, USA) before being analyzed with flow cytometry.

2.2.4 Exposure Methodology: (See supplementary materials, Figure S2.1, for Exposure Diagram) All exposures used exhaust generated from a Yanmar L100V engine (Yanmar, Italy). The engine is a single cylinder, 435cc design coupled with a dynamometer and fitted with Euro V/VI after treatment equipment consisting of an oxidation catalyst and diesel particulate filter (Daimler, Germany). The engine was run at a constant load of 40% and speed of 2000rpm. Exhaust was diluted 1 in 10 with HEPA filtered air inside a dilution/mixing chamber attached to the engine exhaust pipe and then extracted at a rate of 10L per minute through an isokinetic sampling point, leading to a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) containing the cells. The incubator was kept at 36-37°C and exhaust was injected into each cell containing dish via a manifold arrangement. The exhaust then passed through a baffleplate before extraction at 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. Diluting the exhaust 1:10 reflects real world environmental exposure levels of particulate matter (W.H.O., 2006). and produces similar concentrations of both gas and particulate matter pollution compared to recently published studies (Magnusson et al., 2019; Valand et al., 2018; M. C. Zarcone et al., 2018).

2.2.5 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.

2.2.6 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(Amanatidis, Ntziachristos, Giechaskiel, Bergmann, & Samaras, 2014).

2.2.7 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, 100 000 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 (Mullins et al., 2016). Exhaust exposures were normalized to controls before any statistical analysis on viability occurred.

2.2.8 Mediators: Using supernatant, cytokine release was analyzed in duplicate 24 hours after exposure using a BioRad 27plex human cytokine kit following the provided protocol

(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.

2.2.9 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. All statistical analyses were completed using R statistical software (v3.4.3) (R Team, 2018) and p-values less than 0.05 were considered significant. Gas measurements were analyzed using general additive model (GAM) methodologies, using the R package "mgcv". A separate GAM was fitted to each gas measurement, in which the gas measurement was the response variable and time the predictor. Thus allowing for non-parametric fits between gas measurement and time. All other statistical analyses were performed using multivariate linear regression methodology, applying backward elimination approach to remove insignificant predictive variables. Particle concentration was analyzed as total particle number concentration using fuel and time as the predictive variables.

2.3 RESULTS:

2.3.1 Gas Analysis: Combustion gas measurements were taken every 10 minutes over a period of 4 hours (Figure 2.1). 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 2.1a-c and 2.1e) and B20 contained significantly higher NO ($p<0.01$; Figure 2.1d). 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 2.1c and 2.1e), whereas B20 combustion produced greater amounts of NO ($p<0.001$; Figure 2.1d).

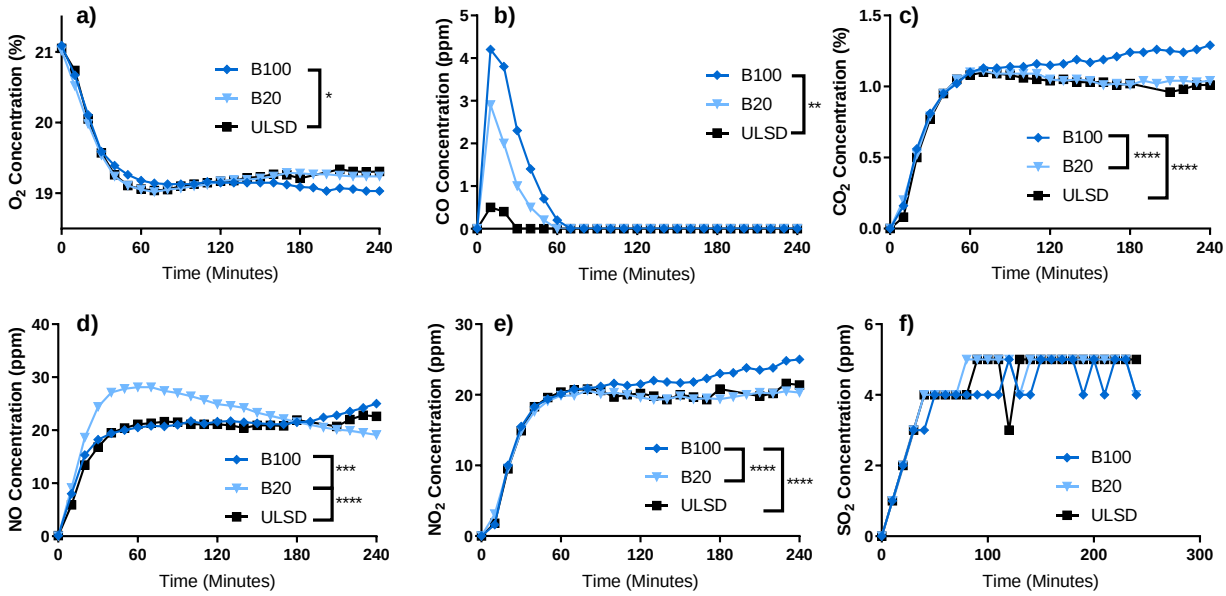


Figure 2.1: Combustion gas analysis from the diluted exhaust of the three different fuels types: a) oxygen concentration, b) carbon monoxide concentration, c) carbon dioxide concentration, d) nitrogen monoxide concentration, e) nitrogen dioxide concentration and f) sulfur dioxide concentration. Measurements were taken every 10 minutes for 4 hours (*=p value<0.05, **=p value<0.01, ***=p value<0.01, ****=p value<0.001). Figure 2.1a) and 2.1c) concentration measurements as a percentage, all other figures show concentration in parts per million (ppm).

2.3.2 Particle Analysis: An average fine particle size spectrum was obtained for each exhaust at each timepoint (Figure 2.2). 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 with the B100 peak being the largest. There was no peak 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 2.1). 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.

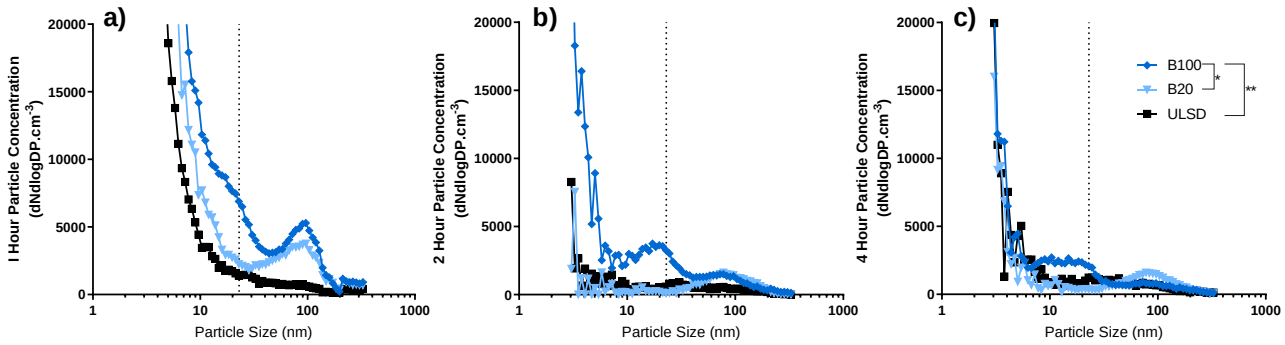


Figure 2.2: Particle size spectra for all three fuels (*=p value<0.05, **=p value<0.01) for the a) 1 hour, b) 2 hour and c) 4 hour timepoints. Data was analyzed using total particle number concentration values for each fuel and timepoint. The dotted line indicates the particle size of 23 nm. Within fuels, 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 ULSD exhaust.

Table 2.1: 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 ($\mu g/m^3$)	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 ($particles/cm^3$)	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 ($particles/cm^3$)	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 ($particles/cm^3$)	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.

2.3.3 Cellular Viability: The B100 exhaust demonstrated the highest toxicity with significantly lower viability compared with the B20 and ULSD exposures (Figure 2.3, 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 2.3b, $p<0.001$), 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 ($p<0.05$).

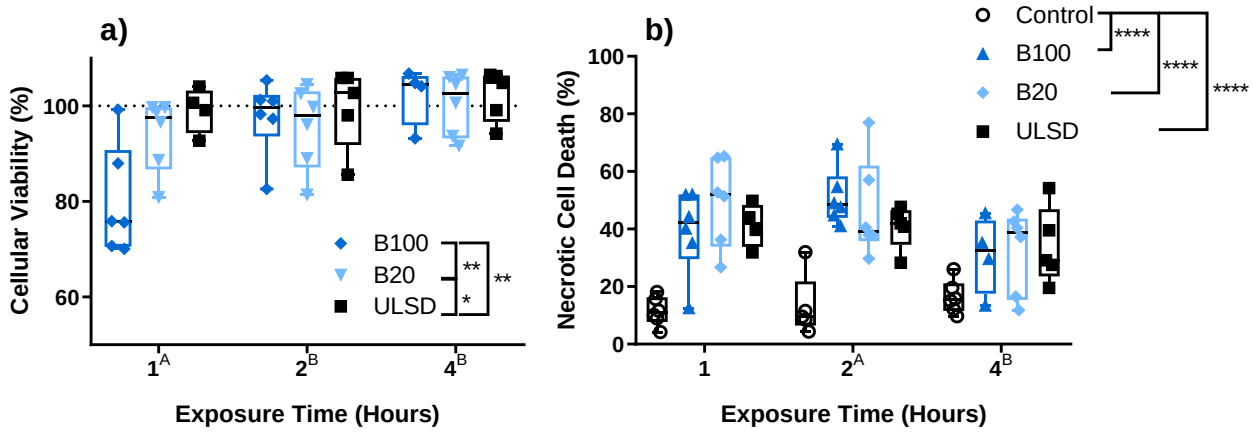


Figure 2.3: a) Cell viability measurements 24 hours after exposure using Annexin V staining. All results are normalized to control measurements (dotted line). The mean viability measurements for the 1, 2 and 4 hour timepoints respectively are: 79.9±11.5%, 97.7±7.9% and 102.2±6.1% for B100, 94.1±7.7%, 95.6±8.9% and 100.5±6.4% for B20 and 99.1±4.8%, 99.6±8.5% and 102.9±5.4% for ULS,. 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) $p<0.05$). Boxplots indicate spread of data and median value is marked by the horizontal line inside the box.

2.3.4 Cytokine Release: Of the 27 cytokines tested, only 11 were released at measurable concentrations; MIP-1β, IL-1β, IL-1RA, IL-6, IL-8, VEGF, G-CSF, GM-CSF, TNF-α, IP-10 and RANTES, which have been displayed in graphical form (Figures 2.4a-k respectively). 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).

We performed univariate linear regression on the effect of individual particle and gas components on viability, necrotic activity and cytokine release. The results of these analyses can be found in Supplementary Materials, Tables S2.1-2. All biological outcomes are significantly associated with and thus likely impacted by a complex mixture of both gaseous and particulate matter components.

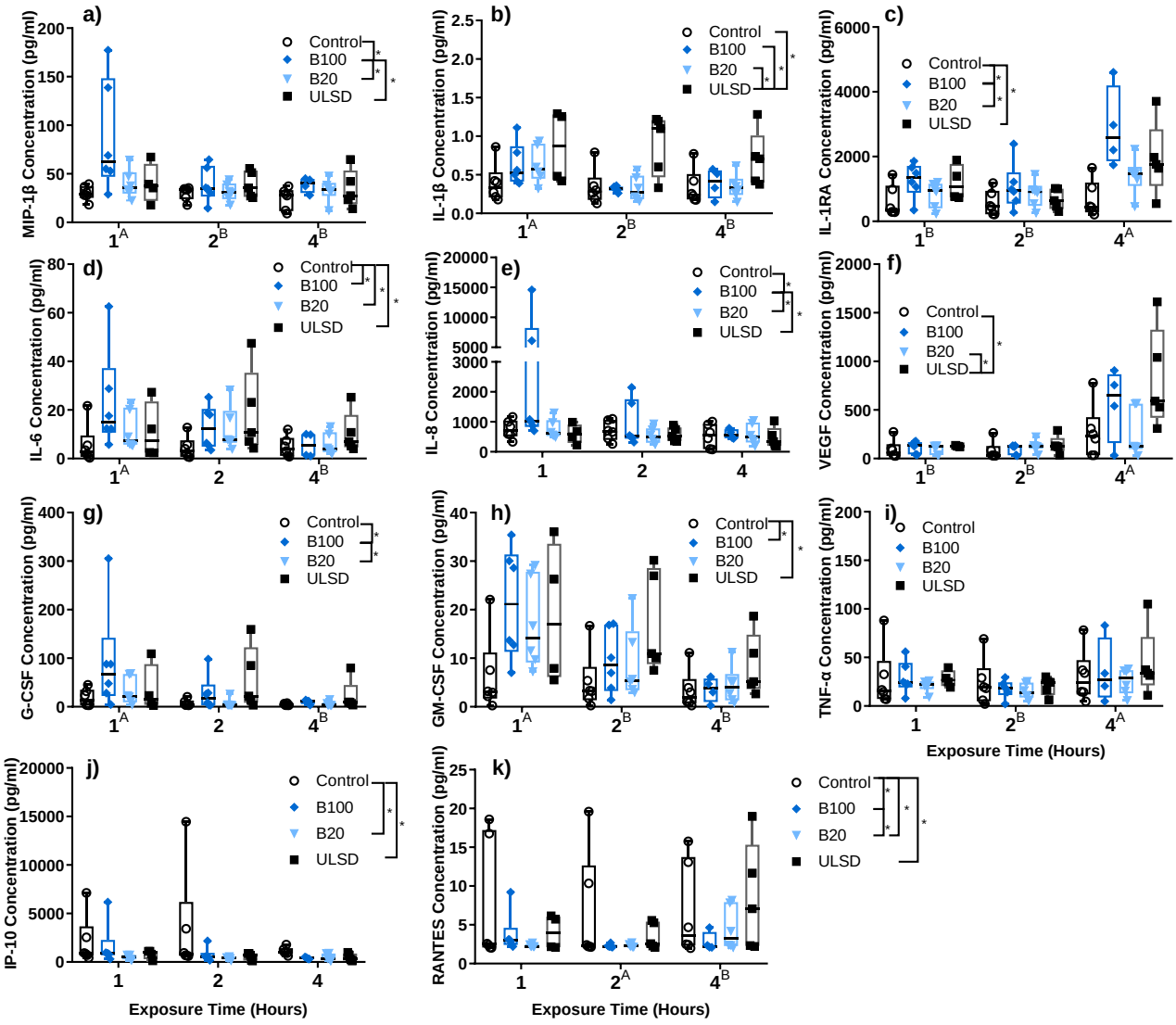


Figure 2.4: Measured cytokine release for all fuels and times for 11 cytokines released above limit of detection. 4a-k) in order: Mip-1β, IL-1β, IL-1RA, IL-6, IL-8, VEGF, G-CSF, GM-CSF, TNF-α, IP-10 and RANTES. A significant difference in the release between fuels is

indicated on the legend of each graph (*=p value<0.05). On the x-axis of each graph, a superscript of A indicates significant increase to a superscript of B between timepoints ($p<0.05$). Boxplots indicate spread of data and median value is marked by the horizontal line inside the box.

2.4 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 associated with the exposure effects of a complex mixture of both gaseous and particulate matter components, displaying why it is vital that exhaust exposure studies use whole exhaust when assessing potential exposure health effects.

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 (Mullins et al., 2016). The results of previous studies comparing the gaseous outputs of the two exhausts vary considerably (Fontaras et al., 2009; Gioda et al., 2016; Graver et al., 2016; Prokopowicz et al., 2015). The majority have found that biodiesel exhaust contains more NO_x as well as a decrease in the average size of the particulate matter (Fontaras et al., 2009; Larcombe et al., 2015; Mullins et al., 2016)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 (T.-M. Chen, Kuschner, Gokhale, & Shofer, 2007). Nitrogen monoxide readily oxidizes into nitrogen dioxide at atmospheric conditions (Chameides et al., 1992) and nitrogen dioxide interacts with moisture to form nitric acid (T.-M. Chen et al., 2007). In addition, NO_x is a major contributor to photochemical smog and ozone pollution (Chameides et al., 1992). Even at environmental levels, NO_x exposure is implicated with increased health risks, including ischemic stroke and cardiovascular disease(T.-M. Chen et al., 2007; Zhu et al., 2012). In children, daily exposure to NO_2 is associated with increased asthmatic symptoms (T.-M. Chen et al., 2007; Smith et al., 2000), 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 (Jaffe, 1968) 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 (Weaver 2009). 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 (J. G. Allen et al., 2018) and negative effects on bone formation and blood pressure have been observed at 12000 ppm (Drummer et al., 1998; Elliott, Prisk, Schollmann, & Hoffmann, 1998). 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 adsorbed to their surface (D. B. Kittelson, 1998); including polyaromatic hydrocarbons, aldehydes, ketones and heavy metals (Gioda et al., 2016; Prokopowicz et al., 2015). Despite the fact that the elemental carbon particles within the particulate matter itself are relatively inert, inhalation causes respiratory irritation (Larcombe et al., 2014) and the inhalation of ultrafine particulate matter is associated with worsening health outcomes including pulmonary inflammation (Oberdörster et al., 1995), exacerbation of existing respiratory diseases (Seaton et al., 1995), 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 (Brook et al., 2010; Goodson et al., 2017). Asthma sufferers are particularly susceptible to adverse effects from inhalation, and exacerbation of childhood asthma has been associated with ultrafine particulate pollution (Evans et al., 2014).

In addition, inhalation of the particulate matter allows the toxic chemicals adsorbed on particles to deposit within the lungs and cardiovascular system. Previous studies have found that biodiesel exhaust contains a greater amount of toxic chemicals and thus exposure has a greater chance for lung deposition, and any subsequent health impacts, to occur (Fontaras et al., 2009; Prokopowicz et al., 2015). Smaller particles have an increased surface to volume ratio, meaning that a greater amount of harmful substances can adsorb to the surface for a given mass (Mullins et al., 2016; Yoza et al., 2002). 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 (Oberdörster et al., 1995). 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 (Amanatidis et al., 2014).

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 (Øvrevik, Refsnes, Låg, Holme, & Schwarze, 2015). Previous studies using adult human airway epithelial cell lines have found similar results, with exposure to diesel exhaust inducing necrotic cell death (Totlandsdal, Cassee, Schwarze, Refsnes, & Låg, 2010). 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 (Larcombe et al., 2015; Mullins et al., 2016; Skuland et al., 2017; Kimberly J Swanson et al., 2009). 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 (Barnes, 2008; Hiraiwa & van Eeden, 2013), while exposure to soy biodiesel exhaust caused significant release of IL-8 and G-CSF, which are associated with neutrophilic inflammation (Barnes, 2008; Xu, Höglund, Håkansson, & Venge, 2000). 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 (Barnes, 2008; Hiraiwa & van Eeden, 2013; Larcombe et al., 2014; Menten, Wuyts, & Van Damme, 2002; Steiner et al., 2016; Xu et al., 2000).

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 (Fisher, Hyde, Baue, Reif, & Kelly, 1969; Ghio et al., 2008), 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 (de Brito et al., 2018; Seriani et al., 2015). 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, cause injury to the cells and deposit the toxic chemicals adsorbed to the surface. As exposure time increases, more and larger particles are added to the media. As diesel particulate matter is known to agglomerate readily in liquid (Larcombe et al., 2014), 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.

2.5 Conclusion

In our study, soy-based biodiesel exhaust contained more, smaller and more toxic 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.

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

AUTHOR INFORMATION

Author Contributions

KRL, JH, AK, BJM and ANL performed the exposures. BJM and RM-H created the fuels. 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 advised on statistical analyses. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

We would like to acknowledge and thank Professor Gerhard Knothe from the USDA Agricultural Research Service for providing input on the creation of the biodiesel fuel. We would like to acknowledge the Australian Research Council for funding via the Discovery Project Grant DP170104346 as well as Curtin University's School of Public Health and 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.

Chapter 3: Fuel feedstock determines biodiesel exhaust toxicity in a human airway epithelial cell exposure model.

*Katherine R. Landwehr[1,2], Jessica Hillas[2], Ryan Mead-Hunter[1], Peter Brooks[3], Andrew King[4], Rebecca A. O'Leary[5], Anthony Kicic[1,2,6,7], Benjamin J. Mullins[1], Alexander N. Larcombe[1,2], on behalf of AusREC[8,9,10], WAERP[8,11]

[1]*Occupation, Environment and Safety, School of Population Health, Curtin University, PO Box U1987, Perth, 6845, Western Australia, Australia*

[2]*Respiratory Environmental Health, Wal-yan Respiratory Research Centre, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia*

[3] *School of Science, Technology and Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia*

[4]*Fluid Dynamics Research Group, School of Civil and Mechanical Engineering, Curtin University, Perth, Western Australia, Australia*

[5]*Department of Primary Industries and Regional Development, Perth, 6151, Western Australia, Australia*

[6]*Department of Respiratory and Sleep Medicine, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia*

[7]*Centre for Cell Therapy and Regenerative Medicine, The University of Western Australia, Perth, 6009, Western Australia, Australia*

[8]*Telethon Kids Institute, Centre for Health Research, The University of Western Australia, Nedlands, Perth, 6009, Western Australia, Australia*

[9]*Priority Research Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, Newcastle, 2305, New South Wales, Australia*

[10]*Robinson Research Institute, University of Adelaide, North Adelaide, 5006, South Australia, Australia*

[11]*St. John of God Hospital, Subiaco, Perth, 6008, Western Australia, Australia*

Corresponding Author*:

Email: katherine.landwehr@telethonkids.org.au

Post: PO Box 855, West Perth, Western Australia, 6872

Published in Journal of Hazardous Materials: with permission

Abstract:

Background: Biodiesel is promoted as a sustainable replacement for commercial diesel. Biodiesel fuel and exhaust properties change depending on the base feedstock oil/fat used during

creation. The aims of this study were, for the first time, to compare the exhaust exposure health impacts of a wide range of biodiesels made from different feedstocks and relate these effects with the corresponding exhaust characteristics.

Method: Primary airway epithelial cells were exposed to diluted exhaust from an engine running on conventional diesel and biodiesel made from Soy, Canola, Waste Cooking Oil, Tallow, Palm and Cottonseed. Exhaust properties and cellular viability and mediator release were analysed post exposure.

Results: The exhaust physico-chemistry of Tallow biodiesel was the most different to diesel as well as the most toxic, with exposure resulting in significantly decreased cellular viability (95.8±6.5%) and increased release of several immune mediators including IL-6 (+223.11±368.83 pg/mL) and IL-8 (+1516.17±2908.79 pg/mL) above Air controls. In contrast Canola biodiesel was the least toxic with exposure only increasing TNF-α (4.91±8.61).

Conclusion: This study, which investigated the toxic effects for the largest range of biodiesels, shows that exposure to different exhausts results in a spectrum of toxic effects *in vitro* when combusted under identical conditions.

Keywords: Biodiesel, Exhaust Exposure, Health, *in Vitro* Exposure Model, Vehicle Emissions

3.1 Introduction:

Due to the current climate crisis and the increasing difficulty and expense of fossil fuel extraction, there is growing pressure to find a cleaner, renewable replacement for commercial mineral diesel fuel. Biodiesel, created through the transesterification of lipids into fatty acid methyl esters (Gerhard Knothe et al., 2015), is a popular alternative since it is made from a wide variety of renewable oils and can be used as a direct replacement for diesel within many engines (Fontaras et al., 2009). Between 2005 and 2019, estimated global biodiesel production increased more than 12 fold, from under 3800 million litres to over 46000 million litres per year (EIA, 2020a). Despite this, the health effects of biodiesel exhaust (BE) exposure are still largely unknown (Larcombe et al., 2015; Madden, 2016; K. J. Swanson et al., 2007), especially when different renewable oils are used to make the biodiesel.

Previous studies comparing the physico-chemical properties of biodiesel and mineral diesel exhaust have mostly used rapeseed or soy based biodiesel (Møller et al., 2020) and generally show that BE contains more toxic gases such as nitrogen oxides (NO_x), more toxic chemicals such as carbonyls and polycyclic aromatic hydrocarbons (PAHs) and more ultrafine particles (<100 nm in size), leading to a smaller median particle size (Fontaras et al., 2009; Gioda et al., 2016; Mullins et al., 2016; Westphal et al., 2013). This is concerning as long-term exposure to NO_x is associated with lung damage, decreased lung function and increased chance of respiratory infections as well as increased risk of stroke even at atmospheric levels (T.-M. Chen et al., 2007). Further, epidemiological associations between inhaled particles and health impact are largely linked to ultrafine particles, rather than larger sizes (Breitner et al., 2011; Oberdörster et al., 1995). This is likely because smaller particles can penetrate deeper into the lungs, potentially bypassing the airway epithelial barrier to enter directly into the cardiovascular system (Brook et

al., 2010; Goodson et al., 2017). In addition, smaller particles have a larger surface area to volume ratio, allowing more toxic chemicals to adhere to the surface for a given mass (Munack et al., 2006). However, despite the potentially more harmful consequences of exposure to BE, little attention has been paid to studying the health effects of exposure (Larcombe et al., 2015). Instead, most studies in the field focus on the effects of biodiesel fuels on engine wear, fuel economy and exhaust outputs, or on the process of streamlining the creation of biodiesel (Fontaras et al., 2009; Ghogare et al., 2020; Graver et al., 2016). Few compare health effects of exhaust exposure between mineral diesel and biodiesel, and those that do generally use older technology engines with little (or no) exhaust after-treatment. This decreases the relevance of their results as newer exhaust after-treatment technologies substantially alter exhaust physico-chemistry (Brito et al., 2010; Larcombe et al., 2015; Madden, 2016; Yanamala et al., 2013).

Importantly, very few studies compare the exhaust output between different types of biodiesels (Graboski et al., 2003; Kado & Kuzmicky, 2003; G. Karavalakis et al., 2011; Omidvarborna et al., 2016) and virtually none comparatively assess exhaust exposure health consequences. This is critical, as biodiesel fuel properties and exhaust outputs change depending on the feedstock used during creation (Graboski et al., 2003). These differences include cetane number, iodine number, viscosity and flashpoint of the fuel, as well as exhaust particle concentrations and combustion gas levels (Graboski et al., 2003; Omidvarborna et al., 2016). With such variation in fuel and exhaust physico-chemical properties, the health effects of exhaust exposure are likely to vary as well (Larcombe et al., 2015). However, very little research has been conducted on BE exposure health effects and that which does exist mostly uses the AMES mutagenic assays (J. Bünger et al., 2000; Westphal et al., 2013) or immortalised cell lines (Cervena et al., 2017; Hemmingsen et al., 2011) to focus on the mutagenic and cytotoxic potential of the particulate matter (PM) exhaust components. Of significance, almost all previous studies have not considered the gaseous component of exhaust and only used the extracted PM collected on a filter (V. André et al., 2015; Larcombe et al., 2015).

Previous studies looking into the toxic consequences of BE exposure provide inconsistent results, with some studies showing mineral diesel to be more mutagenic and cytotoxic (Hemmingsen et al., 2011; Mutlu et al., 2015), others biodiesel (Brito et al., 2010; Mullins et al., 2016) and yet others show no mutagenic effects in either fuel in comparison to air exposed controls (Cervena et al., 2017). Understanding these inconsistent results is hampered by methodological differences between studies and the variety of outcomes measured. For example, exposure methods vary widely, with few studies employing whole or diluted exhaust (V. André et al., 2015; Mullins et al., 2016) and others using particles extracted from filters (Hemmingsen et al., 2011; Libalova et al., 2016; Skuland et al., 2017). Vastly different exhaust particle concentrations (ranging between tens of micrograms to several hundred micrograms for both whole exhaust and particle extracts) are also used (Brito et al., 2010; Douki et al., 2018; Libalova et al., 2016; Magnusson et al., 2019; Yanamala et al., 2013) and the exact make and specifications of the engine used to generate the exhaust are rarely stated (Larcombe et al., 2015). The use of after-treatment technology, which has a significant impact on the physico-chemical makeup of the exhaust (Khalek et al., 2011), is inconsistent (V. André et al., 2015; Gioda et al., 2016; Magnusson et al., 2019). Different health outcomes are measured including

the mutagenicity of diesel particles using the Ames assay, cytotoxicity using cell lines and lung structure and function using animal models (Gioda et al., 2016; Westphal et al., 2012; Yanamala et al., 2013). Different feedstocks including more popular choices such as Soy and Canola, and rarer such as sewerage (de Brito et al., 2018; Møller et al., 2020) are used for the biodiesel, with several studies not stating the exact type (Ackland et al., 2007; Hawley et al., 2014; Magnusson et al., 2019). Even different mineral diesel control fuels have been used depending on when and where the study was conducted with sulfur content ranging from 15 ppm (Yanamala et al., 2013) up to 500 ppm (Brito et al., 2010). In addition, the type of mineral diesel is not always specified with some studies just stating that it was diesel without going into further detail (Magnusson et al., 2019), when it has been found that higher levels of sulfur in the fuel increases toxicity (Jürgen Bünger et al., 2000). These differences within the diesel controls means they cannot always be used as a reference point between studies to compare exhaust toxicity for different biodiesel feedstock types. Combined, these variations in methodologies makes comparing toxicity of different biodiesel feedstocks between different studies difficult (Møller et al., 2020). Thus there is a need for the toxic consequences of exposure to BE from different feedstocks to be directly compared and contrasted within the same experiment, using the same engine and reference diesel fuel in order to minimise experimental variability. Our study, for the first time, achieves this by directly comparing six different biodiesel fuels with ULSD and Air controls, using the same methods and equipment.

Thus, there is an urgent need for a comparative assessment of BE exposure health impacts between different biodiesel feedstocks within the same study to ensure engine parameters as consistent as possible. To address this knowledge gap, in the current study we exposed primary airway epithelial cells to the exhaust generated by a diesel engine fuelled with either ULSD or biodiesel made from six different oils (Soy, Canola, Waste Cooking Oil (WCO), Tallow, Palm or Cottonseed). Fuel characteristics (such as fatty acid methyl ester composition) and the physico-chemical exhaust properties of the different exhausts (including combustion gas levels and particle size spectra) were recorded and 24 hours after a one hour exposure, cells were analysed for viability and the release of mediators. Exposure conditions were kept consistent between treatments with the aim of directly comparing relevant health impacts of exposure to the exhaust from different biodiesel fuels and relating these to the fuel/exhaust physico-chemical properties. To the best of our knowledge, this study is the first to compare the toxic effects of exposure to such a wide range of biodiesel exhausts (in addition to ULSD and air controls). We hypothesised that exposure to BE would cause more toxic outcomes than exposure to ULSD and that different BE exposures would cause a spectrum of health outcomes related to the physico-chemical exhaust properties.

3.2 Materials and Methods:

3.2.1. Fuel Types: Six different pure biodiesel fuels types (B100) were used in this study. Soy, Canola (rapeseed), Tallow, Palm and Cottonseed biodiesel were created using high-quality, food-grade, commercial oils (Campbells Wholesale Reseller, WA, Aus and Range Products, WA, Aus). Waste cooking oil was obtained as used cooking fryer waste from a restaurant in Perth, Western Australia. All oils were converted to fatty acid methyl esters (FAME) using an

established sodium methoxide transesterification process (Gerhard Knothe et al., 2015; Landwehr et al., 2019). Commercial ULSD was obtained from a local supplier (SHELL, WA, AUS, biodiesel free).

3.2.2. Participants: This study was approved by the St John of God Hospital Human Ethics Committee (901) and proof of approval is available on request. Airway epithelial cells were derived from trans-laryngeal, non-bronchoscopic brushings of the tracheal mucosa of children through an endotracheal tube as previously described (Kicic et al., 2006; Lane et al., 2005). With informed parent/guardian permission, brushings were obtained from eight healthy, non-atopic volunteers (2-4yrs, four males) undergoing elective surgery at St John of God Hospital 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.

3.2.3. Sampling and Tissue Culture: Primary airway epithelial cell cultures were established as previously described (Martinovich et al., 2017) and grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. Cells were passaged weekly and used before passage 6 for all experiments. Prior to exposure, cultures were maintained in Basal Epithelial Basal Media supplemented with growth additives (BEGM®; LONZA, Switzerland). Twenty-four hours prior to experimentation, media was changed to starvation media, consisting of BEGM minus the epithelial growth factor additive.

3.2.4. Exposure Methodology: All exposures used exhaust generated from a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) coupled with a dynamometer (Landwehr et al., 2019), which 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. Exhaust was diluted 1:15 with air inside a dilution/mixing chamber attached to the exhaust piping and pumped through an isokinetic sampling point (10 L/min) 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 before being passed over the cell cultures inside the incubator and then vacuumed out to be analysed for physico-chemical properties (Figure 3.1). Exposure to ULSD was used as a positive control and air exposure was used as a negative control to account for any background effect.

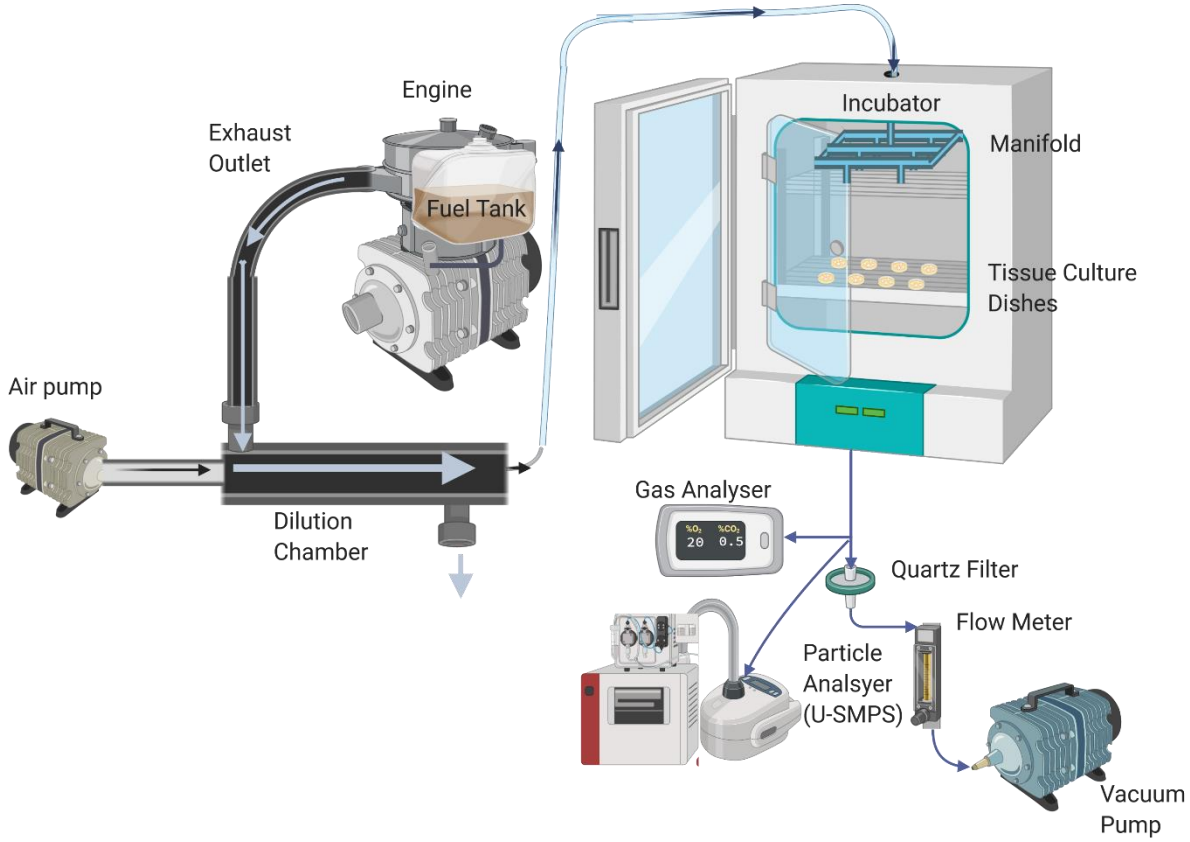


Figure 3.1: A diagram of the exposure set up. Created with Biorender.com.

3.2.5. FAME Analysis: Biodiesels were analysed for their FAME 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). Briefly, biodiesels were diluted 5µL into 1.0mL 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 analysed 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. All biodiesels were found to contain >99% FAME.

3.2.6. Gas and Particle Analyses: For each exposure, exhaust exiting the incubator was analysed every 10 minutes for quantities of common combustion gas products including of oxygen (O_2), carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (nitrogen monoxide (NO) and nitrogen dioxide (NO_2)) and sulfur dioxide (SO_2) using a combustion-gas analyser (TESTO 350, Testo, Lenzkirch, Germany). Similarly, exhaust was analysed every 10 minutes, starting 5 minutes into the exposure, for particle concentrations between the sizes of 3nm-340nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Particles less than 10nm in size were excluded from further calculations due to high variability of

measurements. Count-median particle size was then calculated using the number of particles mean. Particle mass was calculated from particle spectra, assuming sphericity and using the 40% load diesel exhaust particle density as described (Olfert, Symonds, & Collings, 2007). Particle number was either analysed as the total number of particles or separated into two fractions: particles below 23 nm in size and solid particles above 23 nm (Amanatidis et al., 2014).

3.2.7. Cell Viability: Cell viability was analysed 24 hours after exposure as previously described (Landwehr et al., 2019). Briefly, cells were suspended in 1xAnnexin staining buffer and incubated for 15 minutes with a 1/40 dilution of Annexin V, Alexa Fluor^{TM} 488 conjugate solution and 1µg/mL propidium iodide before undergoing flow cytometry analysis on a LSRFortessa flow cytometer (BD Biosciences). 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 (Filograna et al., 2015).

3.2.8. Mediators: Mediator release was analysed 24 hours after exposure using a Bio-Rad 27plx human cytokine kit (Bio-rad, CA, USA) and accompanying software (Bio-Plex Manager, v6.1.1, Bio-Rad, Tokyo, Japan). Of the 27 mediators tested, 14 were found to be released within measurable concentrations; interleukin 1-beta (IL-1 β), interleukin 1 receptor antagonist (IL-1RA), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 9 (IL-9), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-beta (MIP-1β), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), tumor necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF). Results were first normalised to protein content and then background air exposure readings were subtracted for each subject.

3.2.9. Statistical Analysis: The majority of biological data contains results for all fuels (n=8), with the exception of Cottonseed BE (n=7) and Palm BE (n=6). Data are presented as mean ± standard deviation where indicated and data for each biodiesel exposure are analysed using air and/or ULSD as the reference exposure. All statistical analyses were completed using R statistical software (V3.4.3)(R Team, 2018) using the packages “mgcv” and “lme4”. 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, thus allowing for non-parametric fits. All other statistical analyses, including total particle number and biological outcomes, were completed using multivariate general linear modelling 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.

3.3. Results:

3.3.1 Fuel Properties: All fuels showed similar fatty acid methyl ester profiles (Table 3.1). The greatest proportion of shorter chain FAME’s was found in Palm biodiesel with 46.33% of FAME containing 16 carbons in the chain. Canola and WCO biodiesel had the greatest proportions of longer chain FAME’s with 18 carbons in the chain at 97.72% and 93.85% respectively. Since

each FAME molecule contains 2 oxygen atoms, in a given mass of fuel Palm biodiesel would contain the largest number of FAME molecules and thus this would be the most oxygenated fuel and Canola biodiesel the least.

Table 3.1: Fuel properties of the various biodiesels. For FAME percentages, the name of the fatty acid is followed by the carbon chain number (C1:0) number of double bonds (C1:1). All biodiesels were found to contain greater than 99% FAME.

	Fuel					
Fuel Property	Soy	Canola	WCO	Tallow	Palm	Cotton
% Methyl Myristate (C14:0)	0.00	0.00	0.00	1.03	0.35	0.00
% Methyl Palmitate (C16:0)	9.86	2.29	6.15	24.54	46.33	21.99
% Methyl Palmitoleate (C16:1)	0.00	0.00	0.00	0.79	0.03	0.00
% Methyl Stearate (C18:0)	4.83	1.29	1.70	18.19	4.08	3.37
% Methyl Oleate (C18:1 cis)	33.0	80.63	78.27	50.20	42.12	31.73
% Methyl Octadecenoate (C18:1 trans)	0.90	2.80	2.61	3.55	0.76	0.00
% Methyl Linoleate (C18:2)	51.40	13.0	11.27	1.70	6.33	42.91
Molecular Weight (g/mol)	292.98	295.71	294.72	289.67	284.20	289.99
%Oxygen	10.93	10.83	10.86	11.07	11.28	11.05
Estimated Cetane Number	49.33	55.19	56.28	64.63	64.70	52.87
Estimated Iodine Number	111.00	102.93	97.79	57.09	52.41	96.10
% Saturated FAME	14.69	3.58	7.85	43.76	50.76	25.36
% Unsaturated FAME (1 double bond)	33.9	83.43	80.88	54.54	42.91	31.73
% Unsaturated FAME (2 double bonds)	51.4	13.00	11.27	1.70	6.33	42.91

Using our data, it is possible to estimate cetane number and iodine number using previously published models (Bamgboye & Hansen, 2008; Giakoumis & Sarakatsanis, 2018; Kyriakidis &

Katsiloulis, 2000). From the estimated fuel values, Tallow and Palm biodiesel had the highest estimated cetane number at 64.62 and 64.70 respectively and Soy biodiesel the lowest at 49.33. Soy biodiesel had the highest estimated iodine number at 111.0 and Palm biodiesel the lowest at 52.41 (Table 3.1).

3.3.2 Gas Analysis: Mean and standard deviation for each fuel and gas type are shown (Table 3.2), with the exception of CO which shows only the highest reading at the 10 minute mark due to the cold start effect on the performance of the catalytic converter.

All fuels showed similar trends in combustion gas production (Table 3.2). Within the first 20 minutes of exposure NO, NO_2, CO_2 and SO_2 increased rapidly, levelling out by ~30 minutes. The readings for O_2 show the opposite trend, decreasing rapidly for the first 20 minutes before stabilising. Carbon monoxide was highest within the first 10 minutes before the catalytic converter reached optimum working temperature, wherein CO levels decreased rapidly to being almost undetectable (Supplementary Figure S3.2). Tallow BE was found to be the most different to ULSD with significantly increased CO_2 and decreased O_2, CO and SO_2 ($p<0.05$). Soy, Canola and Cotton BE were found to be the most similar to ULSD with only two gases each being significantly different; CO and CO_2 for Soy BE, No and NO_2 for Canola BE and CO and NO for Cotton BE ($p<0.05$).

Table 3.2: Mean (standard deviation) gas measurements for all fuels. All significances displayed are compared to ULSD. Measurements are shown as the mean concentration for the entire exposure, with the exception of CO which is shown as the peak measurement found in the first 10 minutes of exposure before concentration tapers off due to the effect of cold starting on the oxidation catalyst.

	Fuel						
Gas	ULSD	Soy	Canola	WCO	Tallow	Palm	Cotton
O_2 (%)	20.38 (0.15)	20.39 (0.14)	20.37 (0.08)	20.31 ** (0.12)	20.30 *** (0.13)	20.34 (0.15)	20.36 (0.15)
CO (ppm)	3.53 (2.35)	2.07 * (1.23)	3.42 (3.08)	3.77 (3.77)	1.87 ** (1.87)	0.85 *** (0.67)	1.05 ** (1.25)
CO_2 (%)	0.34 (0.11)	0.41 *** (0.10)	0.32 (0.06)	0.38 (0.09)	0.42 *** (0.08)	0.37 (0.10)	0.36 (0.10)
NO_x (ppm)	21.94 (6.11)	20.88 (4.45)	21.43 (3.37)	22.40 (4.19)	20.97 (4.05)	19.75 (5.1)	20.25 (4.52)
NO (ppm)	15.07 (3.85)	14.33 (3.02)	13.65 * (1.57)	16.19 (2.50)	14.07 (2.51)	13.75 * (3.25)	13.63 * (2.68)
NO_2 (ppm)	6.69 (2.21)	6.54 (1.74)	7.99 ** (1.80)	6.21 * (1.70)	6.67 (1.41)	6.08 * (1.86)	6.63 (1.72)
SO_2 (ppm)	1.5 (0.3)	1.6 (0.4)	1.3 (0.3)	1.0 ** (0)	1.0 ** (0)	1.5 (0.6)	1.8 (0.4)

* Significantly different to ULSD (*=p <0.05, **=p <0.01, ***=p <0.001)

3.3.3 Particle Analysis: Particle spectra were obtained for each exhaust between the sizes of 3nm and 340nm (Figure 3.2). All biodiesels were significantly different to ULSD in terms of total particle number ($p<0.05$). All exhausts showed small peaks in particle size between 80-100 nm. This peak was largest in WCO and Canola BE, with concentrations over 1.5 fold that of

ULSD, which was the smallest alongside Palm BE. Both WCO and Canola BE were significantly different to ULSD in terms of particle number at this size range ($p<0.05$) (Table 3.3). The separation of particle sizes into above and below 23 nm in diameter was chosen based on the approximate size of the divide between solid and liquid particles within diesel exhaust (Amanatidis et al., 2014). Between the size of 20 and 35 nm, all biodiesel exhausts showed an additional, substantially larger peak in particle size that was not present in ULSD. This peak was again largest in WCO BE, with an 11 fold greater concentration compared to ULSD, and smallest in Palm BE. All exhausts had significantly increased concentrations compared to ULSD in terms of particle number at this size ($p<0.05$). Median particle size and mass and number concentrations were calculated for particles between the sizes of 10 and 340 nm for all exhausts (Table 3.3).

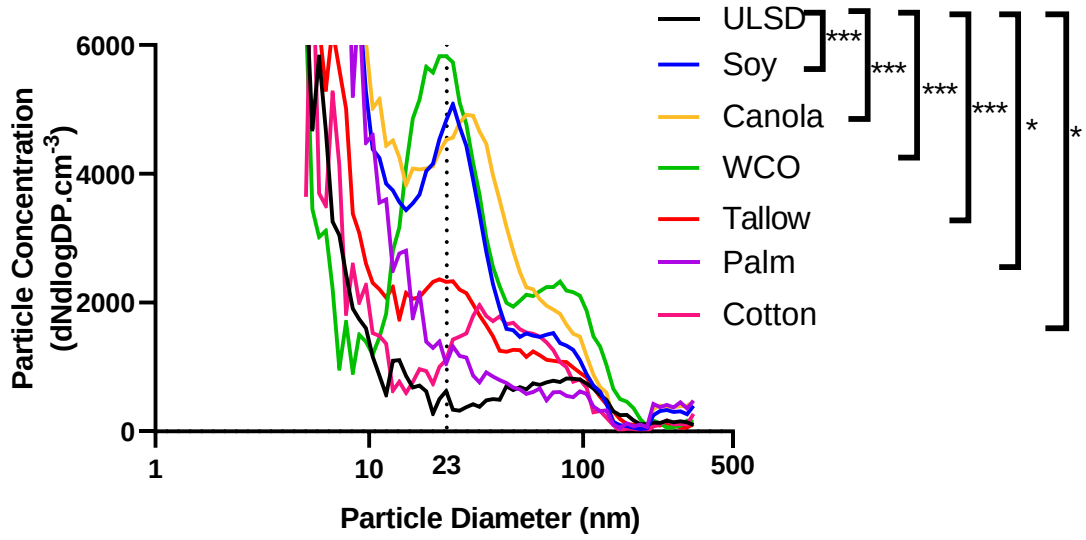


Figure 3.2: Particle size spectra for all fuels. Data were analysed using total particle number concentration between the size of 10 and 340 nm for each fuel (*=p value<0.05, **=p value<0.01, ***=p value<0.001). The dotted line indicates the particle size spectra of 23nm.

Table 3.3: Particle characteristics between the sizes of 10-340 nm for all fuels.

Particle Characteristic	Fuel						
	ULSD	Soy	Canola	WCO	Tallow	Palm	Cotton
Particle Mass Concentration (µg/m3)	12.85	22.40 (1.74)	28.53 (2.22)	19.82 (1.54)	10.69 (0.83)	24.47 (1.90)	11.69 (0.91)
Median Particle Size (nm)	44	25 (0.57)	26 (0.59)	26 (0.59)	26 (0.59)	18 (0.41)	38 (0.86)
Total Particle Number (particles/cm^3)	25993	99040 (3.81)***	120184 (4.62)***	113176 (4.35)***	58586 (2.25)***	49111 (1.89)*	43797 (1.68)*

Particle Concentration Between 80-100 nm (particles/cm^3)	3187	4593 (1.44)	5815 (1.82)*	8330 (2.61)***	3817 (1.20)	2290 (0.72)	3295 (1.03)
Particle Concentration Between 20-35 nm (particles/cm^3)	3453	33695 (9.76)***	37167 (10.76)***	38129 (11.04)***	16789 (4.86)***	8642 (2.50)*	12082 (3.50)***
Particle Number >23 nm (particles/cm^3)	16900 [65.02%]	51028 [51.52%]	67747 [56.37%]	65715 [58.06%]	32490 [55.46%]	20231 [41.19%]	31895 [72.82%]
Particle Number <23 nm (particles/cm^3)	9093 [34.98%]	48012 [48.48%]	52437 [43.63%]	47462 [41.94%]	26096 [44.54%]	28880 [58.81%]	11902 [27.18%]

a Values in round brackets represent proportional changes in comparison to ULSD.
b Values in square brackets represent the percentage of the total particle number concentration within each fuel.
* Significantly different to ULSD (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$)

3.3.4 Cellular Viability: Tallow BE was the most cytotoxic with exposure resulting in average cellular viability decreasing to 95.8±6.5% in comparison to Air ($p<0.05$) (Figure 3.3). Exposure to the remaining six exhausts did not result in either significant increase or decrease in cellular viability in comparison to Air, however exposure to ULSD exhaust resulted in significantly decreased average viability compared to Canola BE ($p<0.05$).

Shifts in cell death mechanisms in comparison to Air were observed with ULSD showing a 1.59± 2.38% increase and WCO BE a 1.16± 0.92% increase in early apoptotic cell death ($p<0.05$) (Figure 3.4a). Exposure to ULSD also caused a significant increase in the populations of early apoptotic cells in comparison to both Soy and Palm BE ($p<0.05$). Exposure to Tallow BE also resulted in a shift in cellular death mechanisms, with an increase of 2.36± 5.25% in the population of late apoptotic cells in comparison to air exposed controls ($p<0.01$) (Figure 3.4b). This was also significantly different to ULSD exposure ($p<0.05$). There was no difference in necrotic cell death mechanism in comparison to Air.

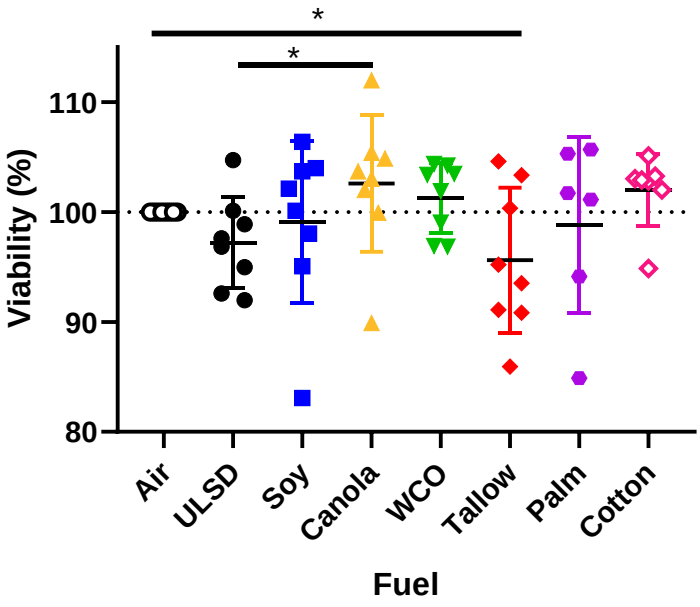


Figure 3.3: Viability measurements normalised to air controls. Mean viability measurements are: 97.0±4.7%, 99.2±7.4%, 102.7±6.2%, 101.4±3.2%, 95.8±6.5%, 102.2±3.4% and 99.0±7.9% for ULSD, Soy, Canola, WCO, Tallow, Palm and Cottonseed BE exposures respectively ($p<0.05$).

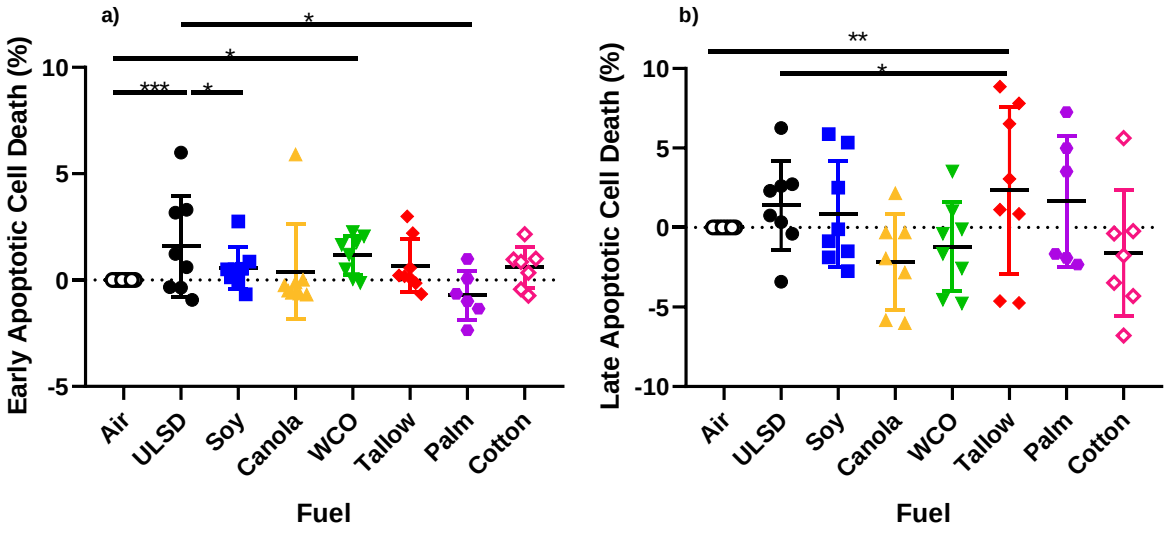


Figure 3.4: a) Percentage change in early apoptotic cell death 24 hours after exposure. b) Percentage change in late apoptotic cell death 24 hours after exposure. All cell death mechanisms were normalised by subtracting Air controls (*=$p<0.05$, **=$p<0.01$).

Table 3.4: Mean (standard deviation) mediator release for the 14 cytokines released above the limits of detection. All values have been normalised by subtracting Air controls for each individual participant. See supplementary Table S3.1 for significant differences between biodiesel fuels.

Mediator Concentration (pg/mL)	Fuel						
	ULSD	Soy	Canola	WCO	Tallow	Palm	Cotton
IL-1β	0.23 (0.65)**	0.32 (0.19)	-0.04 (0.09)##	0.04 (0.11)	-0.03 (0.09)##	0.07 (0.12)	0.07 (0.27)
IL-1RA	10.10 (42.45)	-56.77 (116.49)	82.99 (159.72)	73.61 (97.21)	81.52 (87.52)	12.42 (148.61)	131.94 (173.85)** #
IL-6	-17.04 (68.57)	81.88 (147.78)** ##	-66.18 (81.64)	-30.75 (155.94)	223.11 (368.83)*** ###	-3.06 (67.00)	-17.33 (222.79)
IL-8	255.47 (705.55)	784.43 (1467.67)*** ###	-215.15 (374.62)	-35.93 (587.63)	1516.17 (2908.79)*** ###	1014.91 (1586.81)*** ###	29.75 (622.18)
IL-9	1.98 (4.01)	3.79 (10.63)*** #	-2.71 (4.24)#	-1.78 (5.07)#	6.57 (14.24)*** ###	6.32 (9.55)***	0.62 (7.37)
G-CSF	28.81 (62.43)***	23.35 (33.59)*	-9.71 (24.66)#	7.90 (58.47)##	43.03 (69.50)***	66.16 (79.27)***	12.33 (46.12)##
GM-CSF	8.44 (14.36)**	10.94 (17.06)***	5.05 (9.49)	1.27 (1.36)#	20.23 (30.47)*** ###	14.95 (13.76)*	11.32 (16.73)**
IFN-γ	-1.95 (5.56)	1.93 (6.50)	-0.50 (7.59)	2.15 (4.29)	7.34 (16.20)*** #	5.00 (10.08)*	13.31 (11.12)*** ###
IP-10	-8.76 (200.80)	30.23 (133.54)	32.56 (195.74)	158.95 (170.03)* #	-40.69 (166.35)	16.66 (240.45)	54.66 (205.05)
MCP-1	24.88 (59.99)***	57.43 (97.49)***	2.70 (3.98)###	6.15 (10.19)* ###	66.87 (175.49)***	53.93 (121.01)*** ###	47.58 (124.80)*** ###
MIP-1β	1.23 (1.61)**	1.76 (3.19)***	-0.03 (1.06)#	0.25 (0.91)#	2.46 (4.14)*** ##	2.64 (2.36)***	0.40 (2.20)#
RANTES	-0.29 (1.03)	2.56 (4.09)** #	0.17 (2.04)	0.14 (2.37)	1.66 (2.80)* #	6.02 (6.17)*** ##	0.37 (0.75)
TNF-α	5.16 (4.33)**	6.85 (6.46)***	4.91 (8.61)*	3.51 (3.70)*	7.43 (6.78)**	7.52 (11.51)***	13.48 (10.57)*** ##
VEGF	93.64 (167.33)	7.28 (158.03)	44.07 (161.51)	51.05 (376.80)	48.85 (217.44)	43.16 (164.40)	-131.50 (246.32)* #

* Significantly different to Air (*=p <0.05, **=p <0.01, ***=p <0.001)
# Significantly different to ULSD (#=p <0.05, ##=p <0.01, ###=p <0.001)

3.3.5 Mediator Release: Of the panel of 27 mediators tested, 14 were released at levels above the limit of detection (Table 3.4). Only TNF-α was significantly increased in all exposures compared to Air ($p<0.05$ for all treatments), however both GM-CSF and MCP-1 also significantly increased in at least five of the exposures and G-CSF was significantly increased in four of the exposures. IL-6 and IL-8 were only increased in the 3 fuels with the highest

immunogenic impact; Tallow, Palm and Soy BE. In comparison to Air, Tallow BE induced the largest immune impact with the significant difference in release of ten mediators (7 innate, 3 adaptive), followed by Soy and Palm BE with nine mediators each (7 innate, 2 adaptive and 6 innate, 3 adaptive respectively). Canola BE had the smallest immune impact with a difference found in only 1 innate mediator ($p<0.05$ in all cases).

3.4 Discussion:

The results of this study show that exposure to BE elicits a range of effects on human airway epithelial cells in culture, and that the responses vary depending on the type of fuel used to generate the exhaust. Importantly, there appears to be associations between fuel chemistry/exhaust physico-chemical properties and the innate and adaptive immune responses measured *in vitro*. We found that in comparison to Air exposed controls, all fuels tested caused significant health impacts either cytotoxically and/or immunogenically. Tallow BE was the most toxic, with exposure resulting in significant reductions in cellular viability, increased late apoptotic cell death and significantly higher release of a wide range of mediators in comparison to Air. In contrast, Canola BE was found to be the least toxic, with no significant impact on cell viability or cell death mechanisms and the significantly higher release of only one innate immune mediator (TNFα) when compared with Air.

We found that Tallow BE exposure elicited the most severe response in terms of loss of cellular viability and mediator production. This was closely followed by Palm and Soy BE exposure. These impacts appear to be largely associated with fuel composition. Palm and Tallow biodiesel fuel contained the highest proportions of saturated FAME and thus had the highest predicted cetane numbers, whereas Soy biodiesel contained the highest number of double bonds within the FAME composition and thus had the highest predicted iodine number. Soy biodiesel also contained the greatest proportion of methyl linoleate (C18:2), which indicates that it would also be the most oxidatively unstable of the tested fuels, followed by closely Cottonseed biodiesel (Ramos, Fernández, Casas, Rodríguez, & Pérez, 2009). Previous studies on biodiesel fuel characteristics and resulting engine performance have found that both cetane number and iodine number impact fuel performance, especially NOx and PM concentration (Cardone et al., 2002; Fontaras et al., 2009; G. Knothe & Steidley, 2005; McCormick et al., 2001). Higher iodine number is associated with a more unstable fuel caused by the increased number of chemically reactive double bonds within the FAME molecules (McCormick et al., 2001; Miller & Bowman, 1989) whereas a higher cetane number is associated with a more complete combustion and higher fuel performance (Bamgboye & Hansen, 2008; G. Knothe & Steidley, 2005). However, with ULSD having a cetane number around 49 in our study (SHELL, 2018) the higher cetane numbers found in both Palm and Tallow biodiesel means a larger drift from the physical properties of diesel fuel that a diesel engine is designed to run on. This would likely cause a shift in engine performance (Fontaras et al., 2009; G. Karavalakis et al., 2011), thus impacting exhaust characteristics and the resulting toxicological consequences of exhaust exposure.

The FAME composition of WCO biodiesel matched that of Canola biodiesel and thus it is likely that the WCO collected for this study was composed mostly of Canola oil. However, the biodiesel fuel FAME properties did not translate to exhaust gas and particle properties normally

associated with poorer health outcomes (T.-M. Chen et al., 2007; Reis et al., 2018; Steiner et al., 2016).The three most toxic fuel exhausts (Tallow, Palm and Soy BE) displayed few consistent trends in terms of exhaust gas and particle characteristics, suggesting that the toxic effects are caused by a mix of different exhaust components, possibly including some that are not reported in this study such as PAHs, aldehydes and heavy metals (Fontaras et al., 2009). Indeed, different biological outcomes are associated with different biodiesel exhaust components so no one component is associated with all toxic effects. For example, GM-CSF release is associated with particle concentrations in the 80-100 nm diameter range and TNF-α release is associated with the same as well as median particle size, proportion of particles above 23 nm in size and NO_x concentration. RANTES release is associated with CO concentration, median particle size and particle concentration between 20-35nm while MCP-1 release is not significantly associated with any one exhaust component (see Tables S3.2-3). Our previous study also found that GM-CSF release was significantly associated with particle concentration (Landwehr et al., 2019) and that RANTES was associated with both CO concentration and particle size. Conversely, TNF-α was not associated with any one exhaust component (Landwehr et al., 2019; Mullins et al., 2016).

Tallow BE was the most different to ULSD in terms of exhaust gas characteristics with 4 of the 6 measured combustion gases showing significantly different concentrations. Surprisingly, there were no significant differences in NO_x levels between different exhausts (compared with ULSD), and only minor differences in NO and NO_2. This was unexpected as previous studies have shown that BE generally contains higher levels of these gases compared with mineral diesel exhaust (de Brito et al., 2018; Graboski et al., 2003; Graver et al., 2016). A possibly explanation for this is the fact that we used a dilution of 1/15, which may be too dilute to compare NO_x differences. This is supported by previous studies which have found increases in NO_x in undiluted BE can be subtle enough that a high dilution would remove the differences between fuels (Fontaras et al., 2009; Graver et al., 2016; Kimberly J Swanson et al., 2009). Additionally, the use of biodiesel may be impacting the efficiency of the exhaust after-treatment devices, which are known to alter the conversion of NO to NO_2 and vice versa (Ko et al., 2019). We also found minimal differences in SO_2 levels between ULSD and the various biodiesel exhausts, with only Tallow and WCO exhaust showing significant decreases compared to ULSD. This is likely because the amount of Sulfur in ULSD (<10 ppm) is lower than what has been found in crude feedstock oils (Daun & Hougen, 1976), and transesterification of the oils into biodiesel would likely decrease this amount to <10ppm, similar to that of ULSD (He, Van Gerpen, & Thompson, 2009).

The most toxic biodiesels did not produce the greatest number of ultrafine particles when combusted. Due to the additional peak in particle concentration in the ultrafine particle size range between 20 and 35 nm that was not present in ULSD, all biodiesels had a greater particle number concentration and smaller median particle size in comparison to ULSD, however this was not directly associated with decreased cellular viability and only indirectly associated with levels of certain mediators. In fact, Canola and WCO BE, despite having both the highest particle number concentration and particle mass concentration, were the least toxic amongst the tested fuels, while Palm and Tallow BE (the most toxic) contained some of the lowest fine particle concentrations amongst the tested biodiesels. Tallow BE displayed a lower particle mass compared with ULSD exhaust. This is not the first time that we (Landwehr et al., 2019) and

others (de Brito et al., 2018; Seriani et al., 2015) have found higher exhaust particle concentrations to be the least toxic, both *in vitro* and *in vivo*. The reduced toxicity has previously been attributed to the agglomeration effect of diesel exhaust particles. As PM readily agglomerates to form larger sized particles, both when deposited on a surface (such as a filter) and when dispersed within a liquid (Morin et al., 2008), many of the ultrafine particles are lost and the median particle size is increased. The more particles available the more readily this effect occurs, which may explain why both WCO and Canola BE are found to be the least toxic when they also have the highest particle concentrations. Unfortunately, this effect does not explain why Soy BE, which has the third highest particle number, is the second most toxic, suggesting again that multiple exhaust factors must be important.

Of critical importance, is the fact that all BE contained more and smaller particles than ULSD exhaust (Table 3.3). This was evident as a lower median particle size, greater total particle number and proportion of particle number under <23nm in dimeter. Diesel exhaust particles in this size range are termed liquid or nucleation mode particles, suggesting that a lot of the exhaust particles within biodiesel may be in a liquid state instead of solid (Amanatidis et al., 2014). This is a critical observation as some particles, particularly in the ultrafine range, are capable of migrating through exhaust after-treatment devices such as a DPF due to the pressure drop constraints of the system (Khalek et al., 2011; Wade, White, & Florek, 1981), and a DPF is largely ineffective at controlling this particle size range, or liquid particles in general which can migrate through the filter (or evaporate and recondense) (Sirignano & D'Anna, 2018). Indeed a lot of the more toxic chemicals found in diesel exhaust, such as polycyclic aromatic hydrocarbons, are liquid at the optimal running temperature for a DPF (Choi, Harrison, Komulainen, & Saborit, 2010) and this could explain why this and previous studies have found biodiesel and biodiesel blends to be more toxic even after the use of after treatment devices (V. André et al., 2015; Landwehr et al., 2019; Skuland et al., 2017).

The increase in ultrafine particle number for all biodiesels is concerning since higher ultrafine particle concentrations are associated with a range of adverse health outcomes including increased risk of cardiac events, stroke and asthma (K. Chen et al., 2018; Evans et al., 2014; Seaton et al., 1995; Steiner et al., 2016). Even though the elemental carbon particles within the exhaust are relatively inert, inhalation still causes respiratory irritation (E. André et al., 2006; Ganguly et al., 2017; Larcombe et al., 2014). The increased surface area to volume ratio of smaller particles also means more potentially toxic chemicals can adhere to the particle surface for a given mass (Munack et al., 2006), which has concerning implications for the health impact of BE exposure. Further, ultrafine particles can bypass the airway epithelial barrier of the lungs to enter the bloodstream directly (Brook et al., 2010). This suggests that ultrafine particles are more toxic than larger particles and it is likely that widespread biodiesel usage will lead to an increased risk of adverse health outcomes for already vulnerable populations including those with previously existing respiratory or cardiovascular disease (Evans et al., 2014; Seaton et al., 1995) and children (Heinrich & Slama, 2007).

Although the most significant effect on cellular viability was found after exposure to Tallow BE, a slight increase in early apoptotic cell death was also observed after exposure to both ULSD and

WCO exhaust. This increase, while small, suggests that the effect of exhaust exposure is ongoing with cells shifting towards an apoptotic state even 24 hours after the initial exhaust exposure as the transition from early to late apoptotic cell death is relatively fast (Elmore, 2007). With Tallow BE exposure inducing an increase in late apoptotic populations, this could mean that exhaust exposure can elicit both an immediate impact to cellular viability and have extended consequences over a longer period of time.

Exhaust exposure also elicited a range of altered production among the 27 mediators tested (Table 3.4, Supplementary Table S3.1). The 14 mediators released above the limit of detection represent a spectrum of potential innate and adaptive immune responses (Dayer, Oliviero, & Punzi, 2017; Duffy, Bouchier-Hayes, & Harmey, 2013; Holdsworth & Gan, 2015; Sokol & Luster, 2015) and differences could be found after each BE exposure suggesting that each fuel may induce a unique immune response. For example, even though both Soy and Palm BE caused a significant increase in 9 mediators in comparison to Air and mostly mirror each other in their response, Soy BE exposure caused an increase in IL-6 which is part of the innate acute-phase immune response (along with IL-1β and TNF-α (Holdsworth & Gan, 2015)), whereas Palm BE exposure instead caused an increase in IFN-γ, which helps activate macrophages and promotes antigen presentation as part of the adaptive immune response (Rice et al., 2003; Suda, Sato, Sugiura, & Chida, 1995). Of note, only 3 of the 14 mediators were found to be significantly released in comparison to Air for the majority of BE exposures, all of them classified as part of the innate inflammatory response. These mediators; GM-CSF, MCP-1 and TNF-α are associated with the macrophage and systemic inflammation that is part of the acute phase response (Lloyd, 2002; Mazzon & Cuzzocrea, 2007; Rösler & Herold, 2016), suggesting that these immune responses may be critical for an appropriate reaction to exhaust inhalation. Indeed, these cytokines have been previously found to have increased release after blended BE particle exposure and/or diesel exhaust particle exposure *in vitro* (Boland et al., 1999; Fukagawa et al., 2013). Despite this, it is generally IL-6 and IL-8 that are assessed in previous non-blended BE exposure studies, with only sporadic attention paid to other cytokines (Larcombe et al., 2015; Møller et al., 2020; K. J. Swanson et al., 2007).

The release of mediators associated with the innate neutrophilic and natural killer cell immune response were induced after exposure to ULSD, Tallow, Soy and Palm exhaust with the release of G-CSF and MIP-1β (Cox, Gauldie, & Jordana, 1992; Garofalo & Haeberle, 2000), which have been found previously to increase *in vitro* after diesel exhaust particle exposure in THP-1 differentiated macrophages and in the serum of diesel exhaust exposed workers respectively (Dai et al., 2018; Fukagawa et al., 2013). With the exception of ULSD, these same biodiesels also induced increased release of IL-8, IL-9 and RANTES. IL-8, which has previously been shown to increase after mineral diesel exhaust exposure (Abe et al., 2000) and Canola BE exposure in cell models *in vitro* (Mullins et al., 2016), is a potent chemotactic factor for a range of innate immune cells including neutrophils and eosinophils. IL-9 alters the adaptive immune response and is associated with bronchial hyper-responsiveness and allergic airway inflammation (Little, Cruikshank, & Center, 2001; Zhou, McLane, & Levitt, 2001). To the best of our knowledge, this has not been tested after exposure to diesel outside our group (Landwehr et al., 2019). RANTES affects the adaptive immune response, with some effect on the innate system as well, and is

associated with recruitment and activation of cytotoxic T-cells, eosinophils and natural killer cells (Garofalo & Haeberle, 2000; Olszewska-Pazdrak et al., 1998) and has been shown to be increased after nasal challenge to diesel exhaust particles in human subjects (Diaz-Sanchez, Jyrala, Ng, Nel, & Saxon, 2000). We have previously shown that RANTES is increased after Canola BE exposure *in vitro* (Mullins et al., 2016), however in the present study, we did not measure an increase in IL-8 or RANTES after Canola BE exposure. This is possibly due to methodological differences between this study, and our previous work (Mullins et al., 2016).The remaining adaptive immune cytokine, IFN-γ, is significantly released after exposure to Tallow, Palm and Cottonseed BE and is associated with increased antigen presentation (Mitchell, Provost, Niu, Homer, & Cohn, 2011).

In this study, exposure to Canola BE resulted in the fewest health impacts, with it only inducing a slight acute-phase immune response. Canola biodiesel is one of the worlds most used feedstocks for biodiesel creation (Eea, 2013; OECD/FAO, 2020) so the finding that it is also the least toxic in our study is promising in terms of the health impacts biodiesel use may have already caused. WCO BE (composed mostly of Canola oil) elicited similar responses to Canola BE. WCO is considered to be one of the most environmentally friendly feedstock options since it a waste product of an existing industry and its production would not compete with food resources or require further land clearance (Beer et al., 2007). This suggests that WCO is a viable option for future biodiesel production as lifecycle CO_2 production would be minimal without additional land clearance and as a waste product, the cost of fuel production would also be low (Beer et al., 2007). Additionally, Tallow biodiesel is also considered an environmentally friendly option that would not require additional land clearance and would thus have minimal CO_2 lifecycle production. As it is also a waste product, with lower-grade Tallow being unusable as a food product, cost of fuel production would also be low (Beer et al., 2007). Tallow biodiesel is already used in several countries worldwide including many in Europe, the USA and Australia (ARENA, 2018; EIA, 2020b; Flach, Lieberz, & Bolla, 2019; Toldrá-Reig, Mora, & Toldrá, 2020). Our results suggest that its use in biodiesel fuels may result in greater exhaust exposure induced health impacts and thus it would not be a favourable choice despite the low lifecycle CO_2 production. Similarly, another commonly used biodiesel feedstock, Soy (BR&Di, 2011; OECD/FAO, 2020), was also found to be the second most toxic. This is consistent with ours and others previous work which have found Soy BE to be more toxic than diesel both *in vitro* and *in vivo* (Brito et al., 2010; Landwehr et al., 2019).

Clearly, more research is required before a recommendation as to the healthiest and most environmentally friendly biodiesel option(s) can be made, particularly with respect to third generation feedstocks that will not compete directly with food crops. However, our initial findings suggest that it is unlikely that the best biodiesel option will be Soy, Palm or Tallow. Based on our results, feedstock oils that do not compete with the food industry and best match the FAME profile of Canola in this study should be focused on for future biodiesel development. However inedible oils have their own drawbacks, such as the high content of free fatty acids interfering with the transesterification process and limiting biodiesel creation (Elgharbawy, Sadik, Sadek, & Kasaby, 2021). In saying this, we acknowledge that our study contains several limitations. We have focussed mostly on the toxicological consequences of exposure to the lungs

by using primary epithelial cells grown in cell culture which by its design excludes the ability to measure effects on lung structure and function or to monitor the effects on other organs/tissues which are known to be impacted by diesel exhaust inhalation. We have also only used cells obtained from “healthy” volunteers to establish a baseline, when it is primarily those with an underlying respiratory condition that are likely be the most impacted by BE exposure. We have also studied the toxicological consequences of mostly first generation feedstocks which directly compete with the food industry and are thus likely to be replaced with a feedstock that doesn’t drive up food prices.

3.5. Conclusion:

Despite our exposure being mild, we found a range of significant health impacts for all biodiesel and diesel exhaust exposures. With our exhausts having been generated using newer technologies, including an engine coupled with the latest after treatment technologies which greatly minimise exhaust pollutants, diluted 1/15 with air, which represent real world roadside PM levels (Ginzburg et al., 2015), and an exposure time of one hour, which represent an acute once off exposure, any findings have significant implications for the health impacts caused by biodiesel use. This is particularly true for those who are exposed to higher exhaust concentrations, such as is found in underground mining, or who are exposed chronically, such as those who live near busy roads. We found Tallow BE to be the most toxic amongst the six biodiesels tested and Canola BE to be the least however more research is needed, particularly into different types of feedstocks and health impacts outside the respiratory system, before the least toxic type of feedstock for biodiesel fuel can be found.

Acknowledgements: We would like to acknowledge and thank Professor Gerhard Knothe from the USDA Agricultural Research Service for providing input on the creation of the biodiesel fuel. We would like to acknowledge the Australian Research Council for funding via the Discovery Project Grant, as well as Curtin University’s School of Population Health, 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.

Chapter 4: Comparative toxicity of various 20% blended biodiesel exhausts compared with commercial mineral diesel and pure biodiesel in an *in vitro* human airway epithelial cell model.

Abstract:

Background: Biodiesel is often added to commercial diesel fuel to increase lubricity and address climate change concerns. Future legislative blending mandates are likely to increase biodiesel ratios up to 20% with 80% mineral diesel. Exhaust components of the blended fuels change depending on what the biodiesel fuel is made of, however the health effects of exhaust exposure have not been directly compared between multiple different biodiesel blends and thus it is unknown if health effects change as well.

Methods: Primary human airway epithelial cells (n=8, 2-4 years old, 4 males) were exposed for 1 hour to air or diluted exhaust from an engine running on diesel (ULSD) or 20% blends (B20) of different biodiesels within mineral diesel; Canola, Waste Cooking Oil, Soy, Tallow, Palm and Cottonseed. These biodiesel types are currently used worldwide.

Results: Physico-chemical exhaust characteristics changed significantly between fuels with Cottonseed B20 being the most similar to ULSD and with all other fuels showing significant differences in either exhaust gas concentrations or exhaust particle spectra ($p<0.05$). Early apoptotic cell death was significantly increased 24 hours after exposure to 4 out of 6 B20 exhausts (and ULSD) suggesting that health effects may be ongoing even up to a day post-exposure, however a significant decrease in viability was only found in the Palm B20 exposure group. The results for the B20 fuels mimic those of the B100, with the three most toxic fuels (Palm, Soy and Tallow) and the three least toxic fuels (Canola, Waste Cooking Oil and Cottonseed) being the same.

Conclusion: This study, which has investigated the widest range of toxic effects for the largest amount of biodiesel blends to date, shows that exposure to different exhausts results in a spectrum of toxic effects *in vitro* when combusted under identical conditions and that the health effects of biodiesels made from different source oils can be differentiated even when it makes up only 20% of the total fuel.

4.1 Introduction:

Biodiesel usage is increasing worldwide (EIA, 2020a) due to pressure from the climate change crisis, demand for alternate fuels and the increasing cost of fossil fuel extraction. Currently, the majority of biodiesel use worldwide is in the form of blended fuels where biodiesel is combined with commercial mineral diesel in order to improve the lubricity of low sulfur diesel fuel and manage environmental concerns (EERE, 2020; F. Li et al., 2019; Peng, 2017). Blend types are normally specified by a label B followed by the percentage of biodiesel blended within the fuel, e.g. B20 for 20% biodiesel fuel with 80% mineral diesel or B100 for 100% biodiesel fuel. The

percentage of biodiesel within commercial diesel varies from country to country and is largely dependent on whether biodiesel blending is mandatory or optional (Barros, 2020; Price, 2019). The requirement for biodiesel blends to be labelled as a blend (instead of just diesel) also varies between different countries. For example, in Brazil, B12 became the mandated blend in March 2020 (Barros, 2020), while European Union countries have a legislated maximum amount of B7 (EU, 2016) and the US blend mandates change from state to state with some mandating a blend of B20 with labelling and others with no blending requirements (EERE, 2020; ASTM, 2020a, 2020b). In Australia a maximum blend of B5 is allowed without labelling and B20 can be sold with labelling (ACCC, 2021; Price, 2019). As global climate change concerns increase and air pollution regulations become more stringent, it is likely that mandated blend amounts will increase (EU, 2009; Ragauskas et al., 2006). Thus, most biodiesel research into blended fuel focuses on blends between 20-30% biodiesel, with some testing up to 50% (Larcombe et al., 2015; Møller et al., 2020).

As with studies that investigate the effects of exposure to exhaust generated by the combustion of B100 biodiesels, studies which test blended biodiesel-diesel fuels also produce variable results. This is like due to the use of a wide range of methodologies, and because different studies blend their biodisesl with mineral diesel of varying chemical composition. This makes comparisons between different studies difficult. For example, different engine types (V. André et al., 2015; Magnusson et al., 2019), different exhaust after-treatment systems (Adenuga et al., 2016; (V. André et al., 2015; Magnusson et al., 2019), whether speed and load are kept constant or a drive cycle is used (Fontaras et al., 2009; Magnusson et al., 2017) and whether particulate matter (PM) is measured by mass and/or particle number (Magnusson et al., 2017; Mullins et al., 2016), all contribute to changing the resulting exhaust outputs. This leads to some studies showing the B20 exhaust contains higher concentrations of more toxic pollutants such as oxides of nitrogen (NO_x) and PM (Brito et al., 2010; Graver et al., 2016) when compared with either diesel or B100 fuels, with other studies showing the opposite relationship (Libalova et al., 2016; Mullins et al., 2016).

Another confounding factor in any attempt to compare different biodiesel blend studies is the fact that different countries have different legislative requirements and standards for the chemical properties of commercial diesel fuel (which is subsequently blended with biodiesel). These differences can also significantly impact exhaust physico-chemical properties, and are most easily observed in the amount of biodiesel that may already be present in diesel fuel (without labelling) *prior* to blending and with respect to permitted levels of sulfur. Thus, while biodiesel amounts already present within diesel fuel can range from nothing up to 7% (Magnusson et al., 2019), sulfur levels can also change drastically. For example, some studies use “ultra-low-sulfur-diesel” (ULSD) containing 10 ppm sulfur or less (Mullins et al., 2016), others use fuel containing up to 50 ppm sulfur (V. André et al., 2015) and some up to 500 ppm sulfur (Brito et al., 2010). Sulfur levels are known to alter the toxic effects of diesel exhaust exposure with higher sulfur levels resulting in higher mutagenicity (Jürgen Bünger et al., 2000).

Similarly the toxic effects measured after exposure to exhaust generated from biodiesel blends are also inconsistent, with studies showing biodiesel blends to be more toxic than (in terms of

cytotoxicity and oxidative effects (Adenuga et al., 2016; Betha, Pavagadhi, Sethu, Hande, & Balasubramanian, 2012)), similarly toxic (in terms of DNA damage and mediator release (Cervena et al., 2017; Jalava et al., 2012)) or less toxic (in terms of DNA damage, oxidative stree and mediator release (Steiner, Czerwinski, Comte, Popovicheva, et al., 2013; Yang et al., 2017)) mineral diesel. Biodiesel blends have also been shown to be more toxic than (in terms of oxidative effects and genotoxicity (Ackland et al., 2007; Adenuga et al., 2016)), similarly toxic (in terms of inflammatory response, DNA damage and gene expression dysregulation (Brito et al., 2010; Cervena et al., 2017; Libalova et al., 2016)) or less toxic than (in terms of cytotoxicity and DNA damage (Mullins et al., 2016; Vogel et al., 2019)) B100 fuels generated from the same feedstock type. The effects of different feedstocks being used to make the biodiesels within the blended fuel is rarely considered (Møller et al., 2020), despite the known associations between fuel characteristics and engine performace (Fontaras et al., 2009; G. Knothe & Steidley, 2005; McCormick et al., 2001).

Finally, in previous studies investigating the potential health effects of exposure to biodiesel blend exhaust, whole exhaust, or diluted exhaust are rarely used. Instead, most studies only consider the toxic effects of the exhaust particles extracted from filters using either the Ames assay or an immortalised cell line (V. André et al., 2015; Larcombe et al., 2015; Surawski et al., 2011). As such, both the gaseous component of the exhaust, and the particle size spectra are generally ignored (V. André et al., 2015; Landwehr et al., 2019; Larcombe et al., 2015).

Thus, while there are some published data on the health effects of exposure to exhaust generated from different biodiesel blends, it is impossible to draw any firm conclusions regarding which base-oils may be more or less harmful in blend form (compared with mineral diesel and/or B100). This means that there is an urgent need for a comparative assessment of exhaust exposure health effects of biodiesel blends made from different feedstocks, in which exhaust is generated, and exposures performed in a consistent way. This would allow assessment and direct comparison of the toxicity of different feedstock types and could allow identification of lower toxicity feedstocks before higher biodiesel concentratated diesel blends become mandated in more countries. This comparison needs to be done in such a way that methodological setup including engine paramaters and endpoint measures are kept as consistent as possible. To address this, we exposed primary airway epithelial cells to diluted exhaust generated by an engine running on ULSD or a 20% blend of biodiesel within that same ULSD fuel. Blends were made from several different biodiesel feedstocks including Soy, Canola, Waste Cooking Oil (WCO), Tallow, Palm and Cottonseed. The pure biodiesel exhaust exposure were also assessed alongside the B20 exhaust exposures (Chapter 3) so comparisons between each blend type and its matched B100 could be made. Fuel characteristics (such as fatty acid methyl ester profiles) were measured and exhaust physico-chemical properties for each blend type were recorded. Twenty-four hours after exposure health outcomes including cellular viability and mediator release were analyed. Based on the published literature, and our own previous research, we hypothesised that exposure blended biodiesel exhaust would cause more severe and a wider variety of toxic health effects than exposure to ULSD and B100 exhausts and that the different blended exhausts would cause a spectrum of health impacts, with some being more toxic than others.

4.2 Materials and Methods:

For a detailed description of the methods, please refer to Chapter 3.2 Methods.

The results of this chapter and chapter 3 were obtained as part of the same experiment. Due to the complexity and volume of data generated, the study was split into two: B100 and B20 fuels.

The only difference in the methods for the two chapters can be found below:

4.2.1 Fuel Types: Six different blended biodiesel fuels (B20) were used in this study. Soy, Canola (rapeseed), Tallow, Palm and Cottonseed biodiesel were created using high-quality, food-grade, commercial oils (Campbells Wholesale Reseller, WA, AUS and Range Products, WA, AUS). Waste cooking oil was obtained as used cooking fryer waste from a restaurant in Perth, Western Australia. All oils were converted to fatty acid methyl esters (FAME) using an established sodium methoxide transesterification process (Gerhard Knothe et al., 2015) also used in our previous study (Landwehr et al., 2019). Commercial ULSD was obtained from a local supplier (SHELL, WA, AUS, biodiesel free). All blended fuels used in this chapter were obtained by blending 20% B100 from each biodiesel feedstock type with 80% ULSD.

4.3 Results:

4.3.1 Exhaust gas analysis: Mean exhaust gas levels for each fuel over the 60-minute exposure period are shown (Table 4.1), with the exception of CO, for which the peak measurement (at the 10-minute mark) is shown. This is due to engine cold-start effects whereby CO concentrations peak rapidly, before zeroing by the 20-30-minute mark.

All blends showed similar trends in combustion gas production (Table 4.1, Supplementary Figure S4.1) throughout the 60-minute exposures. Most combustion gases increased rapidly within the first half of the exposure before levelling out around the 30-minute mark. The exceptions were O_2, which instead rapidly decreased until levelling out at ~30 minutes, and CO which peaked ~10 minutes after engine start, before rapidly returning to zero. Cottonseed B20 was the most different to ULSD with significantly increased mean O_2 and significantly decreased CO_2 and NO_x in the form of decreased NO ($p<0.01$ in all cases). In contrast, WCO B20 and Soy B20 were found to be the least different to ULSD with only NO_2 and CO_2 respectively being significantly different to ULSD exhaust ($p<0.05$ in all cases).

Table 4.1: Mean (standard deviation) gas measurements for all fuels. All significances displayed are compared to ULSD. Measurements are shown as the mean concentration for the entire exposure, with the exception of CO which is shown as the peak measurement.

Fuel	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cotton B20
O_2 (%)	20.38 (0.15)	20.43 (0.12)	20.45 (0.13) +	20.33 (0.14) *	20.35 (0.14)	20.36 (0.15)	20.47 (0.11) ** +
CO (ppm)	3.53 (2.35)	2.19 (1.68)	5.10 (5.80) +	1.37 (0.52)	1.22 (0.74)	2.55 (2.48)	1.25 (0.50)
CO_2 (%)	0.34 (0.11)	0.36 (0.09) * -	0.32 (0.10)	0.36 (0.10)	0.37 (0.10) ** -	0.35 (0.11)	0.31 (0.08) -
NO_x (ppm)	21.94 (6.11)	20.25 (4.45)	19.40 (2.07) ** -	22.40 (4.20)	22.21 (5.08)	22.08 (5.60) +	17.74 (3.96) *** -
NO (ppm)	15.07 (3.85)	13.69 (2.95) *	12.79 (3.12) ***	16.26 (3.58)	14.78 (3.28)	15.30 (3.70) +	11.89 (2.46) *** -
NO_2 (ppm)	6.69 (2.21)	6.57 (1.42)	6.47 (1.83) -	7.06 (1.80) +	7.44 (1.80) * +	6.82 (1.92) -	6.02 (1.10)
SO_2 (ppm)	1.5 (0.3)	1.42 (0.20)	1.42 (0.38)	1.25 (0.27)	1.08 (0.38)	1.41 (0.58)	1.17 (0.26) -

* Significantly different to ULSD (*=p <0.05, **=p <0.01, ***=p <0.001)
Shaded values are significantly different to the B100 fuel of the same type, a + sign at the end indicates a significant increase from B100 values, - a significant decrease ($p<0.05$).

4.3.2 Particle Analysis: Particle spectra were obtained for each exhaust between the sizes of 5 nm and 340 nm (Figure 4.1) and key exhaust particle characteristics were analysed (Table 4.2). Canola B20, Tallow B20 and Palm B20 were found to be significantly different to ULSD in terms of total particle number concentration, with Canola B20 and Palm B20 increasing and Tallow B20 decreasing ($p<0.05$ in all cases). All fuels showed peaks in particle concentrations between the sizes of 80-100nm. This peak was largest in Canola B20 and WCO B20 with significantly increased particle number concentrations over 1.4 times that of ULSD ($p<0.05$ in all cases). The separation of particle sizes into above and below 23 nm diameter was chosen based on the approximate size of the divide between solid and liquid particles within diesel exhaust, around the nucleation mode size (Amanatidis et al., 2014). Only Cotton B20 and Palm B20 showed a peak in the blended fuels between the sizes of 20-35 nm. Hence Palm B20 and Cotton B20 exhaust contained significantly more particles compared with ULSD at this size and WCO B20 contained significantly fewer ($p<0.05$). Median particle size and mass and number concentrations were also calculated for particles between the sizes of 10-340 nm (Table 4.2).

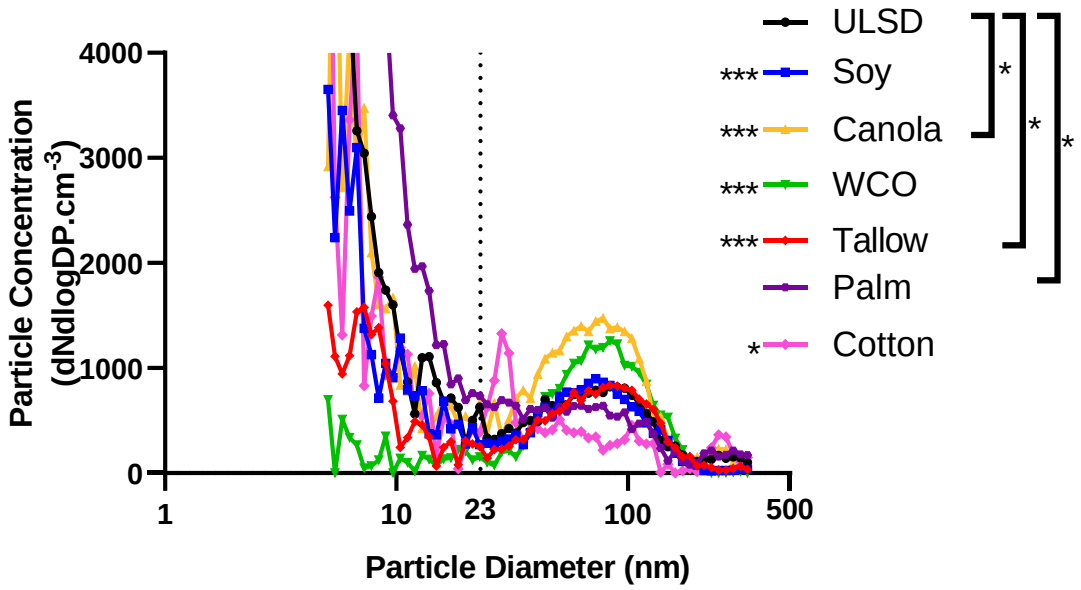


Figure 4.1: Particle size spectra for all fuels. Data were analysed using total particle number concentration between the size of 10 and 340 nm for each fuel (*=p value<0.05, **=p value<0.01, ***=p value<0.001). The dotted line indicates the particle size spectra of 23nm. Bars linking fuel types in the figure key indicate significant difference in particle number between the different fuels. Significance indicators to the left of the figure key indicate significant differences between the B100 and B20 of the same fuel type.

Table 4.2: Particle characteristics between the sizes of 10-340 nm for all fuels.

Particle Characteristic	Fuel						
	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cotton B20
Particle Mass Concentration (µg/m3)	12.85	6.97 (0.54) [0.31]	16.37 (1.27) [0.57]	8.61 (0.67) [0.43]	8.62 (0.67) [0.81]	13.7 (1.07) [0.56]	11.68 (0.91) [0.99]
Median Particle Size (nm)	44	51 (1.16) [2.04]	58 (1.32) [2.23]	72 (1.64) [2.77]	63 (1.43) [2.42]	21 (0.48) [1.17]	31 (0.70) [0.82]
Total Particle Number (particles/cm^3)	25993	22335 (0.86) [0.23] -	34804 (1.34) * [0.29] -	21409 (0.82) [0.19] -	18891 (0.73) * [0.32] -	33809 (1.54) * [0.69]	19165 (1.61) [0.44]-
Particle Concentration Between 80-100 nm (particles/cm^3)	3187	2919 (0.92) [0.64] -	5392 (1.69) * [0.93]	4526 (1.42) * [0.54] -	3267 (1.03) [0.86]	2084 (0.65) [0.91]	1318 (0.41) [0.40] -

Particle Concentration Between 20-35 nm (particles/cm^3)	3453	2525 (0.73) [0.07] -	4524 (1.31) [0.12] -	1293 (0.37) * [0.03] -	2023 (0.59) [0.12] -	5302 (1.54) * [0.61]	5558 (1.61) * [0.46]
Particle Number >23 nm (particles/cm^3)	16900 65.02%	15442 69.14%	27299 78.44%	19776 92.37%	15484 81.96%	16125 47.69%	12711 66.32%
Particle Number <23 nm (particles/cm^3)	9093 34.98%	6893 30.86%	7505 21.56%	1633 7.63%	3407 18.04%	17684 52.31%	6454 33.68%

a Values in round brackets ( ) represent proportional changes in comparison to ULSD.
b Values in square brackets [ ] represent proportional changes in comparison to the B100 of the same feedstock. A shaded cell with a + or – sign after the brackets indicates that this difference was significant, with each sign representing an increase or decrease respectively ($p<0.05$).
c Percentage values in the last two rows represent the percentage of the total particle number concentration within each fuel.
* Significantly different to ULSD (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$).

4.3.3 Cellular Viability: Only exposure to Palm B20 exhaust resulted in a significant reduction in cellular viability compared with Air exposed controls (96.3 ± 1.7%; $p<0.01$) (Figure 4.2). Exposure to exhaust from the remaining five B20 fuels did not significantly alter viability compared to Air. ULSD exhaust exposure resulted in a significant decrease in viability when compared with both Tallow B20 and Cotton B20 ($p<0.01$).

Cell death mechanisms were assessed post exposure, and effects of B20 exhaust exposure were compared with both ULSD and Air exposed controls (Figure 4.3). Compared to Air exposed controls a significant increase in early apoptotic cell death was observed in cells exposed to ULSD, WCO B20, Tallow B20, Palm B20 and Cotton B20 ($p<0.05$). ULSD exhaust exposure also significantly increased early apoptotic cell death when compared with Soy B20 and Canola B20. Late apoptotic cell death was significantly decreased in Tallow B20 exposed cells when compared to both Air and ULSD exposed cells ($p<0.05$). Necrotic cell death was decreased when compared to Air in both WCO B20 and Cotton B20 exposures.

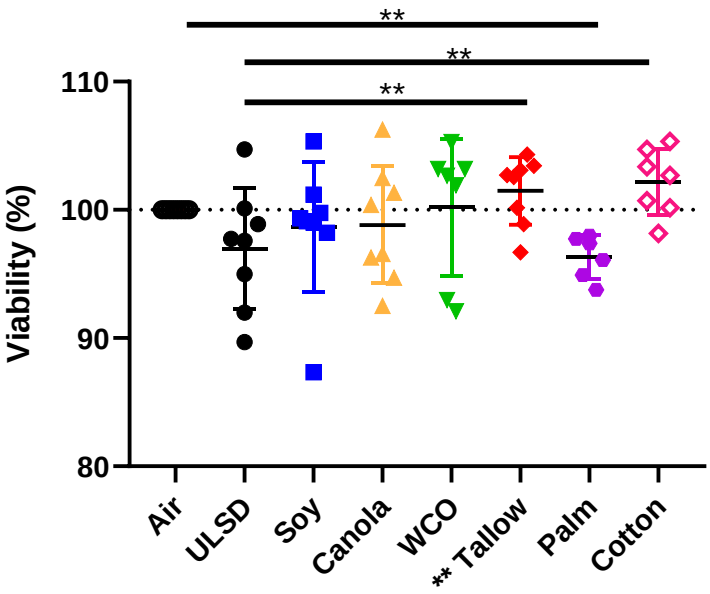


Figure 4.2: Viability measurements normalised to Air controls 24 hours after exposure. Mean viability measurements were: 97.0±4.7%, 98.6±5.1%, 98.8±4.6%, 100.2±5.3%, 101.5±2.6%, 96.3±1.7% and 102.2±2.6% for ULSD, Soy B20, Canola B20, WCO B20, Tallow B20, Palm B20 and Cottonseed B20 exposures respectively. Linking bars on the top of the graph indicate significant differences to Air or Diesel controls compared to the linked fuel (**=p<0.01). Asterisks next to the fuel name on the x-axis denotes a significant difference between B20 and B100 of the same fuel type (p<0.01 in all cases).

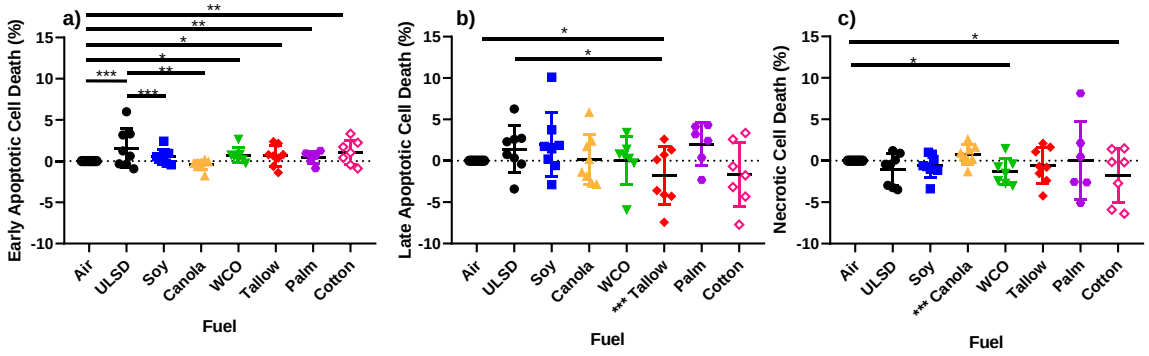


Figure 4.3: a) Percentage change in early apoptotic cell death 24 hours after exposure. b) Percentage change in late apoptotic cell death 24 hours after exposure. c) Percentage change in necrotic cell death 24 hours after exposure. All cell death mechanisms were normalised by subtracting Air controls. Linking bars on the top of the graph indicate significant differences to Air or Diesel controls compared to the linked fuel (*=p<0.05, **=p<0.01, ***=p<0.001). Asterisks next to the fuel name on the x-axis denotes a significant difference between B20 and B100 of the same fuel type (*=p<0.05, **=p<0.01, ***=p<0.001).

4.3.4 Mediator Release: Of the panel of 27 mediators tested, 14 were measured at levels above the limit of detection (Table 4.3, Supplementary Table S4.1). Most of these 14 mediators primarily impacted the innate immune response (IL-1β, IL-6, IL-8, G-CSF, GM-CSF, MCP-1, MIP-1β and TNF-α), with IL-9, IFN-γ, IP-10 and RANTES primarily impacting the adaptive inflammatory immune response. Only TNF-α was significantly increased in all exposures compared to Air ($p<0.05$ for all treatments), however G-CSF, GM-CSF and MCP-1 were all significantly increased for at least four of the exposures. In comparison to Air, Soy B20 exhaust induced the largest immune impact with significant differences in the levels of 9 mediators post exposure, followed by Palm B20 exhaust and Tallow B20 exhaust (Table 4.3). WCO B20 and Canola B20 exhaust induced the fewest significant differences compared with Air.

Table 4.3: Mean (standard deviation) mediator release for the 14 cytokines released above the limits of detection 24 hours after exposure. All values have been normalised by subtracting Air controls for each individual participant so as to minimise variability between samples from different volunteers. See supplementary Table S4.1 for significant differences between biodiesel fuels.

Mediator Concentration (pg/mL)	Fuel						
	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cotton B20
IL-1β	0.23 (0.65) *	0.17 (0.55) ** -	0.15 (0.34) +	0.09 (0.13)	-0.03 (0.13) #	0.05 (0.17)	0.10 (0.35)
IL-1RA	10.09 (42.45)	9.26 (296.96)	-26.56 (87.28)	102.84 (213.82)	-13.29 (121.24)	35.06 (206.63)	143.00 (223.84)
IL-6	-70.79 (117.13)	121.35 (238.44) *** ##	-21.83 (110.25) +	19.84 (147.73)	217.08 (297.55) ** ####	27.43 (269.21)	-0.11 (259.42)
IL-8	255.47 (705.55)	2180.67 (3774.04) **** #### +	-29.93 (151.52)	153.63 (430.81)	3003.06 (5481.21) **** #### +	920.25 (1307.33) *** #	-4.17 (513.87
IL-9	1.98 (4.01)	4.41 (9.52) ***	1.71 (4.07)	2.45 (6.42)	3.68 (6.20) ** -	5.36 (6.58) *	2.28 (7.21)
G-CSF	28.81 (62.43) **	59.17 (113.76) **** ## +	56.19 (69.32) *** +	34.79 (45.71) *	124.63 (196.73) **** #### +	49.90 (46.75) *	23.94 (64.45) #
GM-CSF	8.44 (14.36) *	12.65 (19.46) **	7.22 (9.61)	5.79 (8.22)	19.94 (24.01) ** #	19.07 (23.64)	16.97 (22.59) *
IFN-γ	-1.95 (5.56)	1.83 (8.77)	1.25 (4.23) +	8.45 (13.40)	1.92 (7.07)	6.73 (11.74) *	16.05 (14.20) ** ##
IP-10	-8.76 (200.80)	143.58 (204.32) #	27.21 (157.15)	-22.08 (95.02)	32.37 (112.22)	18.30 (203.81)	68.62 (227.35)

MCP-1	24.80 (56.00) *	61.71 (104.96) *	3.22 (3.31)	6.99 (11.96)	53.50 (126.82) *	57.49 (132.67) *	41.81 (108.48) *
MIP-1β	1.23 (1.61) **	0.38 (1.36)	-0.22 (1.15) ##	0.23 (2.00) ##	0.32 (1.05)	2.42 (1.12) ***	0.18 (1.72) ##
RANTES	-0.30 (1.03)	3.14 (4.4) *** ##	-0.07 (0.95)	0.92 (3.23)	0.34 (0.39)	6.34 (5.26) **** ###	1.17 (0.81)
TNF-α	5.16 (4.33) **	7.08 (7.11) ***	5.07 (2.78) **	8.03 (10.39) ***	4.71 (5.73) **	12.44 (16.74) **** #	15.64 (11.97) **** ###
VEGF	93.64 (167.33)	-39.34 (166.74)	-1.80 (127.09)	16.46 (59.67)	10.05 (237.84)	81.90 (201.20)	-92.75 (197.12) * #

* Significantly different to Air (*=p <0.05, **=p <0.01, ***=p <0.001)
# Significantly different to ULSD (#=p <0.05, ##=p <0.01, ###=p <0.001)
Shaded values are significantly different to the B100 fuel of the same type, a + sign at the end indicates a significant increase from B100 values, - a significant decrease (p<0.05).

4.3.5 Comparisons Between B20 and B100 (Chapter 3)

All B20 exhausts showed significant differences in combustion gas levels when compared to matched B100 exhausts (Table 4.1 in this chapter and Table 3.2 in Chapter 3, p<0.05 in all cases). These differences show no overall trends in CO, NO_x (both NO and NO_2) or SO_2 in that both significant increases and significant decreases are observed in all these combustion gases depending on the fuel type, not the percentage of biodiesel within the combusted fuel. B20 exhaust contained a similar or significantly greater O_2 concentration and a similar or significantly lower CO_2 compared with matched B100 exhaust (p<0.05 in all treatments). Cottonseed B20 and B100 were the most different (when compared within base oil type) with significantly increased O_2 and significantly decreased CO_2, NO_x in the form of NO and SO_2 in Cottonseed B20 exhaust (p<0.05 in all cases) compared with Cottonseed B100. Soy B20 and WCO B20 were the most similar to their matched B100 with only 1 significant difference in CO_2 and NO_2 respectively (p<0.05 in all cases).

In comparison to matched B100 fuels, particle mass was decreased for all B20 exhausts and total particle number was significantly decreased in all B20 exhausts except Palm B20 (p<0.05). Median particle size was also increased for all B20 exhausts except Cotton B20, when compared to matched B100 fuels. Particle concentration between 80-100 nm was significantly lower in Soy B20, WCO B20 and Cotton B20 compared with matched B100 exhausts. Whereas all B100 exhausts (Chapter 3) showed peaks in particle concentration between the sizes of 20-35 nm, the only blends to exhibit this were Palm B20 and Cotton B20. This meant that particle concentration between 20-35 nm was significantly lower in Soy B20, Canola B20, WCO B20 and Tallow B20, when compared to the matched B100 (p<0.05).

There were few significant differences in biological responses when B20 and B100 exhausts made from combusting the same base oil type were assessed. Tallow B100 exhaust exposure however, resulted in significantly decreased viability ($p<0.01$) compared with exposure to Tallow B20. Tallow B100 exposure also resulted in significantly increased late apoptotic cell death compared to Tallow B20. In contrast, Canola B20 resulted in significantly increased necrotic cell death when compared to Canola B100 ($p<0.001$). In terms of mediator response, only Soy B20, Canola B20 and Tallow B20 exhausts displayed some significant differences to their matched B100 fuels, with responses in three mediators being significantly different in both Soy B20 and Tallow B20 and four mediators in Canola B20. Of the 10 significant differences in mediator release found between B20 and B100 matched fuels, only 2 changes were decreases when compared to B100 results; IL-1β in Soy B20 and IL-9 in Tallow B20. The remaining eight responses all increased significantly and were mostly observed in the releases of G-CSF and IL-8, with only Canola B20 showing changes outside those two mediators ($p<0.05$ in all cases, post B20 exhaust exposure compared with post commensurate B100 exhaust exposure).

4.4 Discussion:

The results of this study show that exposure to 20% blended biodiesel exhaust elicits a range of toxic effects on airway epithelial cells and that these changes vary when compared to both ULSD and between different types of B20. Importantly, the three most toxic (Tallow, Soy and Palm) and three least toxic (Cottonseed, Canola and WCO) biodiesel exhaust types are consistent between B100 and B20 fuels (Chapter 3). This again suggests associations between the fatty acid methyl ester (FAME) profile of the fuels and the resulting toxic effects of the exhaust, despite the FAME content being only 20% of the total volume of B20 fuels. No one characteristic can be found in either the exhaust or FAME profile (Chapter 3) that is different in only the three most toxic or only the three least toxic, suggesting that any observed toxic effects are due to a range of exhaust components instead of one component alone, possibly including some not measured in this study (Fontaras et al., 2009).

We found Soy B20 and Palm B20, closely followed by Tallow B20, to be the most toxic fuel types and Canola B20 and WCO B20 to be the least. Previous FAME profile analysis of B100 equivalent fuels (Chapter 3.3.1) showed that WCO is mostly Canola oil, thus it is unsurprising that the toxic effects of these two B20 fuels are similar. We also previously reported that Soy biodiesel had the highest proportion of double-bonded unsaturated FAME molecules and thus the highest predicted iodine number whereas Palm and Tallow biodiesels contained the highest proportion of saturated FAME molecules and thus the highest predicted cetane numbers. Palm B20 induced decreased viability but it was Soy B20 that induced the widest range of mediator responses, followed by Palm B20 and then Tallow B20. As both iodine and cetane numbers increase, exhaust composition is affected, especially PM and NO_x concentrations (Cardone et al., 2002; Fontaras et al., 2009; McCormick et al., 2001). An increased cetane number is associated with more complete combustion (Bamgboye & Hansen, 2008; G. Knothe & Steidley, 2005) whereas higher iodine number is associated with a more reactive and unstable fuel (McCormick et al., 2001; Miller & Bowman, 1989). As the ULSD we used had a cetane number of ~49 (SHELL, 2018), the higher cetane numbers of Tallow and Palm biodiesel and the more unstable

properties of Soy biodiesel indicate a drift from the physical properties of conventional ULSD, which diesel engines are designed use. This will likely alter engine performance and exhaust characteristics (Fontaras et al., 2009; G. Karavalakis et al., 2011) and thus impact the toxicological effects of exhaust exposure.

A key finding of this study was that early apoptotic cell death was significantly increased 24 hours after exposure to 4 out of 6 B20 exhausts (and ULSD). Previous studies have mostly found increases in necrotic and/or late apoptotic cell death (Jalava et al., 2012; Lankoff et al., 2017; J.-S. Wang, Tseng, & Chao, 2017). This suggests that toxic effects in our study may be ongoing 24 hours after a single exposure as the change from early to late apoptotic cell death is quick to occur (Elmore, 2007). This is supported by previous literature showing that mice display effects of exhaust exposure up to 7 days after PM exposure (Yanamala et al., 2013). While we acknowledge that these increases are relatively small, it is important to note that we used diluted exhaust in order to mimic a realistic acute exposure. This means that even small changes could be important for populations exposed regularly to dilute exhaust, or once-off to more concentrated exhaust, such as those who live near busy roads or work with diesel-powered equipment (Rynning et al., 2019; J. J. Zhang et al., 2009).

Exposure to B20 exhaust also elicited alterations in mediator levels, most of which were related to innate and adaptive immune responses (Dayer et al., 2017; Duffy et al., 2013; Holdsworth & Gan, 2015; Sokol & Luster, 2015). Only TNF-α (which is primarily involved in the innate acute inflammatory response (Holdsworth & Gan, 2015)), was significantly released after every exposure, while two others (G-CSF and MCP-1), were significantly increased after the majority of B20 exposures. These mediators stimulate innate neutrophilic and macrophage inflammatory responses (Cox et al., 1992; Holdsworth & Gan, 2015; Lloyd, 2002; Mazzon & Cuzzocrea, 2007), with previous diesel exhaust exposure studies in animals and humans showing macrophages and/or neutrophils increase after exposure (Annelie F Behndig et al., 2011; Karthikeyan et al., 2013; Tong et al., 2014; Yanamala et al., 2013). Similarly, GM-CSF, which is also associated with the innate macrophage response (Rösler & Herold, 2016), was released after exposure to ULSD exhaust and 3 B20 exhaust types (Soy B20, Tallow B20 and Cotton B20).

The three most toxic exhaust exposures in terms of mediator release (Palm B20, Soy B20 and Tallow B20) also resulted in increased release of IL-8 and IL-9. These mediators stimulate the innate neutrophil and adaptive allergic airway responses respectively (Abe et al., 2000; Little et al., 2001; Sokol & Luster, 2015; Zhou et al., 2001). IL-8 has previously been shown to be important in diesel exhaust exposure studies (Dai et al., 2018; Kimberly J Swanson et al., 2009). Conversely, to the best of our knowledge, IL-9 has not been measured in this context outside our group. In addition, IL-6 was released after exposure to both Soy B20 and Tallow B20 exhaust and is associated with the innate acute inflammatory response (Holdsworth & Gan, 2015), Palm B20 and ULSD exhaust induced MIP-1β release, which is associated with the innate neutrophilic and natural killer cell response (Garofalo & Haeberle, 2000; Sokol & Luster, 2015) and Soy B20 and Palm B20 induced RANTES release, which is associated with the adaptive recruitment and activation of T-cells (Garofalo & Haeberle, 2000; Olszewska-Pazdrak et al., 1998; Sokol & Luster, 2015). All three of these mediators have previously been found to be dysregulated in

exhaust exposure studies (Annelie F Behndig et al., 2011; Dai et al., 2018; Matsumoto et al., 2006; Kimberly J Swanson et al., 2009). ULSD, Canola B20 and WCO B20 did not induce the release of mediators that alter adaptive immunity, impacting the release of innate mediators only.

A secondary goal for studying the effects of B20 exhaust exposure was to compare these effects to data presented in Chapter 3. This is important because B20 is already available in some countries (ASTM, 2020b) and others are steadily increasing the blend ratio closer to B20. Unblended biodiesel (B100) is a possible endpoint to this trend, unless alternative energy sources replace combustion engines entirely, as it is capable of directly replacing diesel fuel within many engines (Fontaras et al., 2009). Exhaust physico-chemistry varied significantly between blends and pure biodiesel fuel exhausts generated from the same base-oils, as has been found previously (Graver et al., 2016; Prokopowicz et al., 2015; Steiner, Czerwinski, Comte, Popovicheva, et al., 2013), although few clear trends were identified. For example, CO_2 was lower in three of the B20 exhausts when compared to their respective B100 exhausts, supporting previous findings (Fontaras et al., 2009), while O_2 was higher in two of the B20's. The remaining gases displayed differences only within individual feedstock types with no overall trend in differences between B20 and B100. When comparing trends in particle characteristics, all B20 exhausts had lower particle mass concentrations compared to B100, five of six (all but Cotton B20) had larger median particle sizes and five of six had significantly lower total particle number concentrations (excluding Palm B20). This has important health implications as epidemiological findings have linked ultrafine particles to greater health impact more than larger sized particles (Breitner et al., 2011; Oberdörster et al., 1995).

When comparing the biological outcomes of exhaust exposure, few significant differences in mediator production were identified between B20 and B100 exhaust types generated from the same base-oil. Differences were only found in Canola, Soy and Tallow, suggesting that blended B20 exhaust is only slightly more immunogenic than B100 exhaust for certain feedstocks. Previous studies also show similar mediator release between blends and pure fuels (Jalava et al., 2012; Steiner, Czerwinski, Comte, Popovicheva, et al., 2013). Only Tallow displayed a significant difference in cellular viability with Tallow B100 exhaust exposure reducing cellular viability significantly more than Tallow B20 exhaust. This was largely comprised of increased late apoptotic cell death in Tallow B100. Conversely, Canola B100 exhaust exposure resulted in decreased necrotic cell death when compared to Canola B20 exhaust exposure. This could be due to the order of toxicity we found within the B100 fuels (Chapter 3). Tallow B100 was found to be the most toxic, ULSD was in the middle and Canola B100 was found to be the least toxic. Thus blending ULSD with Tallow would reduce toxicity from the level of the B100, whereas blending ULSD with Canola would increase it. This does not explain why toxicity of Palm B20 and Soy B20 match their respective B100 fuels instead of also decreasing, which may come down to individual exhaust components that cause toxicity or perhaps that they are closer in the toxicity order to ULSD than either Tallow or Canola.

It is difficult to compare the results of our data to those of previous studies for a number of reasons. Firstly, we used the exhaust from an engine equipped with exhaust after-treatment devices (such as a diesel particulate filter and oxidative catalyst), which are known to greatly

impact exhaust output (Khalek et al., 2011; Magnusson et al., 2017). Most previous studies in this field use older engines without these devices, and hence exhausts containing significantly more particles, higher particle mass and higher CO (Larcombe et al., 2015; Valand et al., 2018). Secondly, the findings for blended biodiesel fuel toxicological studies are inconsistent (Larcombe et al., 2015; Møller et al., 2020). This is in part due to the fact that, while B20 is arguably one of the most common blend types in scientific studies, blends of B30 and B50 are also commonly studied (Betha et al., 2012; Gerlofs-Nijland et al., 2013; Libalova et al., 2016). This, combined with the exhaust profile and toxic exposure consequences of steadily increasing biodiesel amounts in blended fuels not being a linear trend between ULSD and pure B100, makes it difficult to draw overarching conclusions. For example, previous studies have found blends to be more toxic in terms of oxidative potential and DNA damage compared with both diesel and B100, and to contain more PM and NO_x (Ackland et al., 2007; Adenuga et al., 2016; Graver et al., 2016).

That said, our results indicate that the toxic results of exposure to B20 exhaust were slightly more inflammatory than exposure to B100 in three of our six biodiesel fuels. This is not the first study to find blended biodiesel fuels can be more toxic than B100, with previous studies finding blends of all ranges between B20-B80 to have more oxidative potential and more DNA damage causing capability than B100 (Ackland et al., 2007; Adenuga et al., 2016; Krahl, Munack, Ruschel, Schröder, & Bünger, 2008). Unfortunately, these studies do not always state the type of feedstock used to create the biodiesel, with only blended rapeseed (Canola) biodiesel known to be more toxic than its B100 counterpart (Krahl et al., 2008). We also found that in the remaining three fuels, toxic consequences of exhaust exposure were similar to that of matched B100 fuels. Again, this has previously been reported with equal levels of DNA damage and gene dysregulation in cell exposure studies and comparable inflammatory responses in mice (Brito et al., 2010; Cervena et al., 2017; Libalova et al., 2016).

4.5 Conclusion:

The results of our study raise the question of whether the toxicological results of B20 exhaust exposures are so inconsistent in the literature because different feedstocks have been used by different studies? Soy and Canola are the most common biodiesel types used in blended fuel studies (Larcombe et al., 2015; Møller et al., 2020) and we have found Soy B20 to be amongst the most toxic, more than that of ULSD, whereas Canola B20 was amongst the least toxic, less than that of ULSD. There are also several studies that use less common feedstock types such as animal fat or corn (Hemmingsen et al., 2011; Yanamala et al., 2013) or don't report the type of feedstock used to create the biodiesel (Ackland et al., 2007; Magnusson et al., 2019), which makes comparisons difficult. This could explain the inconsistencies in toxicological findings, with attempts being made to define the differences between diesel, B20 and B100 while also correlating exposure endpoints of biodiesels made from varyingly toxic feedstock types. Unfortunately, methodological differences make comparisons between different feedstocks unadvisable unless those comparisons are performed within the same study and so previous attempts to review literature and attribute particular toxic effects, such as inflammation or DNA damage, to a particular biodiesel feedstock have been inconclusive (Møller et al., 2020). The

future of biodiesel research needs to become more standardised so that comparisons between different studies can be accurately made. At the very least, engine type, drive cycle type or constant speed and load settings, after-treatment devices, sulfur levels in diesel fuel and the exact type of feedstock, preferably down to the FAME profile, need to be reported consistently before any sort of comparison between studies can be accurately performed. As biodiesel can be made from almost any fat or oil, this will be an undertaking and our study is just a small part of what will be required to find the least toxic feedstock for biodiesel creation.

Chapter 5: Toxicity of different biodiesel exhausts in primary human airway epithelial cells grown at air-liquid interface.

*Katherine R. Landwehr[1,2], Jessica Hillas[2], Ryan Mead-Hunter[1], Peter Brooks[3], Andrew King[4], Rebecca A. O'Leary[5], Anthony Kicic[1,2,6,7], Benjamin J. Mullins[1], Alexander N. Larcombe[1,2], on behalf of AusREC[8,9,10], WAERP[8,11]

[1]*Occupation, Environment and Safety, School of Population Health, Curtin University, PO Box U1987, Perth, 6845, Western Australia, Australia*

[2]*Respiratory Environmental Health, Wal-yan Respiratory Research Centre, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia*

[3] *School of Science, Technology and Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia*

[4]*Fluid Dynamics Research Group, School of Civil and Mechanical Engineering, Curtin University, Perth, Western Australia, Australia*

[5]*Department of Primary Industries and Regional Development, Perth, 6151, Western Australia, Australia*

[6]*Department of Respiratory and Sleep Medicine, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia*

[7]*Centre for Cell Therapy and Regenerative Medicine, The University of Western Australia, Perth, 6009, Western Australia, Australia*

[8]*Telethon Kids Institute, Centre for Health Research, The University of Western Australia, Nedlands, Perth, 6009, Western Australia, Australia*

[9]*Priority Research Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, Newcastle, 2305, New South Wales, Australia*

[10]*Robinson Research Institute, University of Adelaide, North Adelaide, 5006, South Australia, Australia*

[11]*St. John of God Hospital, Subiaco, Perth, 6008, Western Australia, Australia*

Corresponding Author*:

Email: katherine.landwehr@telethonkids.org.au

Post: PO Box 855, West Perth, Western Australia, 6872

Abstract:

Background: Biodiesel usage is increasing worldwide as global warming concerns increase and mineral diesel gets more difficult and expensive to extract. Biodiesel is created through the transesterification of fats/oils and fuel properties change depending on the feedstock used to

create it. The aim of this study was to assess the different toxicological properties of biodiesel exhausts created from different feedstocks using a complex 3D air-liquid interface (ALI) model that mimics human lung formation.

Method: Primary human airway epithelial cells were obtained from nasal brushings from 6 subjects and grown at ALI for 28 days until full differentiation was achieved. Cells were then exposed to 1/20 diluted exhaust from an engine running on Diesel (ULSD), pure or 20% blended Canola biodiesel and pure or 20% blended Tallow biodiesel, or Air as a control. Exhaust was analysed for various physio-chemical properties including combustion gas concentrations and particle size spectra. Then, 24-hours after exposure, ALI cultures were assessed for permeability, protein release and mediator response in the apical and basal compartments.

Results: All measured exhaust components were within Safe Work Australia standards. ULSD contained the highest concentrations of various combustion gases. We found no differences in terms of particle characteristics for any of the tested exhausts, likely due to the high dilution used.

Exposure to Tallow B100 and B20 induced increased permeability in the ALI culture and the greatest increase in mediator response in both the apical and basal compartments. In contrast, Canola B100 and B20 did not impact permeability and induced the smallest mediator response and all exhaust but Canola B20 induced increased protein release, indicating epithelial damage.

Conclusion: Despite the concentrations of all exhausts used in this study meeting industry safety regulations, we found significant toxic effects. Tallow B20 and Tallow B100 were found to be the most toxic of the tested exhausts and Canola B20 and B100 the least, with ULSD toxicity being between the two different biodiesel types.

5.1 Introduction:

Biodiesel is a renewable diesel fuel created through the transesterification of fatty acids found within natural fats and oils into fatty acid methyl esters (FAME) (Gerhard Knothe et al., 2015). It can be used to replace commercial mineral diesel in many diesel engines including those currently on road (Fontaras et al., 2009). The type of fat or oil used to create the biodiesel alters the FAME profile and other properties of the fuel (G. Knothe & Steidley, 2005; Ramos et al., 2009). This in turn changes the combustion exhaust composition (Graboski et al., 2003) and the resulting health impacts of exhaust exposure (Chapter 3, (Landwehr et al., 2021)). Global biodiesel production has increased 50-fold since 2000 (EIA, 2020a) and as diesel fuel gets more difficult and expensive to extract, it is likely that usage will increase even further. This is due to the fact that diesel fuel is still heavily used for long distance transporting due to its economical fuel usage (Suppes & Storvick, 2016) and other renewable energy options are not yet capable of replacing diesel engines in this aspect (Amjad et al., 2010; Camuzeaux et al., 2015).

Currently, biodiesel is often used as a mandated blend with mineral diesel in order to increase lubricative properties and address global warming concerns (EU, 2016, 2019; F. Li et al., 2019). A blend of 20%, labelled B20, is the most common type of blend tested within literature as it is

already in use (EERE, 2020; Hamje et al., 2014; ASTM, 2020b). Biodiesel made from Canola (Rapeseed) and Soy are some of the most commonly used biodiesel types and are thus also the most commonly tested (Møller et al., 2020; OECD/FAO, 2020), however other types such as Palm, Coconut and animal fats such as Tallow are also currently in use worldwide (ARENA, 2018; OECD/FAO, 2020).

As diesel exhaust is generally inhaled, effects of exposure occur primarily in the respiratory and cardiac systems (Annelie F Behndig et al., 2011; Giles, Carlsten, et al., 2018; Mills et al., 2011; Peters et al., 2017), although effects on other organs such as the brain (Nejad et al., 2015) and bladder (Latifovic et al., 2015) have also been reported. Diesel exhaust contains many toxic/irritating compounds including oxides of nitrogen (NO_x), carbon monoxide (CO), elemental carbon particles and polycyclic aromatic hydrocarbons (PAH) which are known to impact health (Fontaras et al., 2009; Gioda et al., 2016; Graver et al., 2016). However, the majority of toxic effects caused by inhalation of diesel exhaust have been attributed to the ultrafine particle component (<100 nm diameter) (Breitner et al., 2011; Oberdörster et al., 1995). Particles under 35 nm in size make up more than 90% of particles found within diesel exhaust and yet only account for approximately 10% of the mass (D. Kittelson et al., 2002; Ris, 2007). Ultrafine particles are considered especially toxic as they are capable of bypassing the epithelial barrier of the lungs and directly entering the blood stream (Brook et al., 2010; Goodson et al., 2017). Additionally, diesel exhaust can contain toxic chemicals such as aromatic hydrocarbons, aldehyde, ketones and heavy metals (Fontaras et al., 2009; Gioda et al., 2016) which are known to readily adsorb/adhere to the surface of these more easily inhaled ultrafine particles (Mullins et al., 2016; Munack et al., 2006).

Biodiesel exhaust is typically similar to mineral diesel exhaust, apart from a few important differences. It generally contains more NO_x and a smaller median particle size (Fontaras et al., 2009; Giakoumis, Rakopoulos, Dimaratos, & Rakopoulos, 2012), which has concerning implications for the toxic effects of exhaust exposure. Previous studies on biodiesel exhaust toxicity have provided conflicting results (Larcombe et al., 2015; Madden, 2016; Møller et al., 2020), with some studies finding biodiesel to be more toxic than mineral diesel in terms of cytotoxicity and inflammatory effects (Skuland et al., 2017; Yanamala et al., 2013), others diesel to be more toxic than biodiesel in terms of mutagenicity and vascular effects (Hemmingsen et al., 2011; Mutlu et al., 2015) and yet others finding blended biodiesel/mineral diesel fuels to be more toxic than either of the pure fuels in terms of mutagenicity and oxidative activity (Adenuga et al., 2016; Krahl et al., 2008). A common limitation in previous literature is the tendency to treat biodiesel as the same regardless of the feedstock used during creation, to the point that the type of biodiesel used is not always stated in previous studies (Ackland et al., 2007; Hawley et al., 2014). Since health impacts are known to change depending on feedstock type (Chapter 3, (Landwehr et al., 2021)), this makes attempts to clarify biodiesel toxicity difficult. Furthermore, methodologies used to test toxicity vary greatly with engine configurations (Brito et al., 2010; de Brito et al., 2018; Hemmingsen et al., 2011; Magnusson et al., 2019), exhaust after-treatment technologies (V. André et al., 2015; Gioda et al., 2016; Magnusson et al., 2017), exhaust dilutions (de Brito et al., 2018; Douki et al., 2018), mineral diesel reference fuels (Brito et al., 2010; Mullins et al., 2016) and toxicological measurements (Adenuga et al., 2016; Gioda et al.,

2016; Mutlu et al., 2015) to the point that meaningful comparisons between different studies are virtually impossible (Larcombe et al., 2015; Møller et al., 2020).

Another limitation of prior literature is the tendency to focus solely on the particle components of the exhaust, ignoring the health impact of the gaseous components entirely (V. André et al., 2015; Larcombe et al., 2015). Exhaust particles are generally collected on filters and extracted using solvents to be added directly to the media of cell lines or the Ames bacterial mutagenicity assay to test cytotoxicity and mutagenicity respectively (J. Bünger et al., 2000; Cervena et al., 2017). A strength of this approach is that the exact deposition amount of the particles added during exposure is known (Cervena et al., 2017), however collecting particles on a filter often removes the ultrafine particles entirely as particles agglomerate to create an artificial particle size spectra (Morin et al., 2008) and the health impact of the gaseous components is removed.

With the aforementioned limitations in mind, the aim of this study was to assess the exhaust toxicity of two different biodiesels and their 20% blends in a 3D primary airway epithelial cell model that accurately mimics human lung formation (Martinovich et al., 2017). We chose Tallow and Canola biodiesel as both feedstock types are amongst the most popular types currently in use (ARENA, 2018; OECD/FAO, 2020) and our previous study found them to be at opposite ends of the toxicity spectrum of six different biodiesel feedstocks, with Tallow being more toxic than ultra-low sulfur diesel (ULSD) and Canola being less (Chapter 3, (Landwehr et al., 2021)). Furthermore, we hypothesised that Tallow would be the most toxic feedstock type and Canola the least and mimic our observations made using monolayer cultures. Collectively, results generated are the first to expose fully differentiated primary human airway epithelial cells to multiple biodiesel exhausts, using an ALI model and exhaust generated from an engine paired with modern exhaust after-treatment devices (both a diesel particulate filter and oxidation catalyst).

5.2 Materials and Methods:

5.2.1 Fuel Types: Commercial ULSD was obtained from local suppliers (SHELL, WA, AUS, biodiesel free, <10ppm sulfur). Two different biodiesel types and their respective 20% blends within ULSD were also used in this study. Canola and Tallow biodiesel were created using high quality, food grade commercial oils/fats (Campbells Wholesale Reseller, WA, AUS). All oils were converted to fatty acid methyl esters (FAME) using an established sodium methoxide transesterification process (Gerhard Knothe et al., 2015, Landwehr et al., 2021).

5.2.2 Participants: This study was approved by the St John of God Hospital Human Ethics Committee (901). Airway epithelial cells were derived from brushings of the nasal mucosa of children as previously described (Kicic et al., 2006; Lane et al., 2005). Informed parent/guardian permission was obtained prior to brushings obtained from healthy, non-atopic volunteers (six total, aged 2-9 years, 3 males) undergoing elective surgery for non-respiratory related conditions. Atopy was determined using a radio-allergo-sorbent test (RAST) for a panel of 8 common childhood allergens. Volunteers positive for atopy, clinically diagnosed with chest infections (bacterial or viral) or any underlying chronic respiratory disease such as asthma were excluded.

5.2.3 Tissue Culture: Primary airway epithelial cell cultures and differentiated ALI models were established as previously described (Martinovich et al., 2017) and grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. All cells tested negative for mycoplasma. Cells were passaged weekly in Corning T75 tissue culture flasks (CLS430720, Corning ®, MERCK, NSW, AUS) and used for differentiation before passage 3 in all cases. For differentiation, cells were seeded at 250 000 cells per membrane onto transwell membranes (Corning® Transwell, 12mm with 0.4µm pore polyester membrane, MERCK, NSW, AUS), allowed to reach confluence over a period of three days and then air lifted. Cells were then differentiated for a minimum of 28 days in UNC-ALI media (K. Looi et al., 2018) before use in exposure experiments. Trans epithelial resistance was tested weekly and the final reading occurred just before exposures began (Supplementary Figure S5.2). Media was refreshed prior to all exposures. ALI models were grown in duplicate for every subject and exposure.

5.2.4 Exposure Methodology: All cultures were exposed for one hour to either air as a control or exhaust generated from a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) coupled with a dynamometer and fitted with Euro V/VI after-treatment technology consisting of a diesel particulate filter and oxidation catalyst (Daimler, Germany) (Landwehr et al., 2019). All exposures included cold start and a constant load of 40% with a speed of 2000 rpm. Exhaust was diluted 1:20 with air inside a mixing chamber attached to the exhaust piping and vacuumed into a sealed incubator at a rate of 10 L/min (Model 1535, Sheldon Manufacturing, OR, USA). The incubator containing the ALI models was maintained at 37ºC. Once the models were exposed, exhaust was vacuumed out for physico-chemical analysis of gas and particle properties (Figure 5.1). Exposure to air was used as a negative control.

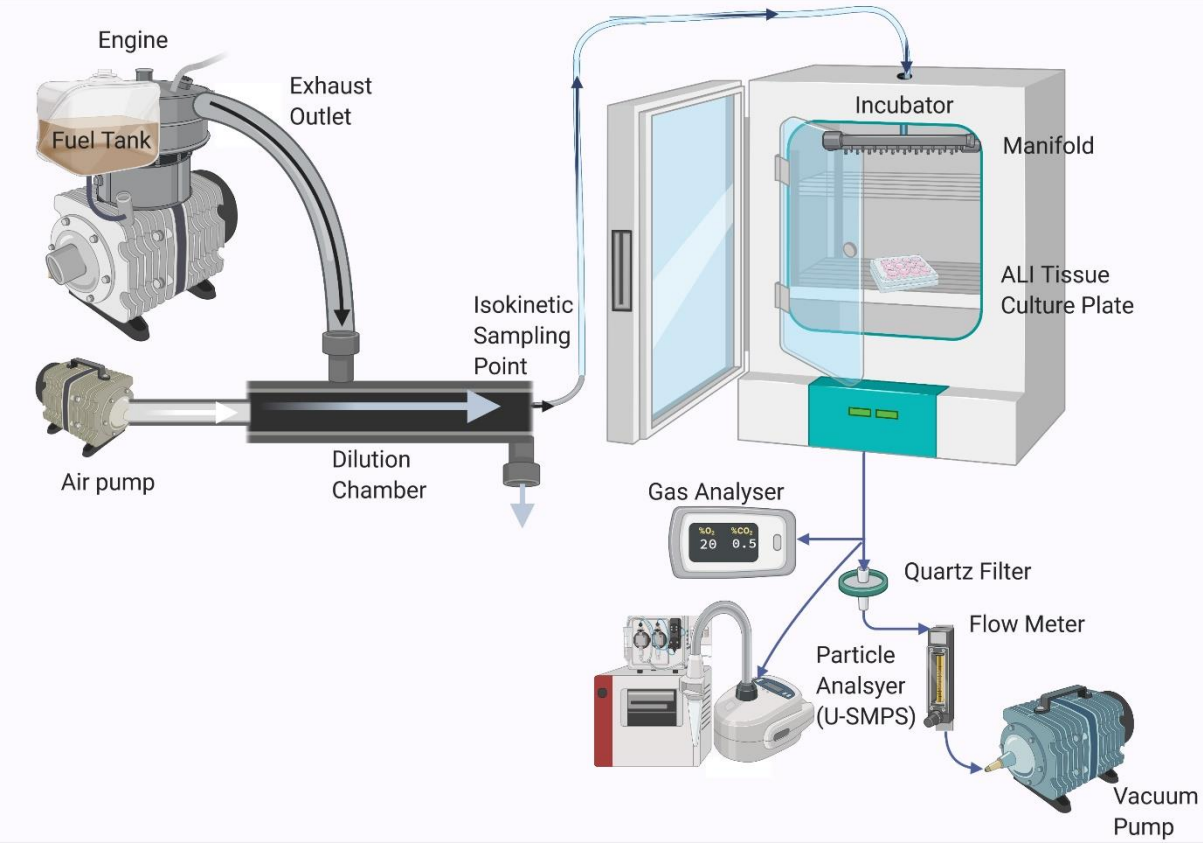


Figure 5.1: A diagram of the exposure set up. Modified from Chapter 2 (Landwehr et al., 2021). Created with Biorender.com.

5.2.5 Gas and Particle Analysis: As previously described (Landwehr et al., 2021), exhaust exiting the sealed incubator was analysed every 10 minutes for concentrations of common combustion gas products including O_2, CO, CO_2, NO_x (NO and NO_2) and SO_2 using a combustion gas analyser (TESTO 350, Testo, Lenzkirch, Germany). Similarly, exhaust was analysed every 10 minutes for particle concentrations between the sizes of 3 nm-340 nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Particles less than 10nm in size were excluded from further calculations due to high variability of measurements. Count-median particle size was calculated using the number of particles mean. Particle mass was calculated from particle spectra, assuming sphericity and using the 40% load diesel exhaust particle density as previously described (Olfert et al., 2007). Particle number was further separated into two fractions: nucleation mode particles below 23 nm in size and solid particles above 23 nm (Amanatidis et al., 2014). Particles for both B100 fuels and ULSD were collected on quartz filters (47mm, SKC, USA) and sent for PAH analysis using Gas Chromatography Tandem Mass Spectrometry at Queensland Health Forensic and Scientific Services (Queensland, Australia).

5.2.6 Permeability: After being left to rest for 24 hours, permeability of the ALI models was analysed as previously described (Kevin Looi et al., 2016). Briefly, transepithelial electrical resistance was measured using a Epithelial Volt/Ohm (TEER) Meter (EVOM2 with chopstick

electrode set, MERCK, AUS) to assess model integrity (Supplementary Figure S5.2) before cultures underwent a fluorescent dextran permeability assay. Fluorescein isothiocynate labelled dextran beads (MERCK, NSW, AUS) were dissolved in HEPES buffered Hank's Balanced Salt Solution (HEPES-HBSS) (4 kDa beads, final concentration 2 mg/ml) and 0.5mL was added to the apical compartment of each ALI insert. 1.5 mL of fresh HEPES-HBSS buffer without dextran beads was added to the basal compartment and cultures were placed on an orbital shaker within an incubator at 37°C in an atmosphere of 5%CO_2/95% air for 6 hours to allow agitation to help beads flow from the apical to the basal compartment. Basal compartment samples of 0.75 mL were taken at 0, 0.5, 1, 2, 3, 4, 5 and 6 hours with fresh buffer replacing the sample. Apparent permeability was then calculated using the equation $P_{app} = (dQ/dt) \times (1/AC_0)$, where dQ/dt is the steady-state flux, A is the surface area of the membrane and C_0 is the initial concentration in the basal compartment.

5.2.7 Histology: After permeability, inserts were fixed in 10% formalin for 30 minutes and stored in 100% ethanol until all samples were collected. Inserts were then embedded in paraffin, and 5-μm thick sections sliced for hematoxylin staining as per manufacturers protocol (Thermofisher Scientific, MA, USA).

5.2.8 Protein Concentration: After being left to rest for 24 hours, protein concentration of the insert lysate and apical and basal supernatant was assessed using a Pierce™ BCA protein assay kit (Thermofisher Scientific, MA, USA). Insert lysate was collected from half the insert after permeability analysis, basal supernatant was collected from the basal compartment of each exposed ALI culture (1.5 mL total) and apical supernatant was collected by performing a 0.5 mL media wash of the apical compartment.

5.2.9 Mediators: Mediator release was assessed 24 hours after exposure for both the apical and basal compartments using a Bio-Rad 27plx human cytokine kit (Bio-rad, CA, USA) and accompanying software (Bio-Plex Manager, v6.1.1, Bio-Rad, Tokyo, Japan). The 27 mediators analysed can be further split into mediators that affect the innate and adaptive immune systems or act as regulators (Dayer et al., 2017; Duffy et al., 2013; Holdsworth & Gan, 2015; Sokol & Luster, 2015). Normalisation was performed to adjust supernatant concentrations to 1ml and all mediator concentrations were normalised to total protein lysate for each exposure group.

5.2.10 Statistical Analysis: Data are presented as mean ± standard deviation where indicated. All statistical analyses were performed using R statistical software (v3.4.3) (R Team, 2018) loaded with the packages “lme4” and “mgcv”. P-values less than 0.05 were considered significant. As previously described (Landwehr et al., 2021), all statistical analyses excluding gas concentration data were completed using multivariate general linear modelling methodologies with the families “Gamma(inverse/log)” and “gaussian(log)” as best fit the data, applying a backwards elimination approach to remove insignificant predictive variables. For combustion gas analysis a separate General Additive Model (GAM) file was fitted to each gas measurement with concentration as the response variable and time as the predictor, thus allowing for non-parametric fits.

5.3 Results:

5.3.1 Gas Analysis: Mean and standard deviation for each fuel and gas type are shown (Table 5.1), with the exception of CO which shows only the highest reading at the 10 minute mark due to the cold start effect on the performance of the catalytic converter. Trends over time can be found in the supplementary materials (Figure S5.1). All fuels displayed similar trends over time with NO_x (NO and NO_2), CO_2 and SO_2 increasing rapidly in the first half of the exposure, O_2 decreasing rapidly in the first 20 minutes and CO peaking in the first 10 minutes before decreasing rapidly to undetectable concentrations. Of the four fuels tested we found Canola B20 to be the most different to ULSD with significantly increased O_2 and significantly decreased SO_2 and NO_x in the form of NO and NO_2 (Table 5.1: $p<0.05$). Canola B20 was also the most different to its B100 counterpart with four significant differences compared to Tallow B20's one (Table 5.1: $p<0.05$).

Table 5.1: Mean (standard deviation) gas measurements for all exhausts. All significances displayed are compared to ULSD. Measurements are shown as the mean concentration for the entire exposure, with the exception of CO which is shown as the peak measurement.

Fuel	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
O_2 (%)	20.63 (0.105) a	20.74 (0.062) ***,b,c	20.64 (0.146) a	20.67 (0.082) a	20.64 (0.077)
CO (ppm)	0.80 (0.20) d	1.23 (0.67)	1.27 (1.19)	0.80 (0.20) d	1.07 (0.50) *,c
CO_2 (%)	0.35 (0.098)	0.25 (0.145)	0.33 (0.068)	0.31 (0.112)	0.34 (0.101)
NO_x (ppm)	11.47 (2.26) a,b,c	7.82 (1.78) ***,b,c,d	7.75 (1.55) ***,a,c,d	10.43 (2.22) * a,b	10.44 (2.21) a,b
NO (ppm)	7.09 (1.52) a,c	4.94 (1.02) ***,c,d	5.51 (1.18) *** c,d	6.83 (1.41) a,b	7.34 (1.62) a,b
NO_2 (ppm)	4.39 (1.06) a,b,c,d	2.89 (0.83) ***,b,c	2.25 (0.41) ***,a,c,d	3.59 (0.90) ***,a,b,d	3.10 (0.68) ***,b,c
SO_2 (ppm)	0.83 (0.38) b	0.89 (0.32) b	0.50 (0.51) ***,a,d	0.94 (0.23)	0.94 (0.24) b

* Significantly different to ULSD (*=p <0.05, **=p <0.01, ***=p <0.001)
a=significantly different to Canola B20 ($p<0.05$)
b=significantly different to Canola B100 ($p<0.05$)
c=significantly different to Tallow B20 ($p<0.05$)
d=significantly different to Tallow B100 ($p<0.05$)

5.3.2 Particle Analysis: Particle spectra between the sizes of 10 nm-340 nm were obtained for each exhaust (Figure 5.2). All fuels displayed small peaks in particle size around the 100 nm mark. In terms of particle number concentration, no significant differences were found between any of the fuels (Figure 5.2). Median particle size and particle mass were also calculated from the particle spectra and found to be similar (Table 5.2). Of the 28 PAHs tested in filter collected particulate matter, only 3 were found at concentrations above the limit of detection (Supplementary Table S5.1).

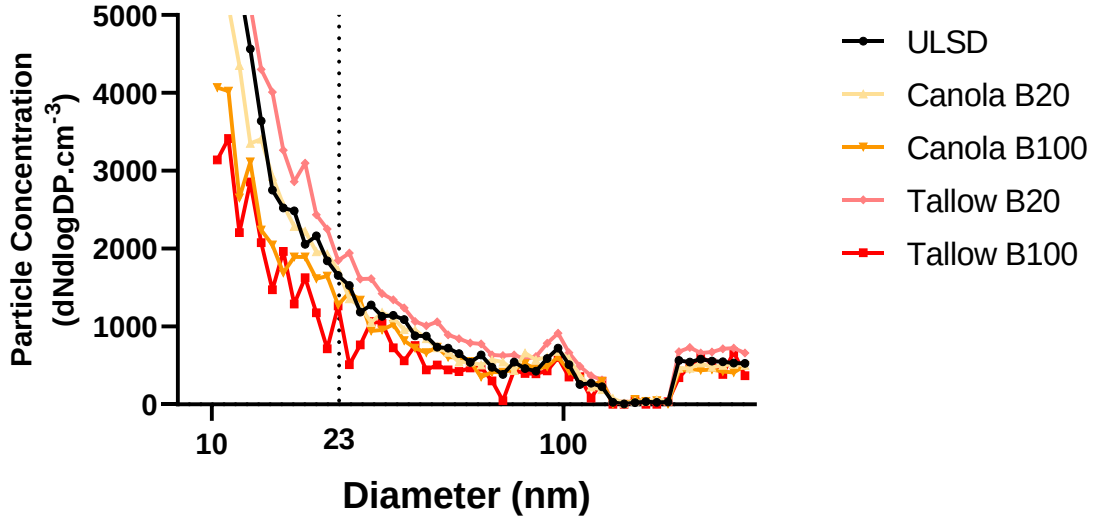


Figure 5.2: Particle size spectra for all fuels. Data were analysed using total particle number concentration between the size of 10 and 340 nm for each fuel. The dotted line indicates the particle size of 23nm. No significant differences were found between total particle number.

5.3.3 Histology, Permeability and Protein Concentration: No morphological differences were observed between Air and exhaust exposed inserts (Figure 5.3, representative images). Exposure to the exhaust of Tallow B100 and Tallow B20 resulted in significantly increased permeability, (1.33±0.67 and 1.52±0.41 fold change respectively) to that of Air exposure ($p<0.05$) (Figure 5.4). Tallow B20 exhaust also significantly increased permeability in comparison to ULSD and Canola B20 ($p<0.01$).

Exposure to both Tallow B100 and Canola B100 also resulted in significantly increased protein concentration in both the basal and apical compartments ($p<0.05$) (Figure 5.5). Exposure to Tallow B20 resulted in increased protein concentration only in the apical supernatant and exposure to ULSD resulted in increased protein concentration only in the basolateral supernatant ($p<0.01$).

Table 5.2: Particle characteristics between the sizes of 10-340 nm for all fuels. Data is displayed as the mean value for all fuels. Data in circular brackets is a ratio in comparison to ULSD, data in square brackets is a percentage of the total within the fuel.

Particle Characteristic	Fuel				
	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
Particle Mass Concentration ($\mu g/m^3$)	30.18	28.10 (0.93)	25.13 (0.89)	38.27 (1.52)	26.74 (0.70)
Median Particle Size (nm)	17	17	18	17	18
Total Particle Number ($particles/cm^3$)	62138	58100 (0.94)	46849 (0.75)	74779 (1.20)	38595 (0.62)
Particle Number >23 nm ($particles/cm^3$)	40864 [65.76%]	37218 [64.06%]	28163 [60.11%]	47567 [63.61%]	23197 [60.10%]
Particle Number <23 nm ($particles/cm^3$)	21274 [34.24%]	20882 [35.94%]	18686 [39.89%]	27212 [36.39%]	15399 [39.90%]

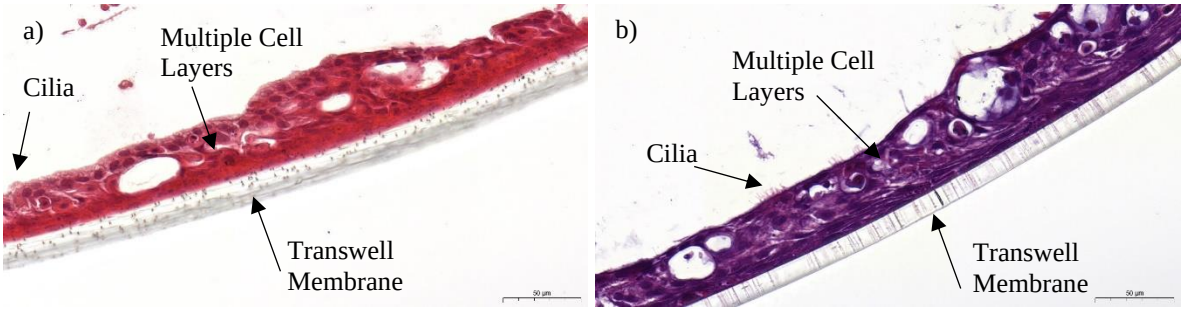


Figure 5.3: Representative morphological images of ALI cultures exposed to a) Air and b) Tallow B100. No differences in morphology were found after any of the exposures. Scale bars on the bottom right of each image indicate 50 µM in size.

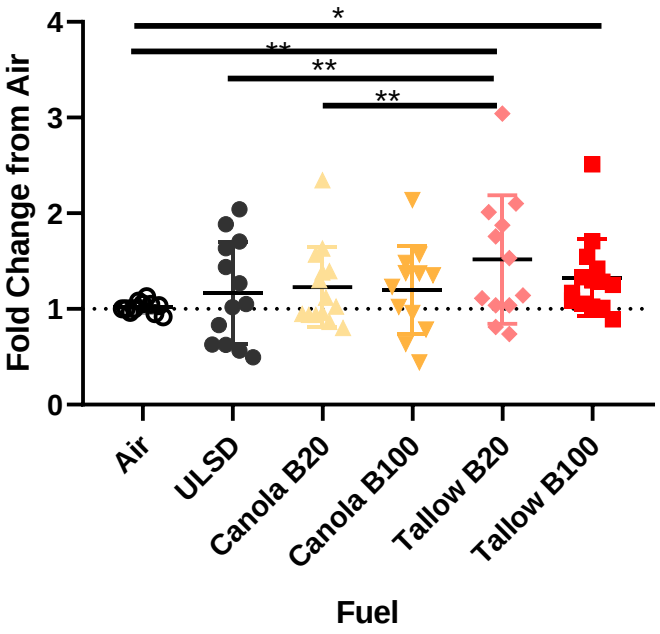


Figure 5.4: Permeability measurements normalised to air controls. Permeability is measured as apparent permeability (Papp Coefficient) and normalised to fold change compared to Air for each fuel (*=p value<0.05, **=p value<0.01).

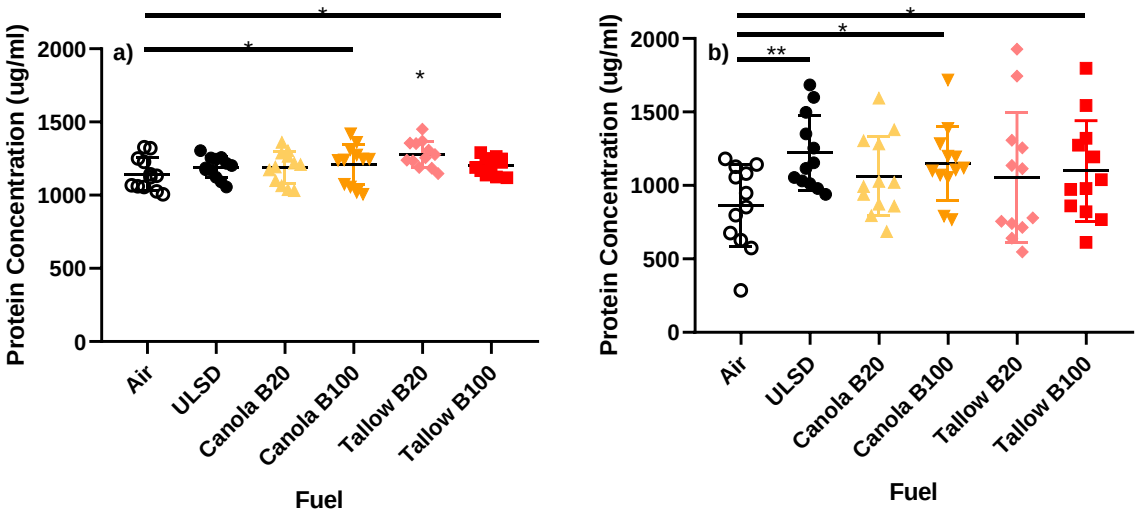


Figure 5.5: Protein concentration in a) apical wash and b) basal supernatant (*= p<0.05, **= p<0.01).

5.3.4: Mediator Release: Mediator release was measured for both the apical and basal supernatants (Tables 5.3 and 5.4). Of the 27 mediators analysed, 16 were released at levels above the limits of detection, 15 for each compartment. PDGF-bb was only measured above the limit of detection in the apical supernatant, whereas VEGF was only measured above the limit of

detection in the basal supernatant. The 15 mediators released in the apical compartment can be translated as local mediator release (Floreth et al., 2011) and significant differences primarily impacted the innate immune system with differences found in 3 regulatory, 4 adaptive and 7 innate mediators ($p<0.05$) (ElKassar & Gress, 2010; Holdsworth & Gan, 2015; Sokol & Luster, 2015). In comparison to Air exposed controls, Tallow B20 was the most immunogenic locally with significantly altered release of 11 mediators, followed by 10 for Tallow B100 whereas Canola B100 was the least with only two altered mediator productions.

The mediator release in the basal compartment (Table 5.4) can be interpreted as systemic mediator production (Floreth et al., 2011) and was also found to primarily impact the innate immune response with 3 regulatory, 4 adaptive and 7 innate mediators ($p<0.05$) (ElKassar & Gress, 2010; Holdsworth & Gan, 2015; Sokol & Luster, 2015). Systemic inflammation was impacted more than local with more exhaust exposures significantly altering mediator production; both IL-1RA and IL-6 were significantly released in all exhaust in the basal compartment compared to Air exposed controls. Tallow B20, Tallow B100 and Canola B20 exhaust exposures induced the greatest mediator responses with the significant increase of 7 mediators each compared to Air exposed controls, although Canola B20 impacted the regulatory response more with significantly increased release of VEGF compared to Tallow B20 and B100 which impacted the innate response more with the increased release of TNF-α ($p<0.05$).

Table 5.3: Mean (standard deviation) mediator release for the 15 cytokines released above the limits of detection for the apical supernatant samples.

Mediator Concentration (pg/mL/mg protein)	Fuel					
	Air	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
IL-1RA	659.37 (987.96)	1264.47 (2113.32)****	1210.17 (1556.21)** d	841.87 (1161.45) ## c,d	1432.94 (1913.46)**** b	1461.20 (2172.96)**** a,b
IL-5	7.23 (8.77)	8.23 (11.56)	10.46 (9.70) b,c	15.76 (23.00)** ## a,c,d	21.91 (37.89)**** ### a,b,d	13.56 (16.31) b,c
IL-6	89.93 (68.02)	130.79 (62.73)	140.25 (79.56)	159.47 (194.00)	234.04 (234.52)*	218.19 (131.65)*
IL-7	23.93 (4.11)	24.30 (6.53)	30.96 (6.72)** # b,d	18.73 (6.88) # a,c	27.59 (8.49) b	22.81 (8.68) a
IL-8	3744.90 (1655.76)	5717.74 (2311.66)*	6311.03 (4263.62)** b	3796.56 (1167.34) # a,c,d	6984.61 (4318.23)** b	7232.75 (4919.93)** b
IL-9	44.04 (7.91)	59.07 (11.55)**	60.27 (16.84)** b	44.24 (11.44) ## a,c,d	59.20 (20.41)** b	60.44 (14.73)** b
G-CSF	40.96 (64.41)	93.08 (107.09)**	61.56 (62.05) d	35.80 (42.43) ## c,d	140.31 (175.58)*** b	130.54 (88.98)*** a,b

GM-CSF	7.17 (5.48)	9.79 (8.34)	8.25 (5.40)	6.18 (4.82) # d	8.55 (6.49)	9.54 (10.42) b
IFN-γ	3.62 (6.35)	8.30 (8.34)	8.32 (5.40)**	5.62 (4.82)	9.52 (6.49)**	9.06 (10.42)*
IP-10	486.74 (276.17)	326.96 (159.21)*	484.45 (250.72) # b	311.37 (292.31) a	403.84 (293.32)	360.41 (163.04)
MCP-1	8.70 (5.38)	11.66 (8.03)	11.70 (7.22) c,d	14.09 (15.07)	22.48 (18.53)** # a	24.25 (29.85)** # a
PDGF-bb	26.57 (26.64)	18.85 (14.41)	44.03 (37.02)** ### b,c,d	13.12 (13.70)* a	9.54 (5.29)* a	14.38 (16.20)* a
MIP-1β	10.95 (2.31)	14.66 (2.28)***	15.53 (3.92)*** b	11.16 (2.89) ## a,c,d	14.40 (4.33)** b	15.31 (4.01)*** b
RANTES	13.80 (8.82)	14.34 (11.47)	12.54 (8.83)	9.80 (5.83)	8.69 (5.48) #	12.00 (7.05)
TNF-α	15.15 (11.88)	24.24 (18.87)*	21.34 (16.19)	16.27 (10.84) c,d	26.01 (18.27)** b	24.14 (21.99)** b

=significantly different to Air (=p <0.05, **=p <0.01, ***=p <0.001)
#=significantly different to ULSD (#=p <0.05, ##=p <0.01, ###=p <0.001)
a=significantly different to Canola B20 (p<0.05)
b=significantly different to Canola B100 (p<0.05)
c=significantly different to Tallow B20 (p<0.05)
d=significantly different to Tallow B100 (p<0.05)

Table 5.4: Mean (standard deviation) mediator release for the 15 cytokines released above the limits of detection for the basal supernatant samples.

Mediator Concentration (pg/mL/mg protein)	Fuel					
	Air	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
IL-1RA	184.66 (69.65)	240.36 (156.58)**	329.16 (124.33)**** d	247.56 (149.94)**	256.57 (139.03)**	253.50 (126.46)* a
IL-5	2.07 (2.50)	1.94 (2.02)	2.40 (2.92)	1.00 (0.27)*	2.16 (2.16)	1.81 (2.29)
IL-6	2.15 (1.37)	11.25 (13.92)**	9.79 (11.36)*	8.29 (8.92)*	14.24 (18.26)**	12.88 (22.07)***
IL-7	2.66 (1.29)	2.15 (1.35)	2.97 (2.00)	2.00 (1.67)	2.44 (1.39)	1.70 (1.27)
IL-8	1417.99 (1425.95)	2798.50 (2455.00)*	2956.48 (2188.96)**	1548.90 (683.90)	3772.63 (1982.51)***	3244.75 (2073.16)**
IL-9	14.61 (5.62)	20.88 (7.07)**	24.92 (8.09)****	16.10 (3.24)	22.19 (5.41)****	22.10 (6.54)****

G-CSF	6.12 (6.40)	19.83 (24.86)***	18.44 (18.37)**	8.72 (7.36)	28.30 (19.17)****	26.68 (19.19)****
GM-CSF	1.34 (1.14)	2.34 (1.74)**	2.55 (1.74)**	1.74 (1.14)	1.76 (0.99)	2.19 (1.65)*
IFN-γ	0.91 (0.50)	1.25 (1.14)*	1.74 (0.80)**	1.09 (0.87)	1.43 (0.89)**	1.19 (0.72)
IP-10	31.74 (20.41)	31.79 (22.51)	41.04 (23.48)	30.10 (31.59)	35.08 (21.11)	35.64 (22.37)
MCP-1	2.02 (1.50)	3.87 (3.45)	3.81 (3.73)	4.36 (5.06)*	6.69 (7.68)**	4.09 (2.45)*
MIP-1β	3.76 (1.25)	5.33 (2.21)**	6.54 (2.07)****	4.13 (1.02)	5.78 (1.40)***	5.64 (1.85)**
RANTES	0.85 (0.74)	1.12 (0.92)	1.42 (1.46)	1.13 (1.00)	1.43 (1.35)	0.92 (0.49)
TNF-α	3.58 (3.98)	5.27 (4.20)	5.90 (4.71)	4.13 (2.94)	7.00 (5.03)**	6.86 (5.13)*
VEGF	97.23 (58.58)	106.18 (63.26	165.32 (45.59)***	93.26 (35.08)	111.19 (51.74)	102.33 (44.20)

=significantly different to Air (=p <0.05, **=p <0.01, ***=p <0.001)
#=significantly different to ULSD (#=p <0.05, ##=p <0.01, ###=p <0.001)
a=significantly different to Canola B20 (p<0.05)
b=significantly different to Canola B100 (p<0.05)
c=significantly different to Tallow B20 (p<0.05)
d=significantly different to Tallow B100 (p<0.05)

5.4 Discussion:

Results of this study show that exposure to highly diluted biodiesel or diesel exhaust elicits a range of health impacts in a primary human airway epithelial ALI exposure model. Of the two biodiesels tested we found Tallow to be the most toxic, with both the 20% blend and the pure biodiesel exhausts inducing increased permeability of the epithelial barrier, increased protein concentrations in the apical and/or basal compartment (suggesting epithelial cell damage) and the broadest range in mediator release in both the apical and basal compartments. This was unexpected considering that ULSD exhaust contained the highest concentrations of exhaust gas components and no differences were found between any of the different fuel types for exhaust particle characteristics. Subsequently, we found Canola biodiesel to be the least toxic of the tested fuels, with no effect on permeability and the smallest impact on mediator release.

In terms of exhaust characteristics, Canola B20 was the most different to diesel exhaust, with three of the six gases tested being significantly different in ways that indicate less toxicity with significantly increased oxygen concentration and significantly decreased NO and NO_2 concentration leading to an overall decrease in NO_x. All B20 and B100 exhausts were found to have decreased NO_2 concentrations (compared with ULSD), and Canola B100 and Canola B20 exhausts displayed a decrease in NO levels. This is in contrast to previous studies which have found NO_x levels to be increased in the exhaust of biodiesel when compared to mineral diesel (de Brito et al., 2018; Graver et al., 2016), although reports on biodiesel blends are contradictory

with studies showing both more and less NO_x (Graver et al., 2016; Mullins et al., 2016). This difference to previous literature observed in our study could be attributed to some effect of biodiesel on exhaust after treatment devices, which are known to impact exhaust NO_x concentrations (Ko et al., 2019), as many of the previous studies that assessed biodiesel health effects used old technology engines not equipped with exhaust after-treatment devices (Larcombe et al., 2015). For example, use of biodiesel in an engine equipped with a diesel particulate filter (DPF) has been found to lower particle loading and shorten regeneration time compared to ULSD, however, biodiesel also reacted more readily with the lubricating oil which in turn caused a slower rise in DPF inlet temperature (Pechout et al., 2019). All these effects would impact the concentrations of various exhaust components.

We found no significant differences in the particle characteristics between any of the tested fuels in the range of 10-340 nm, likely because of the high dilutions used. Previous studies have found differences in particle mass concentrations to be subtle enough that a 1/20 dilution would negate any differences between fuels (de Brito et al., 2018), and this is especially obvious in engines equipped with exhaust after-treatment technology like the one used in this study (Valand et al., 2018). This suggests that the increased toxicity observed after Tallow biodiesel exhaust exposures was not caused by an increase in ultrafine particles, as has been suggested by previous studies (Lankoff et al., 2017; Mullins et al., 2016). Since all exhausts had similar fine particle concentrations and the combustion gas concentrations were highest in ULSD, this suggests that Tallow biodiesel exhaust and Tallow biodiesel blend exhaust toxicity is associated with an exhaust component that has not been broadly tested for in this study, such as PAH's or heavy metals(Fontaras et al., 2009; Kowalska et al., 2017). Although we attempted to analyse PAH concentrations for Diesel and Canola and Tallow B100, collected particle deposits were so low than only 3 of the 28 tested PAH's were found at concentrations above the limit of detection (Supplementary Table S5.1). These concentrations were highest in Tallow B100 exhaust however an analysis of 3 PAHs cannot be considered comprehensive and previous studies have found tallow biodiesel to contain lower levels of non-volatile organic compounds and particulate semi-volatile organic compounds than other biodiesel types (Cheng et al., 2017; Schirmer et al., 2016).

One of the more concerning implications of this study is that we found considerable toxic health effects despite exhaust parameters being within Australian Work Standards, which is also used as a guideline for European standards (EU-OSHA, 2013) and is equal to or stricter than the US Occupational Safety and Health Administration standards (OSHA, 2021). The Safe Work Australia standards for various exhaust components are time weighted 8 hour averages of 3 ppm for NO_2 (with concentrations not exceeding 5 ppm over a 15 minutes average), 25 ppm NO, 2 ppm SO_2 (with concentrations not exceeding 5 ppm over a 15 minutes average), 30 ppm CO and 5000 ppm CO_2 (with concentrations not exceeding 30000 ppm over a 15 minute average) (SWA, 2019). Oxygen must not fall below "safe levels" of 19.5% (SWA, 2018). It is recommended that in Australia, particulate matter exposure from diesel exhaust not exceed 100 $\mu g/m^3$ elemental carbon, although this is not a hard limit (AIOH, 2017). In America the limit for a non-coal mining setting is set at 160 $\mu g/m^3$ total carbon (MSHA, 2016) and the European Union has set a

recent occupational exposure limit of 50 ug/m^3 elemental carbon (EU, 2019; EU, 2004). The diluted exhaust used in this study meets all these limits.

Despite the exhaust used in this study being "safe" in terms of Australian Work Standards, we measured increased airway epithelial barrier permeability after just one hour of exposure to Tallow B100 and B20 in comparison to Air controls. The airway epithelium is designed to act as a first line of defence against insults from viruses, bacteria and other environmental insults such as diesel exhaust (Celebi Sözener, Cevhertas, Nadeau, Akdis, & Akdis, 2020; Faber, McNabb, Ariel, Aungst, & McCullough, 2020; K. Looi et al., 2018). Increased permeability compromises this function and allows these insults to invade the underlying lung tissue (Faber et al., 2020), providing a potential mechanism for entrance into the cardiovascular system (Brook et al., 2010; Cho et al., 2018; Neophytou et al., 2019). Since previous biodiesel exhaust studies have found indications of cytotoxicity in submerged cell line cultures, this indication of increased permeability could provide a functional consequence of that cytotoxicity (Agarwal et al., 2018; J. Bünger et al., 2000). As ultrafine particles are capable of bypassing this barrier to enter the bloodstream directly (Brook et al., 2010; Celebi Sözener et al., 2020) an increase in epithelial barrier permeability would only amplify this effect and likely contribute to even worse health outcomes. Since concentration of ultrafine particles does not change between exhausts and yet we only found increased permeability in the Tallow exposure groups, it is likely one or more different exhaust components are contributing to the increased permeability, which would potentially have a synergistic effect with the ultrafine particles.

In addition, if ULSD exposure at higher concentrations also causes increased barrier permeability then this could help explain why previous studies, including those by Gowdy et al (2010), Zarcone et al (2017) and Shears et al (2020) have found dual insults of diesel exhaust exposure and respiratory pathogens such as influenza, non-typeable *H influenzae* and *S pneumoniae* to increase the severity of disease (Gowdy et al., 2010; Shears et al., 2020; Maria C. Zarcone et al., 2017). Increased permeability would help the virus or bacteria infiltrate the airway epithelium, potentially facilitating infection and increasing disease severity (Fukuoka, Matsushita, Morikawa, Takano, & Yoshimoto, 2016; K. Looi et al., 2018; Shears et al., 2020). Alvarez-Simón et al 2017 have also been successful when using diesel exhaust to sensitise mice to soy protein to simulate an allergic asthma model (Alvarez-Simón et al., 2017). Increased permeability would also facilitate passage of allergens across the epithelial barrier which would be a crucial process in driving allergic responses (Celebi Sözener et al., 2020). Since only small amounts of Tallow biodiesel exhaust is needed to induce increased barrier permeability, this has concerning implication for human exposure in areas where Tallow biodiesel is already in use (ARENA, 2018; EIA, 2020b; Flach et al., 2019; Toldrá-Reig et al., 2020).

We also found altered mediator release in both the apical and basal compartments after exhaust exposure. The mediators measured have a variety of effects and can impact both the innate and adaptive immune responses (Holdsworth & Gan, 2015). As the ALI culture models human lung formation, mediator changes in the apical compartment can be interpreted as changes more relevant to local inflammation of the airway lumen, whereas changes in the basal compartment can be interpreted as relevant to the basement membrane of the airway and thus a more systemic reaction (Floreth et al., 2011). Of the 27 mediators tested, we found 15 were released above

measurable concentrations in both the apical and basal compartments. These 15 cytokines mostly overlapped, however VEGF (which helps promote angiogenesis and lung injury repair (Boussat et al., 2000)) was released only in the basal compartment and PDGF-bb (which helps promote wound repair but is also connected with airway hyperresponsiveness (Kardas et al., 2020)) was released only in the apical compartment. Of the 5 exhausts tested, Tallow B20 and Tallow B100 were the most immunogenic in the apical compartment and Tallow B20, Tallow B100 and Canola B20 were the most immunogenic in the basal compartment. Canola B100 was the least immunogenic in both compartments, which supports our previous work (Landwehr et al., 2021).

The differences seen between apical and basal mediator release, where more mediators are released for all 5 exhaust exposures in the basal compartment, are indicative of the mediator response to exhaust being driven mostly through systemic inflammation, with local airway lumen inflammatory responses in the apical compartment only occurring after exposure to the more inflammatory exhausts. This is understandable as many immune cells, such as neutrophils, would need to be recruited to the site of insult (Sokol & Luster, 2015) and the local inflammation mediators released apically for the more inflammatory Tallow B20 and Tallow B100 exposures primarily impact the innate immune response (Holdsworth & Gan, 2015; Sokol & Luster, 2015). Previous studies looking into the impact of diesel exhaust exposure on workers have found indications of systemic inflammation (H. Wang et al., 2017), and our results indicate that exposure to any of the pure biodiesel or blended exhausts will likely result in similar, or worse, responses.

No cytokine was released apically for all exhausts. In the basal compartment, only two cytokines were released for all exhaust: IL-1RA and IL-6. IL-1RA helps to modulate the release of IL-1, which is classified as an acute phase inflammatory cytokine (Holdsworth & Gan, 2015). This indicates that exposure to any exhaust causes some level of inflammation to the airway epithelium as the release of IL-1 needs to be modulated. A study into the effects of diesel and biodiesel exhaust exposure on mice has previously found the release of IL-1α and IL-1β to be significantly increased after biodiesel exhaust exposure in the lungs (Yanamala et al., 2013), and so this observed increase in IL-1RA to modulate IL-1 in both the apical and basal compartments is unsurprising. The remaining cytokine released for all exposures, IL-6, is also classified as an innate acute inflammatory cytokine and has additional potent local inflammatory effects (Holdsworth & Gan, 2015). Significantly increased release of IL-6 has been previously found in the bronchial wash of non-asthmatics exposed to diesel exhaust (Annelie F Behndig et al., 2011). In addition, IL-8, MIP-1β and G-CSF, which are also released basally in at least 4 of the 5 exhaust exposures, act as innate chemoattractants for natural killer cells and neutrophils (Cox et al., 1992; Garofalo & Haeberle, 2000; Holdsworth & Gan, 2015; Lloyd, 2002) and have previously been found to increase after diesel exhaust exposure in animal and human epithelial cell studies (Boland et al., 1999; Yanamala et al., 2013). Our previous studies (chapters 2, 3 and 4, (Landwehr et al., 2021; Landwehr et al., 2019)) in submerged models also found increased G-CSF to be a driving mediator in the response to diesel and biodiesel exhaust exposures. In the present study, MIP-1β was decreased after exposure to Tallow B100 and Tallow B20 exhaust, but increased after other exposures, suggesting immune dysregulation after Tallow exhaust exposure. A previous study into highly exposed diesel exhaust engine workers also found

dysregulation of serum MIP-1β concentrations, with significant reductions observed in workers exposed to the highest exhaust concentrations (Dai et al., 2018).

The final cytokine released basally in at least 4 of the exhaust exposures is IL-9, which is involved in the adaptive immune response, promoting antigen presentation and increasing bronchial hyperresponsiveness and allergic airway responses (Little et al., 2001; Zhou et al., 2001). To the best of our knowledge IL-9 has not been tested outside of our group. IL-9 has been implicated as one of the driving cytokines in asthma (Zhou et al., 2001) so its release after diesel and biodiesel exhaust exposure could have concerning implications.

Previous studies that assess the health impacts of diesel and biodiesel exhaust focus almost exclusively on the particulate matter components of exhaust, generally ignoring the gaseous components entirely (V. André et al., 2015; Larcombe et al., 2015). In addition, they use filters to collect these particles and then expose submerged cultures directly to extracted particle solutions (Cervena et al., 2017; Gioda et al., 2016). While this method allows for accurate dosing and easier comparison between exposures, it removes both the effects of the gaseous components and the ultrafine particles which agglomerate on the filter leading to skewed particle size spectra (Morin et al., 2008). We exposed our cultures directly to dilute exhaust and found significant health effects after exposure to Tallow biodiesel even though the particle size spectra between the different exhausts did not change. This means that the ALI cultures in each exposure group were likely exposed to similar dosages of particles, so we get all the benefits of a submerged model and none of the weaknesses. This in turn means that the increased toxicity in the Tallow B100 and B20 exposure groups is a direct effect of the different exhaust components being more toxic than those of ULSD, not something that can be attributed to just having more particles within one exposure group. The same can be said for Canola, which we found to be less toxic than ULSD despite having similar exhaust particle profiles.

There are several limitations to our study. We used highly diluted exhaust concentrations in order to simulate real world exposure events, meaning that the health impacts observed are relatively small. Using more concentrated exposures may allow more differences between treatments to be identified but at the detriment to losing “real-world” applicability and one of the strengths of this study is that toxicological differences were observed despite using occupational exhaust concentrations. Our study also lacks a comprehensive particle chemistry analysis, in part because the exhaust concentrations used are so low that we could not collect enough particles for more than one type of analysis. By using airway-epithelial cells, even with an ALI model, we focus mostly on the toxicological effects of exposure on the lungs, missing the potential effects of exposure to other biological systems. We also used primary cells obtained from “healthy” patients, meaning that those with underlying respiratory conditions or diseases could have different health impacts to what was found in our study. Finally, we used two different first-generation biodiesel types chosen based on current biodiesel usage, when in future biodiesel will likely be created from oil crops that do not compete with food prices.

5.5 Conclusion:

To the best of our knowledge, we are the first group to use ALI cultures for biodiesel exposure studies. We are also the first to use a permeability assay to assess exposure impacts for either diesel or biodiesel. We found exposure to Tallow biodiesel exhaust, both B100 and B20, to be the most toxic with increased permeability and the greatest mediator response. This was followed by ULSD and then Canola B100 and B20 exhaust. These results support our previous study into the toxic effects of different biodiesel exhaust exposures where we also found Tallow biodiesel to be the most toxic and Canola the least (Chapter 3) using a submerged culture experimental design which incorporated some additional endpoint measurements. This suggests that a less complicated submerged model can be used to assess the basic toxicity of different biodiesel fuels so long as whole exhaust is used, however for a more comprehensive assessment into the mechanisms of toxicity a more complicated model such as ALI or *in vivo* animal models is needed.

Acknowledgements: We would like to acknowledge and thank Professor Gerhard Knothe from the USDA Agricultural Research Service for providing input on the creation of the biodiesel fuel. We would like to acknowledge the Australian Research Council for funding via the Discovery Project Grant, as well as Curtin University's School of Population Health, 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.

Chapter 6: *In Vivo* Exposure Model

Abstract:

Background: Biodiesel is often promoted as a greener, more sustainable replacement for commercial mineral diesel fuel. As global production increases and usage becomes more widespread, concerns have been raised over the health effects of exhaust exposure. As biodiesel fuel properties and thus exhaust components, change depending on the source oil, the aim of this study was to compare the effects of exposure to exhaust generated by the combustion of diesel and biodiesel made from two different oils, Canola and Tallow. Both of these biodiesel types are currently in use worldwide.

Methods: Adult male BALB/c mice were separated into acute (2hr exposure) or longer term (2hr exposure daily for 8 days) groups and exposed to diluted exhaust from an engine running on diesel or biodiesel created from canola or tallow (or air as a control). Exhaust was characterised for toxic gases and particulate matter physico-chemical properties. Twenty-four hours after the last exposure health outcomes including lung volume, lung function, responsiveness to methacholine, protein and phospholipid concentration within the bronchoalveolar lavage and local and systemic inflammation were assessed.

Results: Exhaust gas composition varied significantly between fuels with diesel and tallow exhaust containing the highest levels of respiratory irritants including nitrogen oxides and carbon dioxide and canola the lowest. Particle size spectra did not differ between exhaust types and no significant differences were found between exhaust particle numbers.

In the acute exposure groups, exposure to tallow and diesel exhaust resulted in increased responsiveness to methacholine ($p<0.05$) compared with air control. In contrast, after chronic exposure only tallow exhaust exposed mice displayed hyperresponsiveness whereas canola were less responsive than the air controls. This is indicative of tallow and canola biodiesel exhaust impacting lung function via different mechanisms.

Bronchoalveolar lavage cellular inflammation was significantly higher in mice exposed acutely to tallow or chronically to diesel or tallow ($p<0.05$) compared with air controls. Higher protein content was also observed in the lavage of mice chronically exposed to tallow exhaust ($p<0.05$), suggesting exposure induced epithelial damage. In contrast, local and systemic mediator release was decreased in biodiesel and diesel exhaust exposed mice compared to air exposed controls, suggesting immune dysregulation and an inability for the mice to respond appropriately to exhaust exposure.

Conclusion: Tallow biodiesel exhaust exposure resulted in significant health impacts post-acute and chronic exposure. Longer exposure to canola biodiesel suppressed typical exhaust exposure responses, including reducing inflammation and responsiveness to methacholine. This suggests that oil type greatly impacts biodiesel exhaust toxicity and changes whether it is more or less toxic than diesel.

6.1 Introduction:

Diesel exhaust exposure is known to lead to negative health impacts on multiple organ systems including, but not limited to, the respiratory, cardiovascular, nervous, endocrine and urinary systems. It has been implicated in lung (Attfield et al., 2012; Silverman et al., 2012), brain (Andersen et al., 2018) and bladder cancer (Latifovic et al., 2015), increased blood pressure (K. E. Cosselman et al., 2012), increased thrombotic risk (Mills et al., 2007), increased stroke risk (Zhu et al., 2012), increased risk of type 2 diabetes (Balti, Echouffo-Tcheugui, Yako, & Kengne, 2014; Eze et al., 2015; Fleisch et al., 2016) and asthma (Evans et al., 2014). Biodiesel exhaust shares many of the same characteristics as diesel exhaust, such as oxides of nitrogen (NO_x), carbon monoxide and dioxide, particulate matter consisting of mostly ultrafine particles (D. Kittelson et al., 2002; Ris, 2007), polycyclic aromatic hydrocarbons (PAHs), aldehydes, ketones and heavy metals (Fontaras et al., 2009; Graver et al., 2016; Prokopowicz et al., 2015) and thus it is suspected it will be associated with many of the same negative health impacts. Previous studies into engine and exhaust characteristics that compared diesel and biodiesel often show that biodiesel exhaust contains more NO_x, PAHs and ultrafine particles (<100 nm) but less overall particulate matter by weight (Fontaras et al., 2009; Giakoumis et al., 2012; Gioda et al., 2016; Graver et al., 2016; Mullins et al., 2016; Westphal et al., 2013) compared with mineral diesel exhaust. This is of concern as ultrafine particles, when compared to larger sizes, are more commonly linked to the negative health effects of air pollution (Breitner et al., 2011; Oberdörster et al., 1995). Up to 90% of diesel exhaust particles by number consist of nucleation mode particles, newly formed from combustion and chemical reactions and under 30 nm in size (D. Kittelson et al., 2002; Ris, 2007), thus a further increase in the proportion of ultrafine particles in biodiesel exhaust is of great concern, in part due to the increased surface area to volume ratios allowing for more dangerous chemicals to be ab/adsorbed onto them for a given particle mass (Mullins et al., 2016). Despite this, biodiesel fuel usage is increasing worldwide (EIA, 2020a).

Previous studies, both *in vitro* and *in vivo*, looking into the health effects of biodiesel exhaust are not comprehensive, mostly because less than optimal exposure models have been used (V. André et al., 2015; Larcombe et al., 2015). Many previous studies have focused solely on the health effects of the exhaust particles by collecting them on a filter and adding a set concentration directly to a flask of cells/bacteria or instilling it into the nose/trachea of rats and mice (Larcombe et al., 2015; Madden, 2016; Møller et al., 2020; K. J. Swanson et al., 2007). This both ignores the effects of the exhaust gases, which have their own set of negative health impacts (T.-M. Chen et al., 2007), but also removes the ultrafine particles, arguably one of the most toxic components of diesel exhaust, which readily agglomerate on filters to form larger sized particles (Morin et al., 2008). This generates an artificial particle size spectrum, with previous studies showing that over a 16 fold increase in particle concentration is needed to generate the similar health impacts as if whole exhaust was used (Lichtveld et al., 2012).

In addition to this, the majority of previous biodiesel exhaust toxicology studies have used bacterial AMES tests to study mutagenic effects, or have exposed immortalised cell lines (J. Bünger et al., 2000; Cervena et al., 2017; Gioda et al., 2016; Westphal et al., 2013) which are not always human or even derived from the respiratory system, the first exposed and likely most

affected tissue (J. Bünger et al., 2000; Jalava et al., 2012). Previous studies that expose animals most often use instillation to expose the mice/rats to the particulate matter in solution, with few studies performing inhalation exposures. These few studies are often divided into several different publications, likely due to the difficulty in conducting them, which artificially inflates the actual number of inhalation studies performed (Chapter 1.1) (Bass et al., 2015; Brito et al., 2010; de Brito et al., 2018; Douki et al., 2018; Dziendzikowska et al., 2018; Farraj et al., 2015; Gavett et al., 2015; Hazari et al., 2015; Magnusson et al., 2019; Magnusson et al., 2017; Valand et al., 2018). Of the few studies that do use inhalation exposures, only half expose mice/rats to pure biodiesel exhausts and the remainder use biodiesel blended with diesel (at ratios of < 30% biodiesel in diesel fuel). With biodiesel concentration being below half of the total fuel content, there is a chance for biodiesel exhaust exposure induced health effects to be masked by those of diesel. That said, blended fuels are highly relevant to what is being used today with biodiesel already being blended up to 20% in some countries (EERE, 2020; Hamje et al., 2014; ASTM, 2020b).

There is also a tendency in the literature to treat all biodiesels as the same, despite evidence that the feedstock used to make the biodiesel vastly affects the fuel and exhaust properties and thus the resulting health impacts of exhaust exposure (Landwehr et al., 2021). Studies often use just one biodiesel type and make claims about biodiesel in general based on the results of that type (Hawley et al., 2014). Some studies do not even disclose the feedstock used to make their biodiesel (Ackland et al., 2007; Hawley et al., 2014). Methodological differences inherent in different study designs; from exact engine type, the use (or not) of exhaust after-treatment systems, differing exhaust concentrations, the use of whole exhaust compared to filter extracted particles and the wide range of health effects measured, including mutagenicity, cytotoxicity and immunological effects (V. André et al., 2015; Brito et al., 2010; de Brito et al., 2018; Douki et al., 2018; Gioda et al., 2016; Hemmingsen et al., 2011; Magnusson et al., 2019; Magnusson et al., 2017; Westphal et al., 2013) makes comparisons of fuel feedstocks between different studies difficult.

Thus, the aim of this study was to compare the respiratory health effects of exposure to one of two different types of biodiesel exhaust, using air and ultra-low sulfur mineral diesel (ULSD) as controls. My goals were to evaluate the impacts of biodiesel exhaust exposure and how these impacts can change between different feedstock types (in comparison with exposure to ULSD exhaust). Tallow and Canola were chosen for study, both because they are commonly used today (ARENA, 2018; OECD/FAO, 2020) and previous research found them to be at extreme ends of the health effects in both a submerged cell-culture model (Chapter 2/ (Landwehr et al., 2021)) and a 3D lung model (Chapter 5). Mice were exposed for two hours each day to one of these four options, either once or for 8 days in a row. This helped to establish if there were differences between acute and ongoing exposures. The main hypotheses were that (i) exposure to Tallow biodiesel exhaust would result in more severe and a greater range of negative health effects than ULSD exhaust exposure, (ii) that exposure to Canola biodiesel exhaust would result in less health impacts, and (iii) the ongoing exposures would result in worse health effects than the acute.

6.2 Materials and Methods:

6.2.1 Animals: 192 seven-week-old male BALB/c mice were purchased from the Animal Resources Centre (Murdoch, WA, Australia) and housed in individually vented cages (IVC Allentown XJ model, ECO FLO air handling unit set at 22-23°C with 30-31% humidity, 50 air changes per hour). They were left to acclimatise for one week before being weighed and randomly assigned into one of 8 different groups (n=24 per group). These groups were either one or 8 days of exposure to Air or the exhaust of an engine running on ULSD, Canola or Tallow biodiesel (Figure 6.1). Thus, there were eight treatment groups: Air 1-day (A1), Air 8-days (A8), ULSD 1-day (U1), ULSD 8-days (U8), Canola 1-day (C1), Canola 8-days (C8), Tallow 1-day (T1) and Tallow 8-days (T8). Twenty-four hours after the last exposure, mice were weighed and prepared for end exposure outcomes as previously described (Larcombe et al., 2008).

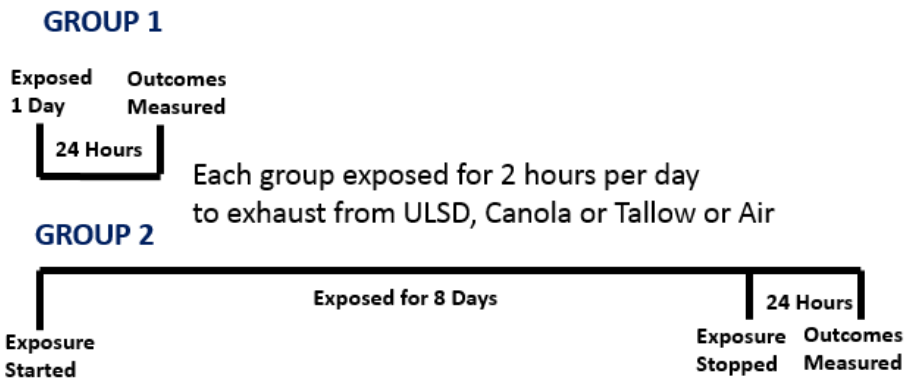


Figure 6.1: Exposure timeline. Group 1 were exposed once to Air or ULSD, Canola or Tallow biodiesel exhaust. Group 2 were exposed once a day for 8 days to the same exhausts. Measurements were taken 24 hours after the last exposure.

6.2.2 Engine Configuration: Exhaust was generated by a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) coupled with a dynamometer and fitted with Euro V/VI after-treatment technology consisting of a diesel particulate filter and oxidation catalyst (Daimler, Germany) (Landwehr et al., 2019). All exposures were run from cold start with a constant load of 40% and a speed of 2000 rpm. Air exposures were done simultaneously alongside exhaust exposures.

6.2.3 Exposure Protocol: To make exposures more realistic to occupational settings, where alternating shifts and work priorities mean that no worker is exposed to a constant level of diesel exhaust, and to account for our previous studies where we found lower levels of exhaust exposure to be more toxic than higher levels (Landwehr et al., 2019), mice were exposed to high and low concentrations of exhaust on alternating days. Higher levels were exhaust diluted 1/5 with air and lower levels were exhaust diluted 1/10 with air. Exhaust was diluted inside a mixing chamber attached to the exhaust piping and pumped through an isokinetic sampling point at a rate of 5 L/min into a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) maintained at 28ºC containing a 27 L exposure chamber with the mice inside divided into individual cubicles to even out each individuals exposures and prevent fighting. The sealed

incubator was used to dampen the sound of the engine and keep chamber temperatures constant, which helped in minimising stress to the animals. During exposures, exhaust was gently vacuumed out of the exposure chamber for physico-chemical analysis of gas and particle properties (Figure 6.2). Simultaneously to the exhaust exposure, a second 4 L exposure chamber was also placed inside the incubator and attached to piping that allowed air to be pumped inside for the Air exposure controls. The difference of pumping air into and vacuuming exhaust out of the different chamber boxes created a pressure gradient that made certain of no chance for cross exposure contamination, in case of any leakages within the sealed exposure chambers. Fewer Air mice were exposed at any one time (i) because of the smaller control exposure chamber and (ii) to ensure that there were control animals on each data acquisition day. All exposure chambers were thoroughly washed and dried between exposures.

Of note, although the high- and low-level exhausts produced different gas and particle concentrations, there was no effect of these different levels in terms of health effects for any of the one-day exhaust exposure groups. As such, high and low exhaust exposed mice for the one-day treatments were combined for each fuel type.

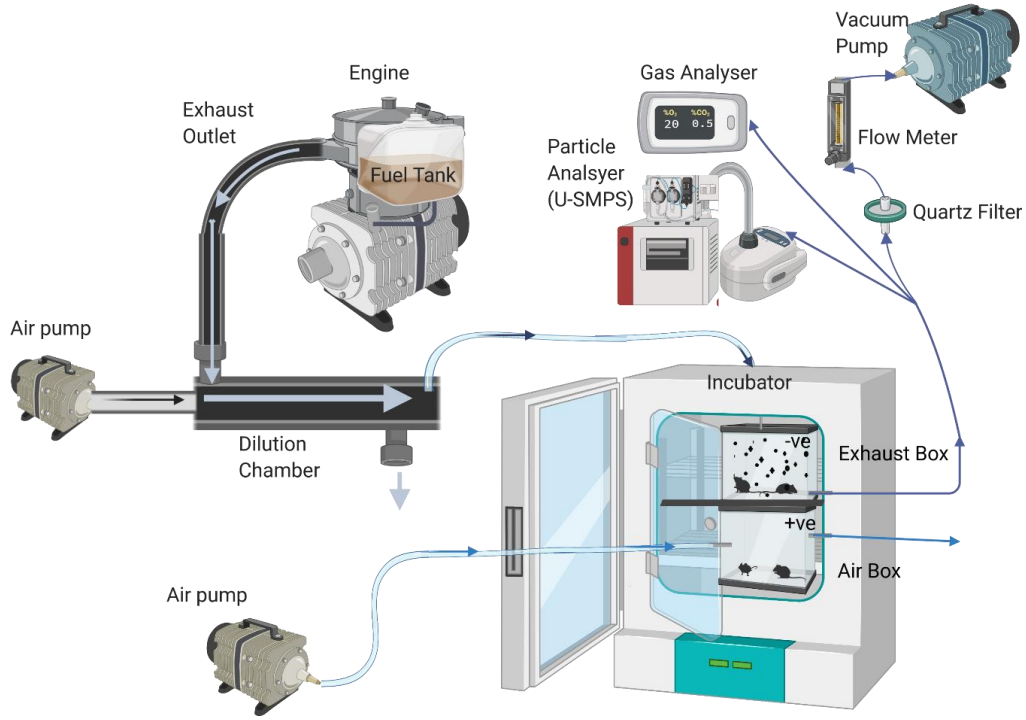


Figure 6.2: Diagram of exposure set-up. As a vacuum is used to transport exhaust into the exhaust chamber and a pump used to transport air into the air chamber, a slight variation in pressure gradient was created so as to ensure there was no chance for cross exposure of groups. Created with Biorender.com.

6.2.4 Gas and Particle Analysis: Exhaust exiting the exposure chamber was analysed every 10 minutes for concentrations of common combustion gas products including O_2, CO, CO_2, NO_x (NO and NO_2) and SO_2 using a combustion gas analyser (TESTO 350, Testo, Lenzkirch, Germany). Similarly, exhaust was analysed every 10 minutes for particle concentrations between

the sizes of 3 nm-340 nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Particles less than 10nm in size were excluded from further calculations due to high variability of measurements. Count-median particle size was calculated using the number of particles mean. Particle mass was calculated from particle spectra, assuming sphericity and using the 40% load diesel exhaust particle density as previously described (Olfert et al., 2007). Particle number was further separated into two fractions: nucleation mode particles below 23 nm in size and solid particles above 23 nm (Amanatidis et al., 2014).

6.2.5 Lung Function Measurements: Measurement of thoracic gas volume (TGV) and lung mechanics were conducted as previously described (Larcombe, Foong, Berry, Zosky, & Sly, 2011; Larcombe et al., 2017). In brief, mice were anesthetized via intraperitoneal injection of a solution containing ketamine (40mg/mL; Troy Laboratories, New South Wales, Australia) and xylazine (2mg/mL; Troy Laboratories, New South Wales, Australia) at a dose 0.1mL/10g body weight, tracheostomized with a 10 mm long cannula with an internal diameter of 0.86 mm, and attached to a mechanical ventilator (HSE Harvard Minivent; Hugo Sachs Harvard Elektronik, March-Hugstetten, Germany). They were ventilated at a rate of 400 breaths/min with a tidal volume of 8 mL/kg and 2 cmH_2O of positive-end expiratory pressure, which is sufficient to allow measurement of lung function parameters without either induction of paralysis or autonomous breathing. Plethysmography was used to measure TGV. At end expiration, the trachea was occluded and the intercostal muscles electrically stimulated (six 2- to 3-ms, 20-V pulses, model S44 electrical stimulator; Grass Instruments, Quincy, MA, USA) to induce inspiration with tracheal pressure and plethysmograph box pressure measured throughout. TGV was then calculated using Boyle's law, after correction for thermal properties and impedance of the plethysmograph (Jánosi et al., 2006). Respiratory system impedance (Z_{rs}) was measured using a wave-tube system adapted for use in small animals (Peták, Hantos, Adamicza, Asztalos, & Sly, 1997; Sly, Collins, Thamrin, Turner, & Hantos, 2003) and a modification of the forced oscillation technique (Sly et al., 2003). The constant phase model was fit to Z_{rs} to generate the parameters of airway resistance (R_{aw}), tissue damping (G), and tissue elastance (H). Z_{rs} was measured at functional respiratory capacity and also during a slow inflation-deflation manoeuvre from 0 to 20 cmH_2O transrespiratory pressure, allowing construction of absolute pressure-volume curves and assessment of the volume dependence of lung mechanics. Specific lung compliance was then measured using lung volume at transrespiratory pressure 8 cm/H_2O minus lung volume at transrespiratory pressure 3 cm/H_2O on the deflationary arm (Limjunyawong, Fallica, Horton, & Mitzner, 2015).

6.2.6 Methacholine Challenge: After measurement of TGV and lung mechanics, a randomised selection of half the mice from each group (n=12) were transferred to a small animal ventilator (Legacy flexiVent; SCIREQ) for assessment of responsiveness to methacholine (MCh; acetyl β-methacholine chloride; Sigma-Aldrich, MO) as previously described (Larcombe, Foong, Bozanich, et al., 2011). Briefly, 5x forced oscillation technique (FOT) measurements were taken at baseline (1 per minute), then after a 10s saline aerosol and again after increasing doses of MCh from 0.1 to 30mg/mL. Peak responses to MCh at each dose were used to construct dose response curves.

6.2.7 Bronchoalveolar Lavage (BAL) Collection and Cell Measurement: At the end of the methacholine challenge, BAL fluid was collected by washing 0.5 mL of chilled saline in and out of the lungs three times via the tracheal cannula (n=12 per group). Lavage samples were processed as previously described for total cell counts (Foong, Sly, Larcombe, & Zosky, 2010) and differential cell counts were obtained using DiffQuik (Thermofisher Scientific) staining as per manufactures protocol. In short, samples were centrifuged at 400 g for 4 minutes to pellet the cells and the supernatant removed and stored at -80°C for future mediator, protein and phospholipid analysis. A total cell count was determined from the cell pellet by staining an aliquot of cells with trypan blue and counting cells with a haemocytometer. Remaining cells were cytospun and stained with DiffQuik and scanned using a Panoramic MIDI® scanner and paired software (3DHISTECH Ltd.) to determine proportion of cell types within a randomised count of 300 cells.

6.2.8 Serum collection: Following completion of BAL collection, blood was obtained through cardiac puncture and placed into tubes containing a lithium heparin serum separator (41.1503.015, micro sample tube, SARSTEDT, USA) and left to clot for a minimum of 30 minutes. It was then centrifuged at 2000 g for 10 minutes to separate serum which was collected and immediately stored at -80°C for future mediator analysis.

6.2.9 Sample collection: In addition to serum and BAL collection, methacholine challenged mice had urine, brain, liver and lung collected and immediately frozen in liquid nitrogen for future analysis. Before being frozen, small pieces of lungs were stored in RNAlater (ThermoFisher Scientific) for later RNAseq analysis. Non-methacholine challenged mice had urine and bladder collected for future analysis before lungs were inflation fixed at 10 cmH_2O transrespiratory pressure using 10% formalin (Hsia, Hyde, Ochs, Weibel, & Structure, 2010) prior to removal *en bloc* for airway morphometry analysis. Thus n=12 for all groups and samples excepting urine where n=24.

6.2.10 Mediator, phospholipid and protein analysis: BAL and serum were analysed for mediators as per kit protocol using Bio-Rad Mouse Cytokine 23plx kits (Bio-rad) and accompanying software (Bio-Plex Manager, v6.1.1, Bio-Rad, Tokyo, Japan). Protein concentration of the BALs was assessed as per kit protocol using a Pierce™ BCA protein assay kit (Thermofisher Scientific). Phospholipid (choline containing) concentration within the BAL was analysed as per kit protocol using a Colorimetric Phospholipid Assay Kit (Abcam). Serum immunoglobulin concentration was analysed as per kit protocol using Mouse Immunoglobulin Isotyping Magnetic Bead Panel (Milliplex, MERCK).

6.2.11 Airway morphometry: The left lung of the non-methacholine challenged mice was embedded in paraffin, and 5-μm thick sections were taken from the proximal region, where the primary bronchi is first fully enclosed by tissue. Three section from each mouse were stained using Masson's trichome and the most intact sections were imaged using a Panoramic MIDI® scanner and paired software (3DHISTECH Ltd.). Semiautomated assessment of chord length was performed (Crowley et al., 2019; Larcombe et al., 2021) and collagen content was quantified as a percentage of total tissue in the cross-sectional area using ImageJ (Schneider, Rasband, & Eliceiri, 2012). The cross-sectional area of outside bronchi wall, airway smooth muscle, the gap

between smooth muscle and epithelium and the airway epithelium were measured. The square root of all areas was normalized to the internal perimeter of the basement membrane to correct for differences in airway size (James, Hogg, Dunn, & Paré, 1988).

6.2.12 Statistical analysis: Data are presented as mean ± standard deviation. All statistical analyses were performed using R statistical software (v3.4.3) (R Team, 2018) loaded with the packages "lme4" and "mgcv". P-values less than 0.05 were considered significant. As previously described (Landwehr et al., 2021), all statistical analyses excluding gas concentration data were completed using multivariate general linear modelling methodologies with the families "Gamma(inverse/log)" and "gaussian(identity/log)" as best fit the data, applying a backwards elimination approach to remove insignificant predictive variables. For combustion gas analysis a separate General Additive Model (GAM) file was fitted to each gas measurement with concentration as the response variable and time as the predictor, thus allowing for non-parametric fits as caused by cold start effects.

6.3 Results:

6.3.1 Exhaust Gas Characteristics: Mean and standard deviation for each fuel and gas type are shown (Table 6.1), with the exception of CO which shows only the highest reading at 10 minutes for each of the repeated exposures due to the cold start effect on the performance of the catalytic converter. Trends over time can be found in the supplementary materials (Figure S6.1). All fuels displayed similar trends over time with NO_x (NO and NO_2), CO_2 and SO_2 increasing rapidly in the first 30 minutes of the exposure, O_2 decreasing rapidly in the first 20 minutes and CO peaking in the first 10 minutes before decreasing rapidly to undetectable concentrations. Canola was found to be the most different of the tested fuels with significant changes in each of the measured combustion gases except for CO when compared to both Tallow biodiesel diesel exhaust ($p<0.05$). In contrast, Tallow and ULSD exhaust were only different for O_2, CO_2 and SO_2.

6.3.2 Exhaust Particle Characteristics: Particle size spectra were obtained for all of the fuels between the sizes of 3-340 nm however no differences were observed for any of the fuels for total particle number concentrations (Figure 6.3). Particle mass concentrations (Table 6.1) were highest in ULSD however the concentrations in the Canola and Tallow biodiesels were 78% and 92% of that measurement respectively, showing little differences between fuels.

Table 6.1: Mean (standard deviation) combustion gas concentrations and mean particle characteristics for the three measured exhausts. Measurements are shown as the mean concentrations for all of the exposures, with the exception of CO which is shown as the mean peak measurement. Data in square brackets is a ratio in comparison to ULSD, particle data in parentheses is a percentage of the total within the fuel.

	ULSD	Canola	Tallow
O_2 (%)	19.45 (0.53)	19.58 (0.43)* ####	19.20 (0.49)**** ####
CO (ppm)	0.99 0.76)	1.87 (0.90)	2.06 (1.15)
CO_2 (%)	0.95 (0.35)	0.84 (0.27)** ####	1.11 (0.32)**** ####
NO_x (ppm)	33.30 (14.52)	24.73 (9.35) **** ####	32.37 (13.37) ####
NO (ppm)	28.23 (11.48)	22.71 (8.42) **** ###	27.11 (9.95) ###
NO_2 (ppm)	5.07 (3.27)	2.17 (1.47) **** ####	5.47 (3.87) ####
SO_2 (ppm)	1.64 (0.73)	1.21 (0.53) **** #	1.38 (0.72) ** #
Particle Mass Concentration ($\mu g/m^3$)	54.42	42.58 [0.78]	50.17 [0.92]
Median Particle Size (nm)	18	20	20
Total Particle Number (particles/cm^3)	101788	89086 [0.88]	98418 [0.97]
Particle Number >23 nm (particles/cm^3)	39035 (38.55%)	39141 (43.94%)	41191 (41.85%)
Particle Number <23 nm (particles/cm^3)	62753 (61.65%)	49945 (56.06%)	57228 (58.15%)

=Different to ULSD (=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)
#=Different to the other biodiesel (#=p<0.05, ##=p<0.01, ###=p<0.001, ####=p<0.0001)

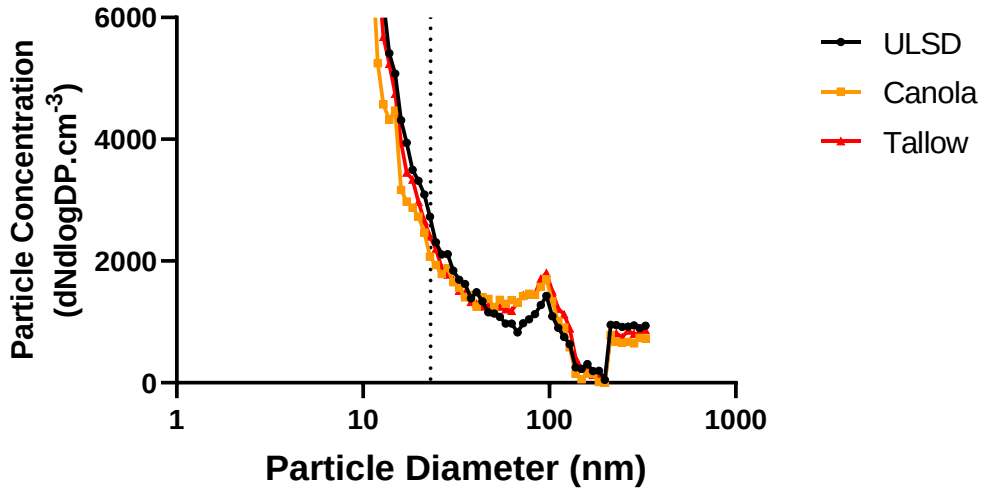


Figure 6.3: Particle size spectra for all fuels. Data were analysed using total particle number concentration between the size of 10 and 340 nm for each fuel. No significant differences in particle number were found.

6.3.3 Mouse Weights: Mice were weighed before lung function assessment, allowing for calculation of weight changes between the 1 and 8-day exposure groups (Figure 6.4). Significant differences in weight were found for A1 groups compared to T1. C8 weights were significantly less than U8 and T8. Importantly, A8 and U8 mice were heavier than A1 and U1 mice respectively ($p<0.05$) but no changes were found between T8 and T1 or C8 and C1, suggesting that mice exposed to biodiesel exhaust did not increase in weight unlike those exposed to Air and ULSD. No significant differences were found in % weight increase for any of the 8 day groups.

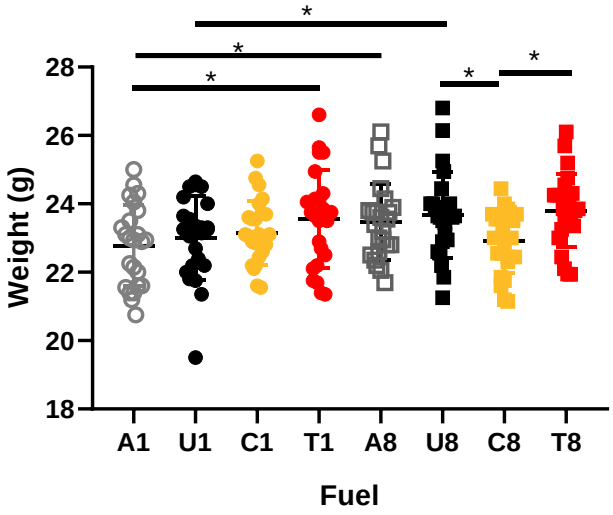


Figure 6.4: Mouse weights measured 24 hours after the last exposure for each group. (* indicates $p<0.05$). n = 24 per treatment.

6.3.4 Lung Function at Functional Residual Capacity: Thoracic gas volume (TGV) and lung mechanics (R_{aw}, G, H and η/hysteresivity) at functional residual capacity (FRC) were measured (Table 6.2). TGV was significantly higher in C8 mice compared with both C1 mice and all other 8-day groups. Due to this, lung function parameters at FRC were normalised to TGV to generate specific lung function measurements. Specific R_{aw} was higher in all 8-day groups compared to 1-day (although this was not always statistically significant), and C8 mice had significantly higher R_{aw} in comparison to C1, U8 and T8 groups ($p<0.01$). Specific G, H and hysteresivity were significantly higher for C8 mice in comparison to C1 and all other 8-day groups ($p<0.01$). C1 also had significantly lower hysteresivity in comparison to A1 ($p<0.05$).

Table 6.2: Mean (standard deviation) thoracic gas volume (TGV) and specific lung function at FRC for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8 days as measured by plethysmography and the forced oscillation technique (n=22-24 per group). As significant differences in TGV were measured between groups, FRC lung function measurements have been normalised to TGV.

FRC Lung Function Measure	1 Day				8 Days			
	Air	ULSD	Canola	Tallow	Air	ULSD	Canola	Tallow
TGV (mL)	0.277 (0.043)	0.268 (0.031)	0.260 (0.033)b	0.280 (0.041)	0.291 (0.043)	0.279 (0.042)	0.328 (0.049) **###ab	0.283 (0.035)a
sR_{aw}, $hPa \cdot s^{-1}$	97.36 (17.26)	98.58 (11.39)	99.37 (14.64)b	102.8 (23.14)	108.3 (23.80)	102.6 (20.82)	120.0 (21.08) ##ab	103.6 (22.32)a
sG, hPa	2302 (313)	2244 (300)	2264 (210)b	2286 (406)	2267 (421)	2281 (255)	2865 (398) ****#### ab	2160 (322)a
sH, hPa	9493 (1478)	9132 (955)	9612 (1235)b	9457 (1570)	9446 (1734)	9656 (1248)	11362 (1716) ***##ab	9217 (1835)a
η	0.244 (0.022)	0.241 (0.017)	0.233 (0.014) **ab	0.242 (0.022)	0.243 (0.020)	0.237 (0.015)	0.253 (0.024) **####ab	0.236 (0.027)

TGV = thoracic gas volume; sR_{aw} = specific airway resistance; sG= specific tissue damping; sH= specific tissue elastance; η = hysteresivity.
=Different to Air controls of same exposure duration (=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)
#=Different to ULSD of same exposure duration (#=p<0.05, ##=p<0.01, ###=p<0.001, ####=p<0.0001)
a=Different to other biodiesel of the same exposure duration (p<0.05)
b=Different to same fuel exposure between 1 day and 8 days groups (p<0.05)

6.3.5 Volume Dependence of Lung Function: Respiratory pressure-volume curves and specific lung function compliance (Figure 6.5) and volume dependant R_{aw}, G and H (Figure 6.6) were

measured throughout a slow, induced inflation-deflation manoeuvre for each mouse. At 20 cm H_2O transrespiratory pressure (P_{rs}), the lung volumes of A1 and C1 were significantly less than A8 and C8 respectively ($p<0.05$ in all cases). C1 also had a significantly lower volume than T1 ($p<0.01$). Specific compliance was significantly lower for C8 mice compared to all other groups ($p<0.0001$). At a volume of 0.7 mL (chosen as it is the largest lung volume with data for all mice) Canola exposed mice were the most different to every other group, whereas Air, ULSD and Tallow exposed mice were similar. A8 had significantly higher R_{aw} and lower H than A1, U8 had significantly higher R_{aw} and G than U1 and A8 respectively and C8 had significantly lower G and H than C1. C8 was also found to have significantly lower R_{aw} than all other 8 Day groups whereas C1 had significantly higher G and H than T1 ($p<0.05$).

6.3.6 Responsiveness to Methacholine: R_{aw}, G and H were measured after exposure to increasing doses of methacholine with statistical analysis using the highest response for every dose (Figure 6.7). There were significant effects of treatment on responsiveness to MCh with respect to airway resistance at both timepoints. After 1 exposure, T1 mice were significantly more responsive than A1 and C1 mice ($p<0.05$ in both cases), but not U1 mice ($p = 0.21$). U1 mice were also more responsive than A1 at this timepoint ($p<0.05$). After 8 exposures, T8 mice were significantly more responsive than A8 ($p<0.018$) but C8 mice were significantly less responsive than A8, U8 and T8 ($p<0.001$ in all cases). For G, tissue damping, T8 mice had a significantly higher readings than A8 mice and C8 mice had significantly lower responses than all other 8-day groups as well as C1 mice ($p<0.01$ in all cases). For H, tissue elastance, all 8-day mice were significantly different to each other with T8 having the highest response and C8 the lowest ($p<0.0001$ in all cases). The C8 and A8 mice were also different to the C1 and A1 groups respectively ($p<0.0001$ in both cases). This pattern was repeated in terms of sensitivity to MCh (evocative concentration needed to reach a 30% increase in R_{aw}, G and H from saline; Figure 6.8). The dose of MCh required to elicit a 30% increase in response was significantly lower in the T8 mice for R_{aw} and H, significantly lower in the U8 mice for H and significantly higher in the C8 mice for R_{aw}, G and H when compared to the A8 mice ($p<0.05$ in all cases). The C8 mice also need significantly lower doses than the C1 mice for G and H ($p<0.001$).

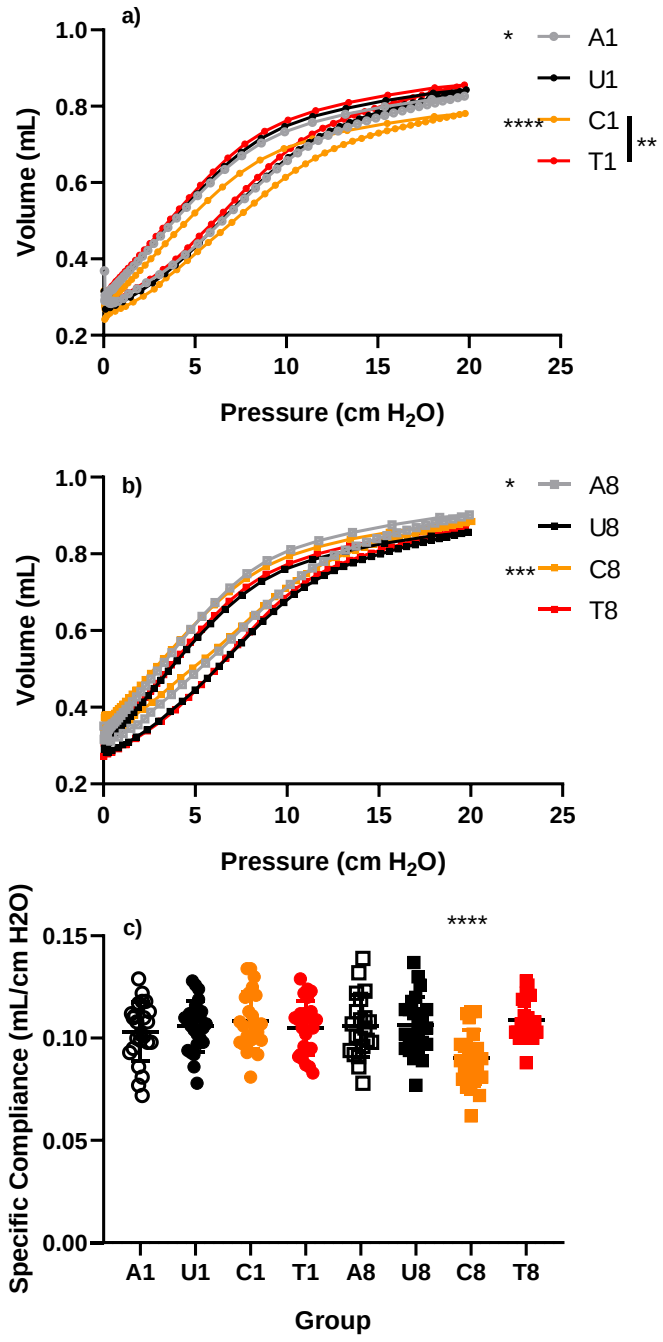


Figure 6.5: Pressure-volume loops for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for a) 1 Day and b) 8 Days and c) specific compliance obtained from the deflationary arm. Data are group means; n=23 for all groups except U1 and U8 (n=22) and T1 and T8 (n=21). Changes in thoracic gas volume were analysed statistically at P_{rs} = 20 cm H_2O. * on the left of the figure legend indicates differences between exposure groups with the same fuel type, * on the right of the figure legend indicates differences within 1 and 8 day exposure groups and above the group means in comparison to every other group (*=p<0.05, *=p<0.01, ****=p<0.0001).

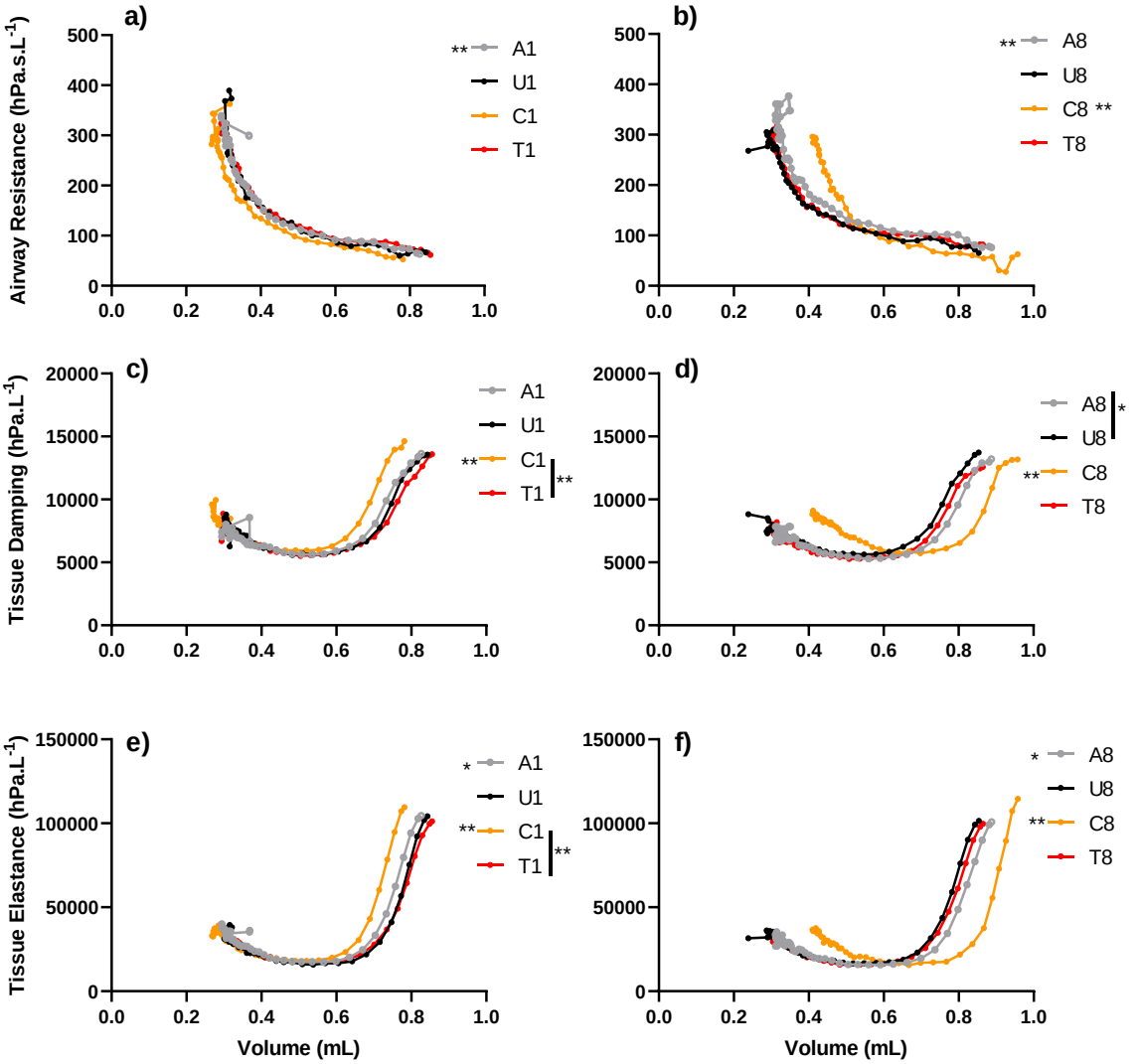


Figure 6.6: Volume dependence of lung function for a-b) airway resistance, c-d) tissue damping and e-f) tissue elastance for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8 days. Data are group means; n=23 for all groups except U1 and U8 (n=22) and T1 and T8 (n=21). Differences between groups were analysed statistically at a lung volume of 0.7mL, representing the highest volume for which data was available for each individual. * on the left of the figure legend indicate differences between exposure groups with the same fuel type, * on the right of the figure legend indicate differences within 1 and 8-day exposure groups (*=$p<0.05$, **=$p<0.01$).

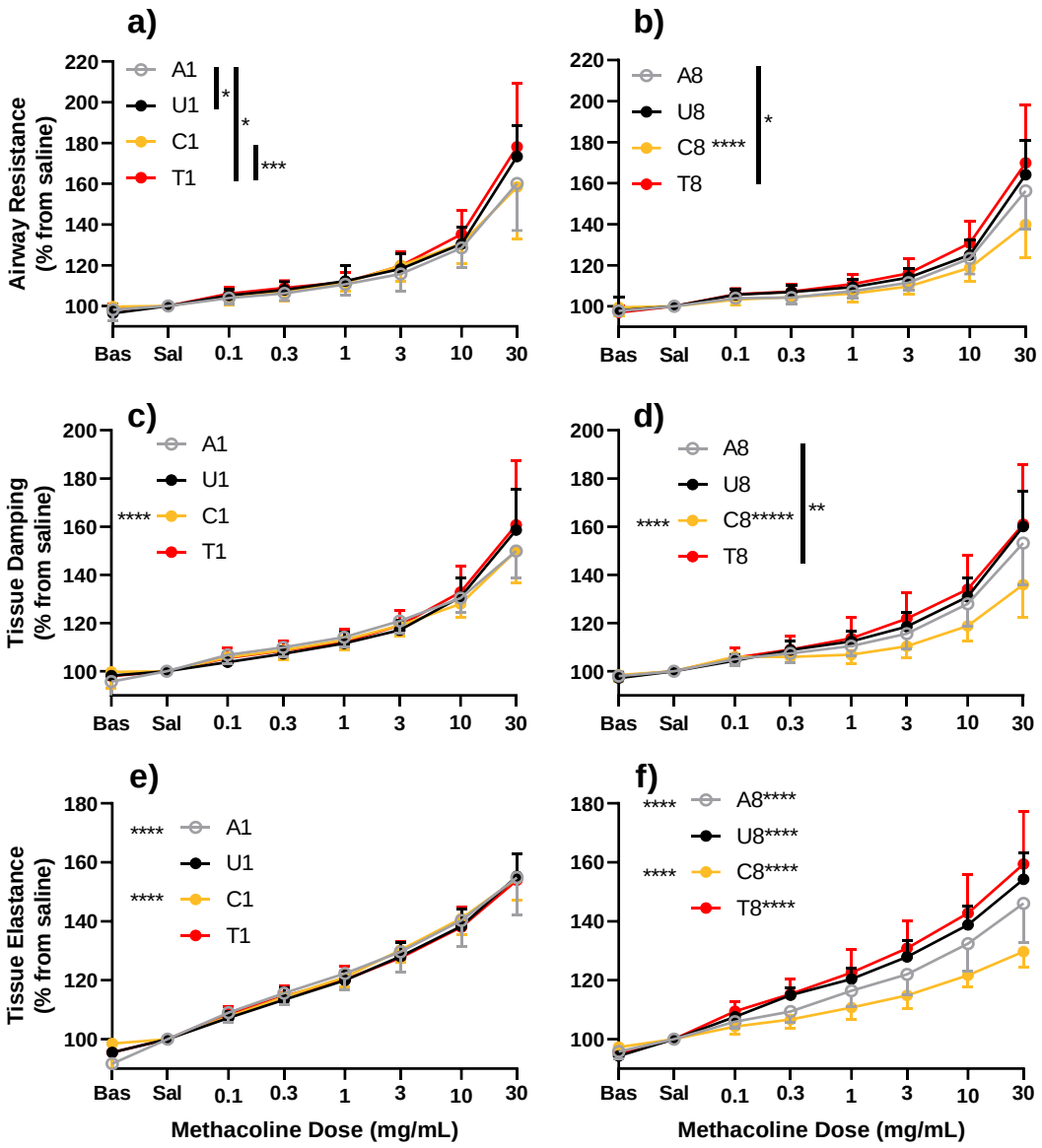


Figure 6.7: Responsiveness to methacholine for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 (left –panels a, c and e) or 8-days (right-panels b, d and f). Data shown are (a and b) airway resistance, (c and d) tissue damping and (e and f) tissue elastance for all exposure groups (n=12, except A8 and U8 where n=11). Bas stands for baseline (FRC) readings, Sal for saline. All data are shown as increase/decrease from saline. * on the left of the figure legend indicates differences between exposure groups with the same fuel type, * on the right of the figure legend indicates differences within 1 and 8-day exposure groups (*=p<0.05, **=p<0.01, ***=p<0.001, ***=p<0.0001).

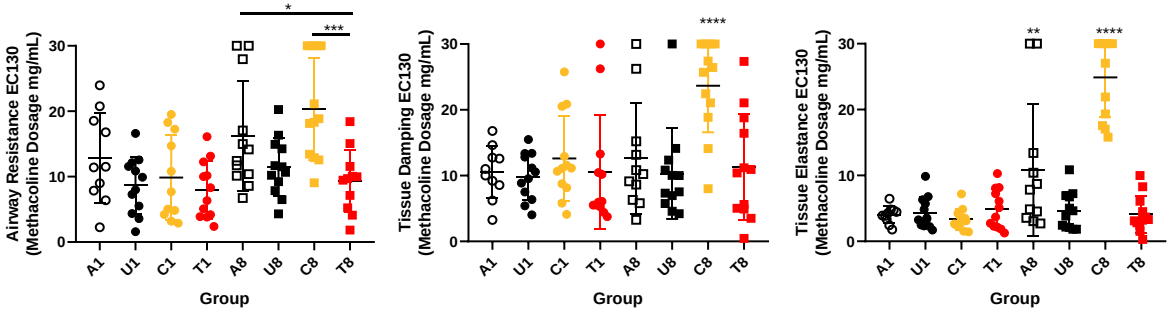


Figure 6.8: Evocative concentration showing methacholine dose needed to produce a 130% increase from saline in a) airway resistance, b) tissue damping and c) tissue elastance in mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8 days (n=12, except A8 and U8 where n=11) (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001).

6.3.7 Bronchoalveolar Lavage Cells, Mediators, Protein and Phospholipid Concentrations: Total and differential cell counts were performed on bronchoalveolar lavage (Figure 6.9). Significantly more immune cells were found in the BAL of T1 mice compared to A1 mice, and T8 and U8 mice compared to A8 ($p<0.05$). The U8 mice also had significantly more cells than the U1 mice and the T8 group also had more immune cells than the T1, C8 and U8 mice ($p<0.05$). This pattern was seen again in the macrophage cell counts, with the addition of U8 having more macrophages in the BAL than C8 ($p<0.05$). The neutrophil count was higher in both T1 and C1 mice compared to A1, however in contrast U8 and C8 had significantly less neutrophils than A8 mice ($p<0.05$). T8 mice had less neutrophils than T1 mice ($p<0.05$). For lymphocytes, all 8-day groups had significantly more in their BAL than their respective 1-day groups and T1 mice had more lymphocytes than C1 and U1 mice, whereas T8 had more lymphocytes than the A8, C8, U8 and T1 mice ($p<0.05$). No other cell types were detected.

In terms of BAL mediator concentrations (Table 6.4), the majority of significant differences were found in T1 compared to A1 with 9 out of 21 mediators being significantly different ($p<0.05$). All 9 mediators were significantly decreased after Tallow biodiesel exhaust exposure. The next biggest change in BAL mediator levels was found between T8 and A8, with 4 significant differences ($p<0.05$). Of those 4, 3 were significant decreases after Tallow biodiesel exhaust exposure.

Total protein and phospholipid concentrations within the BAL were also measured (Figure 6.10). There were few effects of exposure on either of these parameters, however T1 mice had significantly higher phospholipid concentrations than A1 mice whereas T8 mice had significantly increased protein concentration in comparison with A8 mice ($p<0.05$).

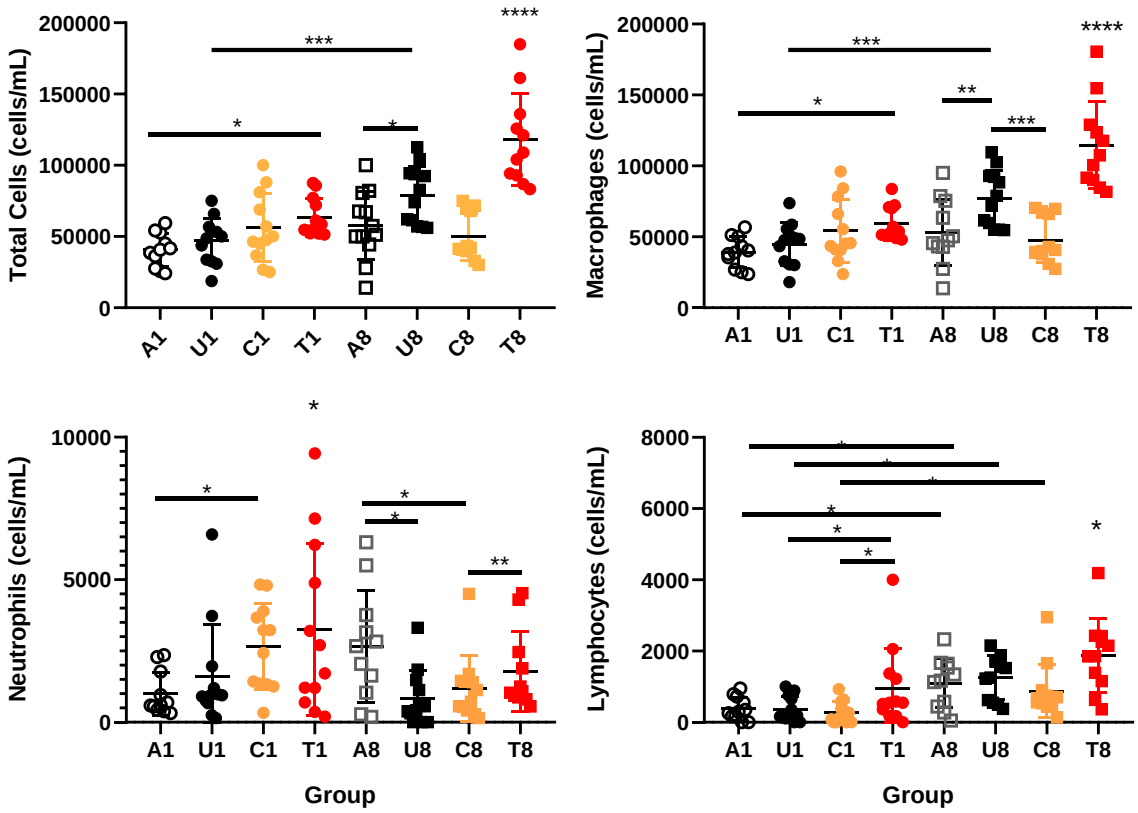


Figure 6.9: Cellular inflammation in bronchoalveolar lavage. Results are shown for a) total cells, b) macrophages, c) neutrophils and d) lymphocytes (n=12, except A1 and T8 where n=11) (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001). Note different scales.

Table 6.4: Mean (standard deviation) mediator levels in bronchoalveolar lavage fluid for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8 days (n=12).

BAL Mediator	1 Day Exposure				8 Days Exposure			
	Air	ULSD	Canola	Tallow	Air	ULSD	Canola	Tallow
IL-1α (pg/mL)	3.173 (1.863)	2.583 (0.651)	2.758 (0.710)	2.326 (0.682)	2.407 (0.806)	2.278 (0.967)	2.805 (1.097) a	1.976 (0.767) a
IL-2 (pg/mL)	3.166 (1.235)	2.813 (1.001)	2.916 (0.870)	2.586 (0.822)	2.835 (1.129)	2.642 (1.242)	3.643 (1.458) #a	2.176 (0.771) a
IL-4 (pg/mL)	0.390 (0.138)	0.294 (0.113)	0.298 (0.101)	0.255 (0.111)*	0.338 (0.120)	0.305 (0.130)	0.357 (0.176)a	0.220 (0.091)*a
IL-5 (pg/mL)	0.948 (0.427)	0.692 (0.338)	0.675 (0.315)	0.695 (0.332)	0.900 (0.401)	0.697 (0.366)	0.990 (0.417)	0.795 (0.615)
IL-6 (pg/mL)	1.922 (1.285)b	1.180 (1.085)	1.694 (0.814)	0.991 (0.930)*	0.937 (0.744)b	1.052 (0.743)	0.207 (0.070)	0.811 (0.722)
IL-9 (pg/mL)	4.319 (1.765)	3.272 (1.490)	3.546 (1.347)	2.780 (1.430)*	3.364 (2.069)	3.351 (1.958)	3.795 (2.239)	2.749 (1.380)
IL-10 (pg/mL)	5.386 (1.502)	4.121 (1.114)*	4.412 (1.394)a	3.149 (1.350)***a	4.540 (1.297)	3.036 (1.365)*	4.160 (1.940)a	2.890 (1.461)**a

IL-12(p40) (pg/mL)	43.234 (6.989)	51.991 (28.060)b	47.163 (28.882)	38.00 (10.190)	39.924 (16.53)	37.253 (7.705)b	36.263 (8.970)	43.284 (17.725)
IL-12(p70) (pg/mL)	12.074 (5.163)	9.529 (5.099)	10.708 (3.223)	7.160 (4.536)*	11.478 (2.881)	8.457 (4.369)	10.218 (4.897)	7.948 (5.217)
IL-13 (pg/mL)	27.139 (14.320)b	18.399 (6.412)*	19.843 (7.798)*	18.593 (2.876)*	17.758 (5.711)b	15.983 (6.676)	21.243 (10.470)	15.875 (6.435)
IL-17 (pg/mL)	1.785 (0.590)	1.568 (0.614)	1.523 (0.513)b	1.369 (0.578)	1.760 (0.343)	1.464 (0.487)	2.005 (0.864)#ab	1.198 (0.470)*
Eotaxin(pg/mL)	5.606 (1.809)	4.902 (1.007)	4.901 (1.120)	4.781 (1.405)	5.084 (1.469)	4.215 (1.585)	5.359 (2.630)	4.625 (1.226)
G-CSF(pg/mL)	3.846 (1.488)	3.288 (0.907)	3.325 (1.043)	3.623 (1.399)	4.447 (2.192)	3.342 (1.101)	3.024 (0.009)*a	4.707 (3.623)a
GM-CSF(pg/mL)	5.228 (2.221)	3.811 (1.565)	3.570 (1.455)*	3.412 (1.161)*	4.494 (2.114)	3.903 (2.182)	4.725 (2.281)	3.495 (1.313)
IFN-γ (pg/mL)	3.569 (1.164)	2.925 (1.018)	3.233 (0.949)	2.615 (0.924)*	3.201 (1.257)	2.711 (1.083)	3.152 (1.112)	2.495 (1.140)
KC (pg/mL)	37.466 (8.131)	47.665 (11.315)b	41.541 (14.035)b	46.682 (15.752)	39.244 (12.641)	30.240 (7.664)*b	30.379 (2.714)*ab	41.86 (21.676)#a
MCP-1 (pg/mL)	18.00 (12.725)	17.119 (6.479)	13.502 (6.549)	14.587 (8.137)	13.565 (8.868)	13.646 (7.047)	13.594 (8.997)	14.862 (12.742)
MIP-1α (pg/mL)	2.205 (0.555)	2.596 (0.804)	2.574 (0.699)	2.325 (0.974)b	2.258 (0.584)	1.545 (0.457)	2.299 (1.297)a	3.945 (3.918)*#ab
MIP-1β (pg/mL)	13.754 (5.341)	9.912 (4.322)	11.734 (6.019)	7.531 (5.267)*	11.348 (4.568)	8.628 (6.085)	9.672 (6.022)	8.876 (7.383)
RANTES (pg/mL)	9.315 (3.046)	8.578 (1.607)	8.180 (2.142)	7.259 (2.077)	8.276 (2.247)	7.515 (3.282)	9.357 (3.376)a	6.410 (2.617)a
TNF-α (pg/mL)	9.713 (3.011)	8.093 (2.330)	8.030 (2.144)	7.652 (2.523)	8.913 (2.940)	7.345 (2.861)	9.451 (4.023)	7.329 (1.951)

=Different to Air controls of same exposure duration (=p<0.05)
#=Different to ULSD of same exposure duration (#=p<0.05)
a=Different to other biodiesel of the same exposure duration (p<0.05)
b=Different to same fuel exposure between 1-day and 8-day groups (p<0.05)

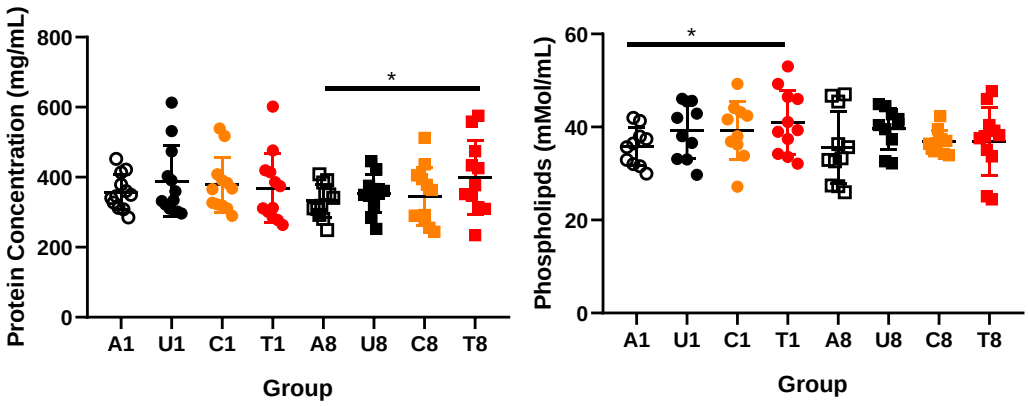


Figure 6.10: a) Total protein concentration in the bronchoalveolar lavage and b) total phospholipid concentration in the bronchoalveolar lavage for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8-days. For protein, n=12 except T8 where n=11. For

phospholipid concentration, n=11 except for A1, C1, U1 and U8 where n=10 (*=p<0.05). Data are individual mice with mean ± SD.

6.3.8 Systemic Mediators: Similar to what was observed in the BAL, changes in systemic mediator levels (Table 6.5) after exhaust exposures were mostly decreases compared with Air controls. The biggest changes were observed in T1 compared to A1 with 4 of 20 mediators being significantly different. Of those 4 mediators, 3 were significantly decreased (p<0.05) after Tallow biodiesel exhaust exposure. In contrast, in the 8-Day groups, only 1 significant difference was observed, between C8 and A8. This significant difference was also found to be a decrease (p<0.05). There was no effect of treatment on the concentrations of the three tested immunoglobulins (p > 0.05 in all cases).

Table 6.5: Mean (standard deviation) mediator levels in serum for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8-days (n=12 for the majority of mediators except the immunoglobulins where n=10).

Serum Mediator	1 Day Exposure				8 Days Exposure			
	Air	ULSD	Canola	Tallow	Air	ULSD	Canola	Tallow
IL-1α (pg/mL)	4.168 (3.021)	2.362 (1.928)	1.484 (1.627)	4.290 (5.430)	3.050 (2.494)	2.622 (2.007)	2.278 (1.724)	2.560 (3.091)
IL-5 (pg/mL)	4.967 (3.129)	7.667 (10.370)	2.790 (2.098)	11.228 (21.436)	3.118 (5.118)	8.727 (16.477)	2.771 (4.310)	3.300 (3.413)
IL-6 (pg/mL)	7.748 (5.915)	4.669 (3.654)	6.972 (6.089)b	4.700 (5.118)	4.295 (4.245)	3.671 (4.239)	0.511 (0.804)*#ab	4.579 (5.378)a
IL-9 (pg/mL)	46.893 (31.069)b	42.464 (29.900)	28.239 (15.415)*	29.558 (15.495)*	18.328 (6.510)b	20.998 (4.647)	13.705 (9.122)	17.736 (15.883)
IL-10 (pg/mL)	10.822 (9.955)	4.902 (8.054)	9.786 (13.306)	8.740 (15.543)b	4.170 (5.950)	1.684 (0.027)	3.845 (5.083)	1.684 (0.027)b
IL-12(p40) (pg/mL)	1239.088 (233.455)b	827.667 (445.103)*	2488.227 (4996.530)	1144.150 (383.125)#	930.752 (2052.625)b	851.766 (377.673)	963.610 (291.857)	953.015 (289.705)
IL-12(p70) (pg/mL)	15.280 (8.621)	11.862 (12.810)	14.123 (13.065)	16.371 (20.513)	4.587 (4.527)	4.178 (6.091)	4.668 (5.546)	8.580 (9.805)
IL-17 (pg/mL)	5.975 (5.142)	5.593 (4.587)	2.941 (1.490)	7.930 (15.429)	2.835 (3.166)	3.572 (3.775)	3.509 (4.920)	2.926 (1.741)
Eotaxin (pg/mL)	1205.448 (307.882)	1115.083 (236.074)	1452.833 (266.304)*#	1452.841 (295.915)*#	1385.121 (383.687)	1292.903 (341.166)	1366.058 (234.815)	1502.420 (300.573)
G-CSF (pg/mL)	32.330 (18.714)b	17.531 (15.667)*	29.033 (14.362)	15.921 (16.149)*	7.345 (10.019)b	5.243 (7.569)	16.843 (14.684)	16.118 (24.351)
IFN-γ (pg/mL)	26.587 (17.933)b	24.206 (19.903)	15.531 (8.746)*	17.932 (10.355)	10.503 (4.087)b	11.295 (4.157)	9.478 (5.137)	12.057 (8.552)
KC (pg/mL)	23.995 (14.910)	22.595 (15.052)	30.898 (52.189)b	26.651 (28.129)	14.908 (8.463)	23.723 (34.510)	9.451 (7.390)b	16.627 (11.299)
MCP-1 (pg/mL)	289.678 (161.533)	254.899 (137.987)	269.168 (67.92)b	327.529 (197.229)	147.968 (174.082)	181.910 (174.302)	68.552 (58.994)*#ab	214.671 (183.223)a
MIP-1α (pg/mL)	2.068 (2.012)	0.955 (1.092)	0.605 (0.833)	1.008 (1.384)	0.580 (0.788)	0.833 (1.540)	0.557 (0.724)	1.133 (1.524)
MIP-1β (pg/mL)	226.024 (106.792)b	191.033 (100.063)b	201.929 (116.093)b	154.813 (45.642)*	116.520 (42.692)b	105.259 (33.598)b	122.993 (40.117)b	116.000 (58.784)

RANTES (pg/mL)	163.896 (51.417)b	155.070 (45.872)	144.333 (34.312)	158.062 (33.415)	121.368 (40.559)b	131.668 (67.362)	137.140 (45.577)	147.987 (50.136)
TNF-α (pg/mL)	21.135 (31.153)	14.864 (18.093)	4.029 (5.223)	26.004 (56.437)	5.988 (7.752)	9.694 (13.288)	5.891 (10.055)	4.872 (7.173)
IgA (ng/mL)	96108 (20736)	10791 (25287)	87178 (15318)#	94587 (15518)	98675 (18050)	11184 (18838)	10732 (28382)	10400 (33247)
IgG1 (ng/mL)	39187 (16104)	46820 (16652)	35648 (15799)	45273 (13010)	49782 (14353)	51774 (15710)	47663 (17247)	42978 (16274)
IgM (ng/mL)	20815 (40938)	21182 (42933)	18648 (37966)	22031 (85873)	23501 (41941)	21472 (23482)	20611 (50014)	23222 (59841)

=Different to Air controls of same exposure duration (=p<0.05)
#=Different to ULSD of same exposure duration (#=p<0.05)
a=Different to other biodiesel of the same exposure duration (p<0.05)
b=Different to same fuel exposure between 1-day and 8-day groups (p<0.05)

6.3.9 Airway Morphometry: Size corrected total airway wall, airway smooth muscle mass and airway epithelial thickness were measured (Table 6.6, representative images in Figure 6.11). Chord length, collagen and total tissue % were also measured (Table 6). There was no effect of treatment on any airway morphometry parameter ($p > 0.05$ in all cases). Chord length was significantly higher in T8 mice compared to A8 mice and T8 mice had significantly more collagen than C8 and U8 mice ($p<0.05$).

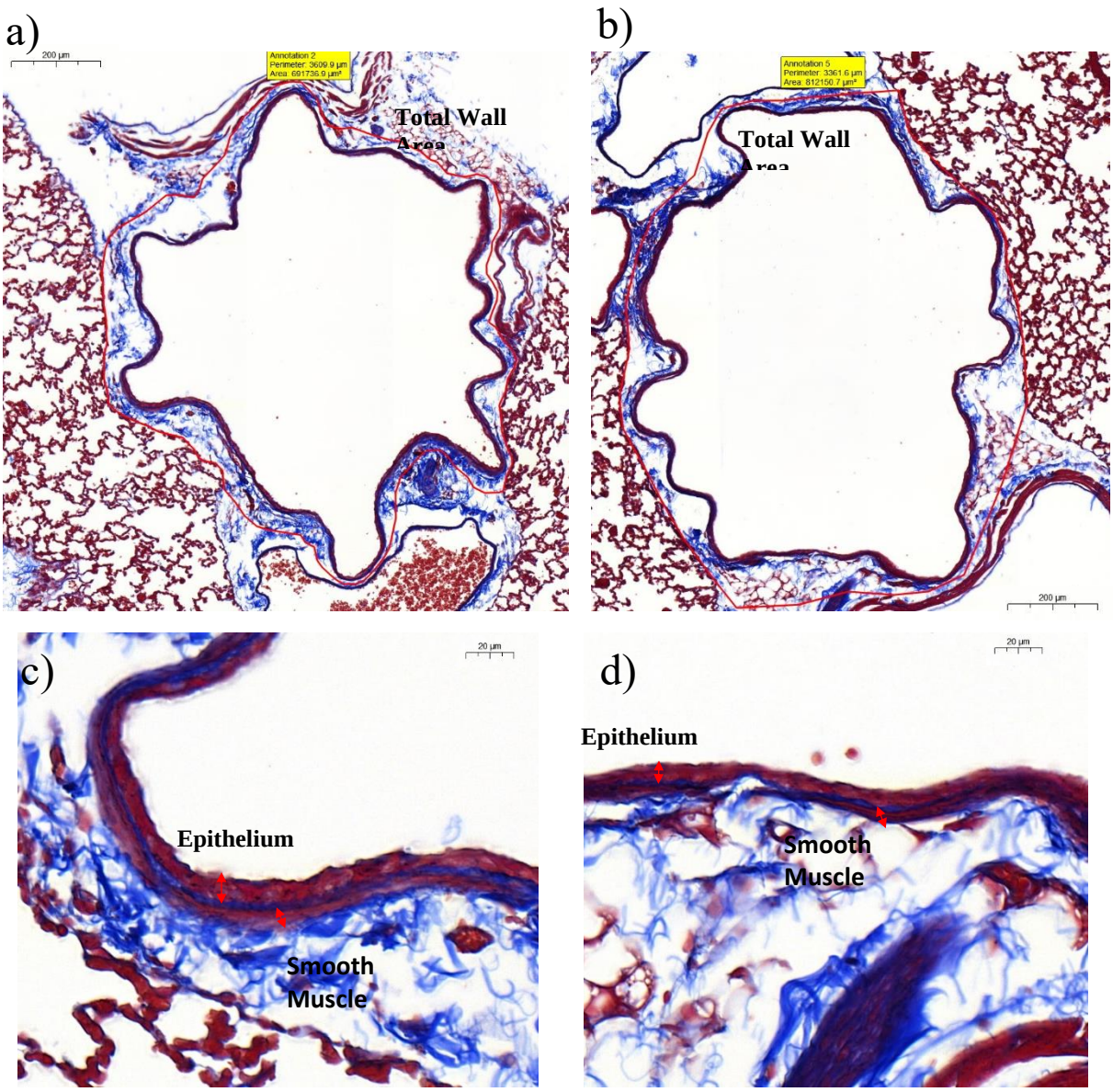


Figure 6.11: Representative images of airway sections stained with Masson's Trichrome. Images are taken from air and ULSD exhaust exposed mice. a) and c) are whole and zoomed airway images of a representative air exposed mouse while b) and d) are the same for an exhaust exposed mouse. The different airway measurements are outlined in red.

Table 6.6: Mean (standard deviation) measurements for airway morphology and chord length and collagen % for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust for 1 or 8 days (n = 10-12 for all measurements).

Measurement	Air	ULSD	Canola	Tallow
Total Wall Thickness (μm^2)	0.123 (0.015)	0.130 (0.013)	0.128 (0.013)	0.127 (0.007)
True Wall Thickness (μm^2)	0.060 (0.007)	0.057 (0.006)	0.058 (0.005)	0.058 (0.008)
Airway Smooth Muscle Thickness (μm^2)	0.036 (0.003)	0.033 (0.003)	0.033 (0.003)	0.035 (0.004)
Epithelial Thickness (μm^2)	0.040 (0.003)	0.039 (0.003)	0.037 (0.002)	0.038 (0.003)
Chord Length (μM)	21.07 (2.31)	21.89 (1.54)	22.42 (2.39)	23.33 (1.74)*
Collagen (%)	2.718 (0.534)	2.604 (0.230)	2.456 (0.487)a	3.262 (1.197)#a

=Different to Air controls of same exposure duration (=$p<0.05$)
#=Different to ULSD of same exposure duration (#=$p<0.05$)
a=Different to other biodiesel of the same exposure duration ($p<0.05$)

6.4 Discussion:

The results of this study show that exposure to diluted diesel or biodiesel exhaust causes a range of negative health impacts in a murine exposure model. These include impacts on weight gain, lung function, cellular inflammation, small changes to lung structure and large impacts to the immune response. Of the two biodiesels tested, Tallow biodiesel exhaust exposure was associated with the widest range of negative health effects with no increase in weight between the 1 and 8 day groups, a greater increase in responsiveness to methacholine, a greater than two-fold increase in inflammatory cell numbers in the lungs, a wider disruption in the mediator release both locally and systemically, increased protein and phospholipid concentrations in the BAL and a small impact on lung structure with significantly increased chord length. In contrast, Canola biodiesel exhaust exposure only led to negative impacts on weight gain, lung function at FRC, specific compliance, some decreases in mediator release and increased neutrophilic (but not total) inflammation. Mice exposed to Canola biodiesel exhaust were less responsive to MCh than Air exposed controls. The impacts of exposure to ULSD exhaust were generally between those of Canola and Tallow, with increased tissue damping in volume dependant lung mechanics, several increases in methacholine responses, some decreases in mediator release and increased immune cell numbers in the lungs of 8-day exposed mice.

A concerning implication for this study is that negative health impacts (with implications for wide reaching consequences) were identified, yet the exhaust used mostly met Safe Work Australia standards (SWA, 2019). These standards are equivalent to, or more stringent than, the standards used in Europe and USA (OSHA, 2021; EU-OSHA, 2013). The Safe Work Australia standards for various exhaust components are time weighted 8-hour averages of 30 ppm CO, 5000 ppm CO_2 (peak concentration not exceeding 30000 ppm), 25 ppm NO, 3 ppm NO_2 (with peak concentrations not exceeding 5 ppm) and 2 ppm SO_2 (peak concentration not exceeding 5 ppm). Oxygen levels below 19.5% are considered "unsafe" (SWA, 2018). Table 6.1 shows that almost all exhaust gases in this study (with the exception of a slightly too high NO_2 and a slightly too low oxygen concentration) met these standards. The European Union has set a recent particulate matter occupational exposure limit of 50 ug/m^3 elemental carbon (EU, 2019; EU, 2004) whereas in America the limit for a non-coal mining setting is set at 160 µg/m^3 total carbon (MSHA, 2016) and in Australia it is recommended that diesel exhaust not exceed 100 µg/m^3 elemental carbon (AIOH, 2017). In this study, particle mass concentrations were between 42.6 and 54.4 µg/m^3 of which less than 75% is elemental carbon (Chapter 1), again showing that common exposure standards were not exceeded.

In the current study, biodiesel exhaust did not contain higher levels of NO_x or lower levels of PM compared with ULSD exhaust, as is commonly reported in the literature (Fontaras et al., 2009; Graver et al., 2016; Mullins et al., 2016; Prokopowicz et al., 2015; Unosson et al., 2021). That said, other studies that measured NO_x and PM have also reported either no differences between biodiesel and mineral diesel exhausts, or a decrease in the biodiesel exhaust (Hawley et al., 2014; Prokopowicz et al., 2015; Valand et al., 2018). Previous studies from my laboratory have also found a wide variation in NO_x and PM concentrations in biodiesel exhaust compared to ULSD, with differences dependent on feedstock type used to make the biodiesel (Landwehr et al., 2021; Landwehr et al., 2019; Mullins et al., 2016). This suggests that differences in NO_x and PM concentrations between diesel and biodiesel are subtle enough that the dilutions used in toxicology studies to make concentrations "real world" relevant can mask the changes (Fontaras et al., 2009; Graver et al., 2016; Landwehr et al., 2021) and/or that the differences are related to feedstock type. The overarching idea in literature that biodiesel exhaust overall contains more NO_x and less PM is feedstock specific and should thus be viewed critically (Landwehr et al., 2021). This idea is further supported by the findings that Tallow biodiesel exhaust is no different to ULSD in terms of PM and NO_x but that Canola biodiesel exhaust contained significantly less NO_x. Another potential explanation is that many previously published exhaust-only comparisons have been conducted using old technology engines not equipped with a diesel particulate filter and/or diesel oxidation catalyst (Fontaras et al., 2009; Graver et al., 2016; Westphal et al., 2013) and thus the prevailing idea in the literature of increased NO_x and decreased PM in biodiesel exhaust is true only for older technology engines (Hawley et al., 2014; Prokopowicz et al., 2015; Valand et al., 2018).

A key finding of this study is that both the 1-day and 8-day Tallow biodiesel exhaust exposed mice were both hyperresponsive to MCh with respect to airway resistance. This was also observed for the 1-day ULSD exposed mice, suggesting that very little exhaust is needed to induce a hyperresponsive phenotype and that a single exposure can result in similar effects as

multiple exposures. The mice in this study were exposed for only two hours per day, to exhaust that largely met Safe Work Australia Standards, and yet airway resistance responsiveness to methacholine increased significantly for two of the 1-day exposures and 1 of the 8-day exposures. It is possible that differences between treatments would be observed if the 8-day exposure were extended, but in this study, 1-day of exposure and 8-days of exposure resulted in similar effects for Tallow biodiesel exhaust. The response measured is smaller compared to similar exposure studies in smoking, asthmatic and respiratory viral infection mouse models (Collins et al., 2005; Larcombe et al., 2017; Kimberley C. W. Wang et al., 2018) however comparisons between models which employ a variety of environmental exposures are difficult. Previous studies testing response to methacholine in mice after intranasal instillation of black carbon or diesel exhaust found greater hyperresponsiveness than was measured in our study, although differences in diesel exhaust exposure protocols and methacholine dosages make direct comparisons difficult (Lambert, Mangum, DeLorme, & Everitt, 2003; Nemmar et al., 2011). Studies that co-exposed house dust mite and diesel exhaust also found increased responsiveness to methacholine, although only in the co-exposed group and not in the diesel exhaust alone exposed group (Brandt et al., 2015; Brandt et al., 2013). Studies testing response to methacholine after diesel exhaust exposure in asthma and atopy also found increased hyperresponsiveness, although once again these studies cannot be directly compared due to difference in subject type and measurements (Chris Carlsten, MacNutt, Zhang, Sava, & Pui, 2014; Nordenhäll et al., 2001). Furthermore this finding of airway hyperresponsiveness has concerning implications for those with asthma and allergy exposed either acutely or for prolonged periods of time to diesel or biodiesel exhaust, especially as diesel exhaust can act as an sensitiser for aeroallergens (Alvarez-Simón et al., 2017) and the responsiveness to methacholine suggests that biodiesel exhaust exposure may elicit worse responses.

Thus, the finding that mice exposed to Canola biodiesel exhaust for 2 hours per day for 8 days were less responsive to methacholine than Air controls was unexpected. Despite being the least toxic in terms of methacholine response and immune cell counts, the Canola biodiesel exposure groups are the stand-outs in this experiment with indications of both positive and negative health impacts. While the lower 8-day methacholine responsiveness compared to Air, increased thoracic gas volume measurements (despite the Canola mice being significantly smaller than the other groups) and decreased airway resistance in the volume dependant measurements could be interpreted as positive findings (i.e. “improvements” compared with Air controls), when combined with the negative indications of increased specific R_{aw}, G and H at FRC in the C8 and the increased G and H in the C1 volume dependant measurements, it instead suggests that the complete picture is much more complicated. Diesel (and thus likely biodiesel) exhaust is a highly complex mixture made up of thousands of different chemicals (J. Bünger et al., 2000; Fontaras et al., 2009; Hemmingsen et al., 2011; Hesterberg et al., 2011; George Karavalakis et al., 2009; Khalek et al., 2011; Kisin et al., 2013) and it is possible (and indeed likely, from the results of this study) that exposure to such a mixture could lead to both “positive” and “negative” health impacts as seen for Canola biodiesel exhaust. Further experiments are needed to explore what makes the Canola biodiesel exhaust exposure group so unique and whether those potentially positive effects can be isolated to not only what is changing in the lungs of the exposed mice to induce them, but also what part of the Canola biodiesel exhaust is causing them. To address this,

BAL choline containing phospholipid concentration was measured to assess whether Canola biodiesel exhaust altered surfactant levels in the lungs. Surfactant is comprised of approximately 70% of phosphatidylcholine, which in turn makes up approximately 80% of phosphatidylcholine in the lungs (Bernhard, 2016; Chakraborty & Kotecha, 2013). It is both produced naturally and also used medicinally to improve breathing in preterm children and other children at risk of respiratory failure, as it acts to acts to decrease surface tension at the air–liquid interface of the lung alveoli (Chakraborty & Kotecha, 2013). However, only the T1 exposed group showed any difference in phospholipid concentrations, an interesting finding in of itself with potential to suggest that acute exhaust exposure can rapidly change lung chemistry. Thus, reasons for why the C8 mice responded as they did are difficult to elucidate and warrants further investigation.

Another key finding of this study was the increased cell numbers in the BAL of both T1 and T8 biodiesel exhaust exposed mice and the U8 exhaust exposed mice. This increase mostly consisted of an increase in macrophages, and an increase in lymphocytes in the 8-day exposed groups. Interestingly, neutrophil numbers decreased between T1 and T8 exposed mice, even though the total BAL cell concentration show the opposite trend. A decrease in neutrophils was also observed between the A8 group compared to both the C8 and U8 groups. This suggests some immune dysregulation might be occurring in mice exposed to exhaust for the longer duration, a finding that is supported by the local (BAL) and systemic (serum) mediator response both of which show significant decreases in the T1 and/or T8 exposed mice compared to their respective Air controls. Due to kinetics of immune mediator release after exhaust exposure, wherein the greatest immune responses in a previous study were found 3-6 hours after exposure with decreases back to baseline levels observed by 24 hours (Boylen, Sly, Zosky, & Larcombe, 2011), a decrease in the 1-day exposed mice was expected and may be due to immune mediators being “used up” by the time 24-hour end point measurements were conducted. However, with the depletion effect ongoing even in the 8-day exhaust exposure groups, combined with the decrease in neutrophil numbers (especially when those numbers are increased after 1-day of exposure to both biodiesel exhausts), this instead suggests an inability for the mouse immune system to cope with ongoing exhaust exposure, which could have serious consequences for cancer and infection (Dai et al., 2018; Gowdy et al., 2010; Shears et al., 2020; Maria C. Zarcone et al., 2017). These findings have been mirrored in a diesel exhaust human exposure study of occupationally exposed workers (Dai et al., 2018), which found workers exposed to high amounts of exhaust for prolonged periods showed immune dysregulation and decreases in serum inflammatory mediators, such as IL-8 and MIP-1β. In addition, previous studies co-exposing mice to both a respiratory infection and diesel exhaust found that exposure increased infection susceptibility (Larcombe, Foong, Boylen, & Zosky, 2012; Shears et al., 2020). Studies have also been able to induce allergic airways disease using diesel exhaust (Alvarez-Simón et al., 2017) and human exposure studies on populations with allergic rhinitis found that diesel exhaust exacerbated allergic inflammation, likely by dysregulating the immune systems’ ability to remove eosinophils (Pawlak et al., 2016).

There were also minor changes in the lung structure of Tallow biodiesel exhaust exposed mice. The T8 exposed mice showed an increased protein content in the BAL, which is a marker of increased lung permeability and epithelial damage (Lambert et al., 2003; Maria C. Zarcone et al.,

2016) and further supports the finding of increased epithelial cell damage and increased permeability in air-liquid interface cell cultures (Chapter 5). This finding is also supported by the small, but statistically significant increase in chord length in T8 mice. Chord length, also known as mean linear intercept, is a measure of the mean space between airway structures (Crowley et al., 2019; Hsia et al., 2010) and increased chord length has been linked to airway damage and disease such as emphysema (Mitzner, 2008) although it is not a direct measurement of airway size as airway morphology is complex and never just a single shape (Hsia et al., 2010). Increased epithelial damage and increased chord length would indicate damage to the airways by Tallow biodiesel exhaust exposure (Crowley et al., 2019; Mitzner, 2008), which is concerning after such a relatively short duration exposure period. There were no other indications of changes to airway morphometry, however very mild exposures were used in comparison to some previous studies (de Brito et al., 2018; Gavett et al., 2015; Larcombe et al., 2014).

6.5 Conclusion:

Exposure to diesel and/or biodiesel exhaust impacted to lung function measured at FRC, volume dependant lung function, methacholine responsiveness, inflammation and airway morphometry in a mouse model. In line with previous research (Chapter 3/(Landwehr et al., 2021)), Tallow biodiesel exhaust exposure resulted in the widest range of negative health impacts, followed by ULSD exhaust with Canola biodiesel exhaust causing the most limited impacts and arguably even having a positive effect on methacholine response. More research is needed to parse out reasons for this.

Chapter 7: The Screening Model

Abstract:

Background: The majority of biodiesel exhaust literature focuses on its effects on engine wear, fuel efficiency and exhaust characteristics. Few studies assess how the toxicology of exhaust exposure changes between diesel and biodiesel, let alone between different biodiesel fuels. This is likely because the toxicology studies are expensive, time consuming and require experts from a wide range of vastly different fields. Thus, the aim of this study was to use recently generated data that tested the toxicological outcomes of exhaust exposure for 6 different biodiesel fuels to generate an *in silico* screening model. This screening model could then be used to predict health effects of exposure to new biodiesel exhaust types and directly compare them to the generated data.

Methods: The statistical program R and the packages "vegan", "rpart", "rpart.plot" and "lmrrpp" were used to generate the model. First, redundancy analysis was performed to assess and compare the suitability of both the biodiesel exhaust components and the fuels fatty acid methyl ester (FAME) profile for their ability to predict toxicological outcomes. Next, linear regression trees were generated to analyse how different fuel components impact toxicity. Finally, the screening model was generated using linear modelling with a randomized residual permutation procedure and data from five of the tested fuels so that the sixth could be used for quality control analysis.

Results: The redundancy analysis showed that the biodiesel FAME profile explained more of the variance observed in the toxicological outcomes than the exhaust physico-chemical characteristics. Linear regression tree analyses showed that the number of double bonds greatly impacted toxicity with saturated FAMEs having the greatest impact. The screening model was successfully generated and accurately predicted the toxicity of the 6^{th} biodiesel fuel for the majority of toxicological outcomes. Biodiesels from other studies were also successfully inputted and predicted results were biologically feasible, however the more dissimilar the FAME profile to the ones used in this study the more caution should be taken.

Conclusion: An *in silico* screening model was successfully generated that can use the FAME profile of new biodiesels to predict toxicological outcomes. The model ultimately needs strengthening using toxicity data from biodiesels with more extreme FAME profiles however it is the first of its kind to date.

7.1 Introduction:

Biodiesel is a renewable diesel fuel that can be made from almost any fat or oil (Beer et al., 2007; Graboski et al., 2003). The feedstock type used during creation alters the fatty acid methyl ester (FAME) profile (Jafarihaghighi, Ardjmand, Salar Hassani, Mirzajanzadeh, & Bahrami, 2020; Landwehr et al., 2021). This profile greatly determines fuel properties such as cetane number, iodine number, viscosity and density (G. Knothe & Steidley, 2005; McCormick et al., 2001), which in turn alters exhaust physico-chemical properties and thus the health impacts of

exhaust exposure (Landwehr et al., 2021). Unfortunately, previous studies on the health impacts of biodiesel exhaust exposure rarely take this into account, often testing only one type of biodiesel and drawing broad conclusions based off that type (Hawley et al., 2014). Some studies do not state the feedstock type used for their biodiesel fuel (Ackland et al., 2007; Hawley et al., 2014), thus incorrectly implying that all biodiesels are the same.

In addition, different exhaust toxicology studies have different limitations and variabilities inherent in the methodologies used. Exhaust physico-chemistry can be greatly altered by different engine parameters such as the use (or not) of after-treatment technologies such as particulate matter filters (Magnusson et al., 2019; Westphal et al., 2013), different loads (Fontaras et al., 2009; Olfert et al., 2007) and the use of a drive-cycle (of which there are many) compared to a constant speed (Bass et al., 2015; Douki et al., 2018). Different reference diesel fuel controls, which can have vastly different sulfur levels (ranging from <10 ppm to >500 ppm) are also used, which can impact outcomes such as mutagenicity (Brito et al., 2010; de Brito et al., 2018; Farraj et al., 2015; Mullins et al., 2016). Different end point health outcomes are also measured ranging from mutagenicity, cytotoxicity, oxidative stress, circulatory impacts and immune responses. These are tested in exposure models ranging from enzymatic response, bacterial assays, to complex *in vitro* and *in vivo* models (Douki et al., 2018; Hazari et al., 2015; Jalava et al., 2012; Lankoff et al., 2017; Westphal et al., 2013). Due to these wide range of methodological differences, it is unadvisable to directly compare biodiesel from different feedstocks if the health effects measured come are obtained from different studies (Larcombe et al., 2015). As different diesel fuels are used between different studies (Jürgen Bünger et al., 2000), it is not possible to use diesel controls as reference fuels to "normalise" studies.

Another inherent methodological difference present in both biodiesel and diesel exhaust exposure studies is that many studies only explore the resulting health effects of the diesel particulate matter, ignoring the gaseous components entirely (Gioda et al., 2016; Skuland et al., 2017). Both diesel and biodiesel share many similarities in exhaust components, although exact concentrations of the various components vary greatly (Fontaras et al., 2009; Graver et al., 2016; Prokopowicz et al., 2015). A common method for toxicological studies of exhausts is to collect only the particulate matter on a filter and then resuspend it in solution to be added directly to cell/bacterial media or instilled directly into the nose/trachea of a mouse or rat (Bendtsen et al., 2020; Gioda et al., 2016; Skuland et al., 2017; Yanamala et al., 2013). This method is often easier than using whole exhaust, and yet studies have found that up to 16 time more particles are needed to generate the same health impact as if whole exhaust was used from the start (Lichtveld et al., 2012).

In Chapter 3 I recently performed a study testing the health impacts of whole exhaust exposure on primary human airway epithelial cells obtained from eight different volunteers using a submerged cell culture model (Landwehr et al., 2021). Although a wide range of fuels were tested before the limited cell stocks depleted, this study still suffered from the inherent limitations found in all exhaust exposure models; it cannot be directly compared to other studies. Thus, the aim was to use the data obtained to generate a mathematical screening model that would allow the potential health impacts of exposure to new biodiesels (not tested in our study)

to be estimated from the fuel or exhaust characteristics alone. This would allow new fuels to be tested and compared without the need for time-consuming and expensive biological assessments of exhaust toxicology, and allow comparison between studies regardless of methodological differences. Although both exhaust components and fuel FAME profiles were tested for their suitability in predicting biological outcomes of exhaust exposure, the mathematical screening model was ultimately developed with the latter due to variability in exhaust outputs related to the methodological differences listed above.

7.2 Materials and Methods:

7.2.1 Sampling: All results used in these analyses were taken from Chapter 3 as it gave the broadest data set and the widest range of fuels with which to build the model. Submerged primary airway epithelial cells from 8 volunteers were exposed to diluted exhaust from an engine running on various diesel or biodiesel fuels: Ultra-Low Sulfur Diesel (ULSD) or Canola, Soy, Waste Cooking Oil (WCO), Tallow, Palm or Cottonseed biodiesel. Biodiesel fatty acid methyl ester (FAME) content was analysed and a list of six particle and seven gas exhaust components were characterised for each of the fuels (Chapter 3). The impact on viability, cell death mechanisms and mediator release was measured for end point toxicological assessment. All results have been normalised to Air controls so as to remove any background effects from the model.

7.2.2 Statistical Analysis: Statistical analysis was run using the R packages "Vegan", "rpart", "rpart.plot" and finally "lmrrpp". First, using the R package "vegan" (Oksanen et al., 2020), redundancy analysis (RDA) was run on the toxicological endpoints (Table S7.1) compared to exhaust outputs (Table S7.2) of the both ULSD as well as the 6 different biodiesel fuels to get a picture of how the different exhaust variables impacted the exposed cells. Second, RDA was run on the toxicological endpoints compared to the various FAME variables (Table S7.3) for all of the biodiesel fuels. As ULSD is not a biodiesel, the results for that exposure were excluded from the second RDA as well as all subsequent analysis. Third, linear regression trees were run using the R package "rpart" (Therneau, Atkinson, Ripley, & Ripley, 2019) to analyse the importance of the various FAME variables for all toxic outcomes. This was done after a correlation analysis on the FAME profiles to exclude redundant FAME variables with a correlation value above 0.95. Using the additional package "rpart.plot" (Milborrow, 2020), the most important splits in the created trees were plotted.

Linear regression modelling was performed on the whole dataset from the six tested biodiesel exhausts. This includes each of the replicate results for each fuel and toxicological endpoint of the exposed cells for each of the eight volunteers. Since the data were high-dimensional (i.e. when only using averages, the number of variables exceeds the number of observations), the linear model was evaluated with a randomized residual permutation procedure using the "lmrrpp" R package (Collyer & Adams, 2021). To account for biological variability when first testing the fit of the various linear regression models, each of the volunteers was assigned a random code which was added to the linear model as a factor variable. To obtain the coefficients, the biological variability was removed by rerunning the same model in "lmrrpp" on only the averages of each biological result obtained from the 8 different volunteers' cells, excluding the

need for the factor code. The following were excluded from calculating the coefficients: toxic outcomes where the results from some volunteers were out of range of the testing kits used (Chapter 3) (IL-1RA, IP-10) and where the linear model did not provide a significantly relevant fit (early apoptosis, IL-1β) or where no significant differences were observed after exposure to at least one of the biodiesel exhausts (necrosis). As the remaining cell death variable, late apoptosis, was mostly described by the viability results, that endpoint was also excluded for being redundant. After generating the model using the results from five of the six tested fuels (Canola, Cottonseed, Palm, Tallow and Soy), Tallow and Canola were rerun through the model to test the fit between predicted outcomes and measured outcomes. The sixth fuel, WCO (which was left out of model building) was then run through the model in order to further test the differences between results predicted from the model compared to the results actually measured. An additional three biodiesel FAME profiles were also run through the model to test its robustness: one (Bran) generated at our lab without any accompanying toxic outcomes and an additional two (*Fritillaria persica* and *Viola odorata*) provided by another study (Jafarihaghighi et al., 2020).

7.3 Results:

7.3.1 RDA of Biological Results compared to Exhaust Variables: First a redundancy analysis model (RDA) was fitted to the exhaust variables and toxicological outcomes (Tables S7.1 and S7.2) in order to assess how well the exhaust components explained the resulting biological results. The fitted RDA model explained 40.82% of the overall variability observed within the toxicological results, with the first two axes explaining 69.91% of the variability observed within the fitted model (28.76% of the variability overall) (Figure 7.1) and the addition of a third axis explaining 84.75% (34.59% of the variability overall). Axis 1 was strongly related to SO_2 and particle number, particularly the number of particles below 35 nm in size, whereas axis 2 was strongly related to CO_2 and NO_x, particularly NO_2, and axis 3 to particle size. For the exhaust components, CO and the various particle number characteristics were highly correlated, NO_x was highly correlated with NO and NO_2 and negatively correlated with CO_2. In terms of biological components, viability was negatively correlated with many of the mediator responses, which were mostly correlated with each other.

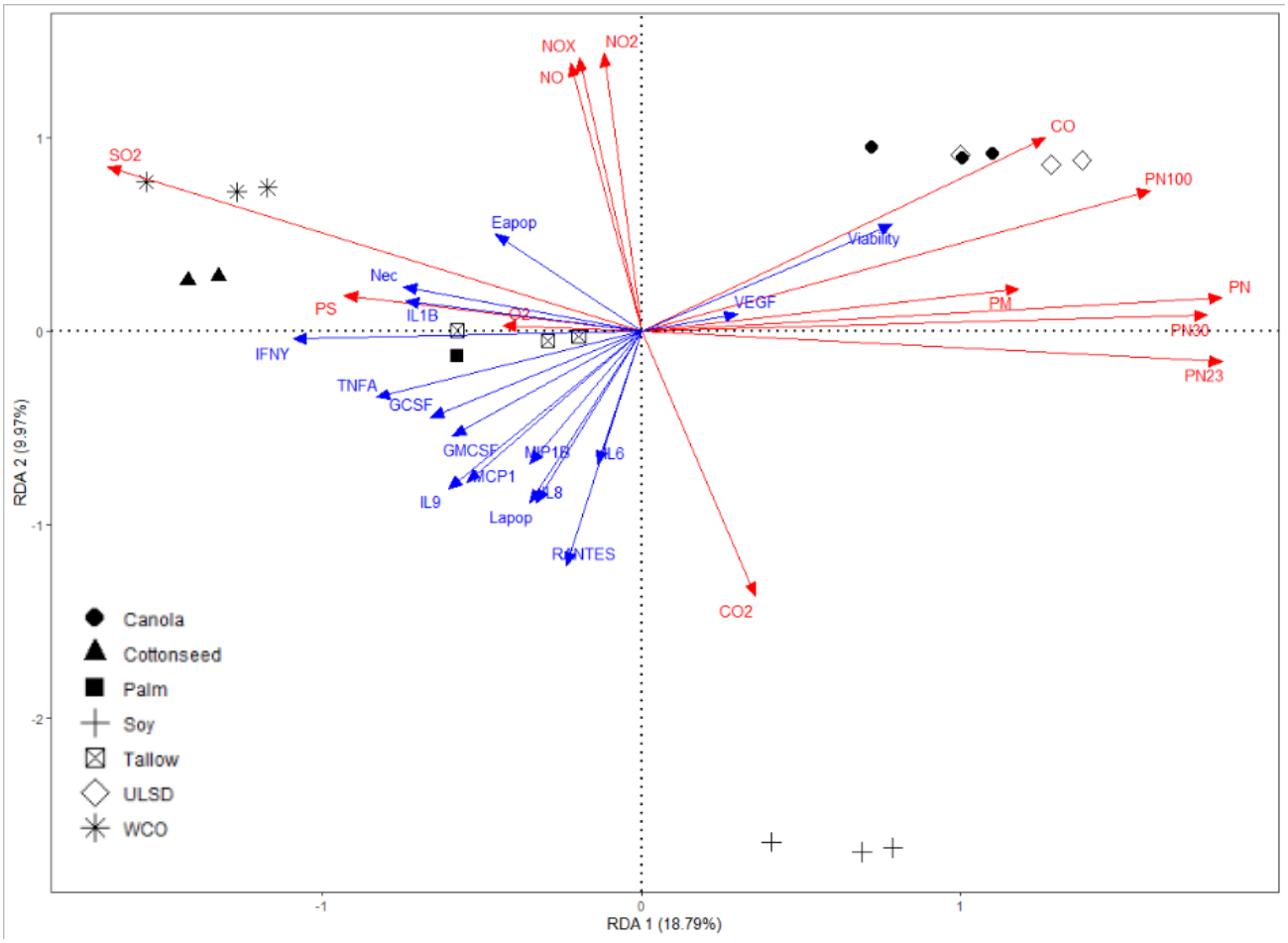


Figure 7.1: Redundancy analysis model plot showing the results for the analysis of the exhaust components impact on the various toxicological endpoints. The percentages for each axis are the proportion of variability explained by that axis for the entire dataset.

7.3.2 RDA of Biological Results Compared to Fuel FAME Analysis: Another RDA model was fitted using the biodiesel FAME compositions and the toxicological variables (Tables S7.1 and S7.3) in order to assess how well the FAME components explained the biological results. The fitted RDA model explained 50.95% of the overall variability observed within the toxicological results, with the first two axes explaining 84.97% of the variability observed within the fitted model (43.73% of the variability overall) (Figure 7.2) and the addition of a third axis explaining 99.92% (50.54% of the variability overall). Axis 1 was strongly related to C14:0 and C18:0 whereas axis 2 was strongly related to C18:1 and axis 3 to C18:2. For the FAME components themselves, C18:0 was highly correlated with C14:0 and C16:1 and C18:1 was negatively correlated with C16:0. The biological components show a similar pattern as seen with the exhaust RDA with viability negatively correlated with many of the mediator responses which were in turn correlated with each other.

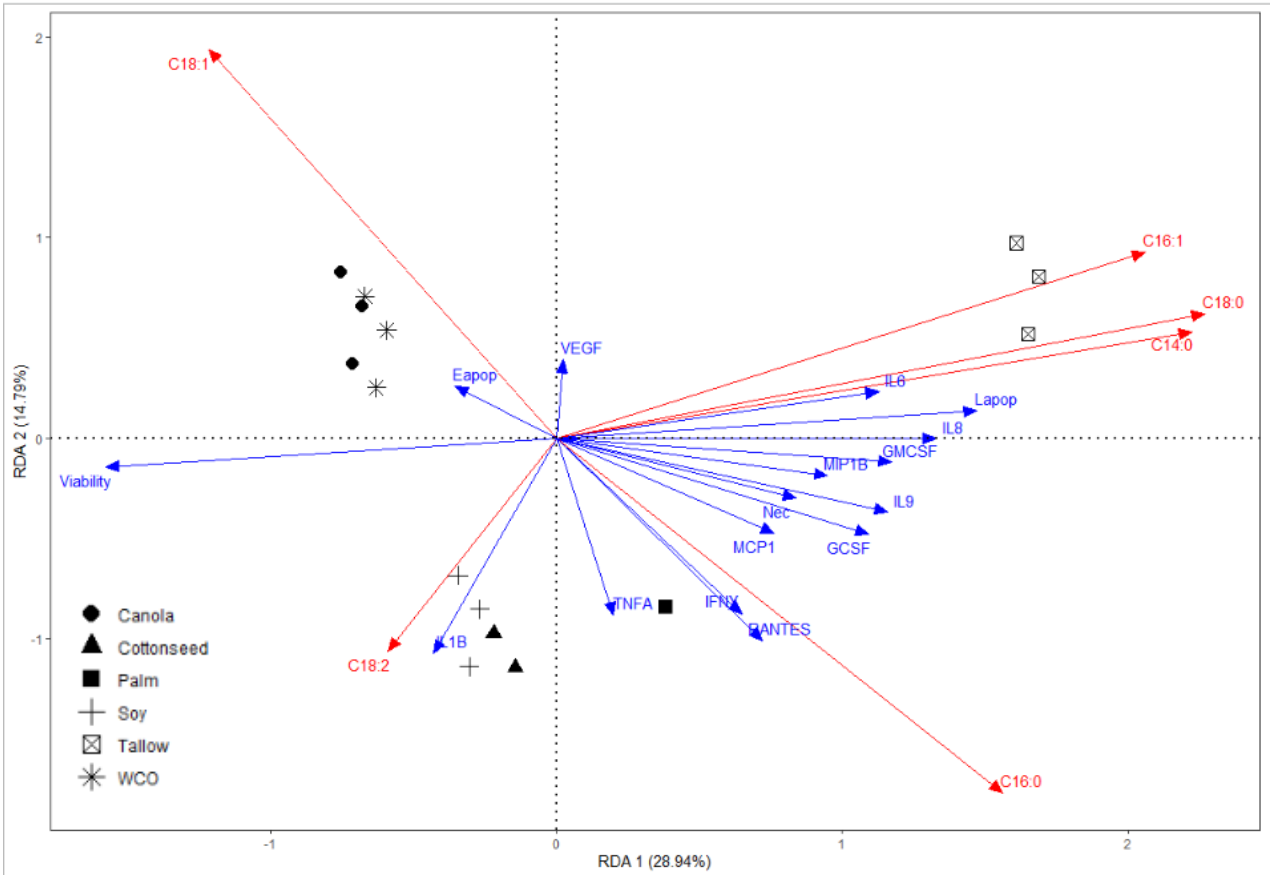


Figure 7.2: Redundancy analysis model plot showing the results for the analysis of the FAME composition impact on the various toxicological endpoints. The percentages for each axis are the proportion of variability explained by that axis for the entire dataset.

7.3.3 Linear Regression Trees Modelling of FAME: Before the regression tree analysis was begun, a correlation analysis of all the FAME components was performed (Table 7.1). C14:0, C16:1 and C18:0 were found to have correlations over 0.90 with each other. As C18:0 makes up a greater proportion of the FAME content for all fuels, C14:0 and C16:1 were subsequently removed from further analysis for being redundant.

The linear regression trees ranked FAME components by order of importance for their impact on the toxicological outcomes (Table 7.2). C18:0 was the most important component followed by C18:1 and C16:0. The trees for each toxic outcome can be found in Figure 7.3 and show that despite C18:2 having minimal importance in the ranked order of effects, a lot of the nodes in the linear tree use this component to define where to split and group results. C18:2 is often the second or lower node.

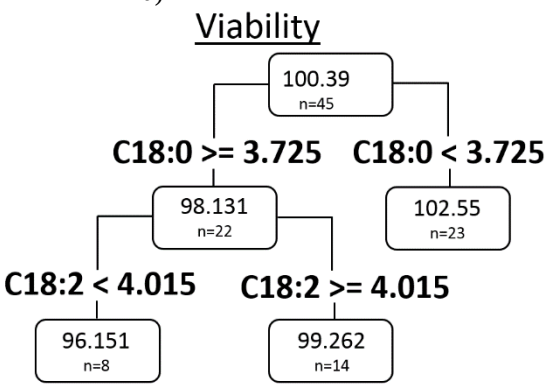
Table 7.1: FAME correlation results. Correlations over 0.90 (bold) were considered redundant and the FAME removed from further analyses.

	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
C14:0	**1**	0.472	**0.954**	**0.947**	-0.100	-0.583
C16:0	0.472	**1**	0.215	0.294	-0.553	-0.274
C16:1	**0.954**	0.215	**1**	**0.979**	-0.024	-0.475
C18:0	**0.947**	0.294	**0.979**	**1**	-0.211	-0.336
C18:1	-0.100	-0.553	-0.024	-0.211	**1**	-0.603
C18:2	-0.583	-0.274	-0.475	-0.336	-0.603	**1**

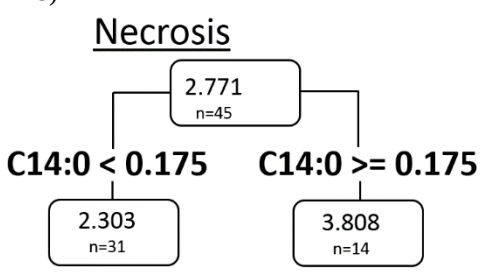
Table 7.2: Linear tree regression models for each toxic outcome (first column), showing the ranked importance of various FAME variables. A higher number indicates a more important variable for predicting the outcome measured. Highlighted variables for each model are considered the most important for the particular biological outcome tested.

Variable	C16:0	C18:0	C18:1	C18:2
Viability	20	33	24	23
Necrosis	39	39	0	22
Early Apoptosis	39	39	0	22
Late Apoptosis	12	48	12	29
IL-1β	22	22	32	24
IL-6	9	45	1	45
IL-8	21	33	24	22
IL-9	23	32	23	22
G-CSF	38	24	6	32
GM-CSF	31	28	31	9
INF-γ	20	13	52	15
MCP-1	32	32	32	4
MIP-1β	23	33	23	21
RANTES	19	33	25	24
TNF-α	12	12	69	6
VEGF	13	15	60	12
Number of times most important FAME variable	5/16	10/16	6/16	1/16

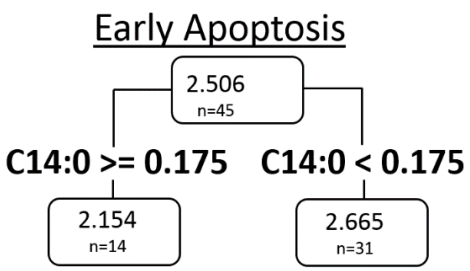
a)



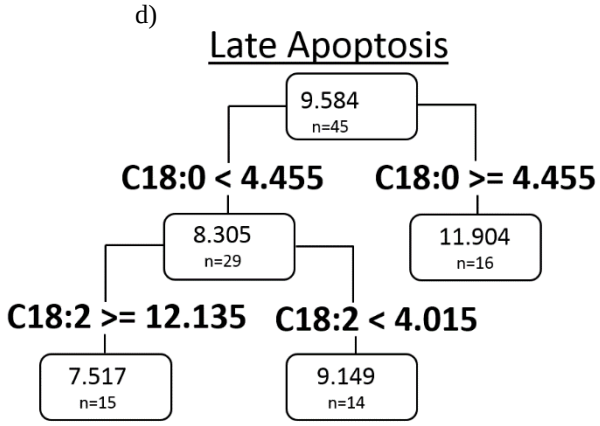
b)



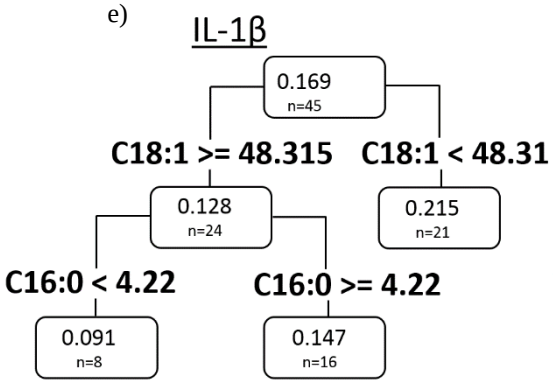
c)


Early Apoptosis
2.506
n=45
C14:0 >= 0.175
C14:0 < 0.175
2.154
n=14
2.665
n=31

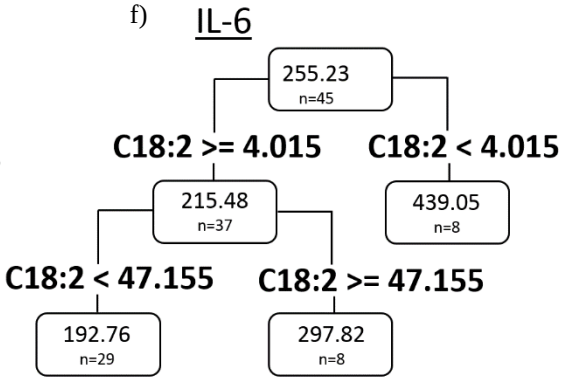
d)


Late Apoptosis
9.584
n=45
C18:0 < 4.455
C18:0 >= 4.455
8.305
n=29
11.904
n=16
C18:2 >= 12.135
C18:2 < 4.015
7.517
n=15
9.149
n=14

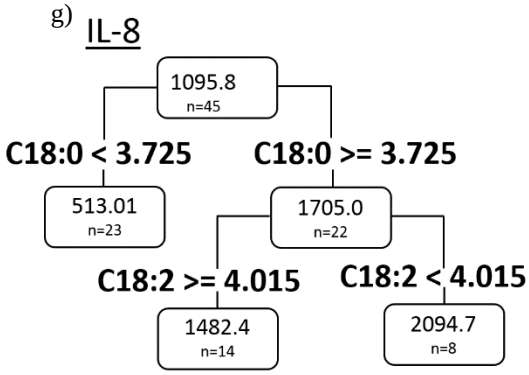
e)


IL-1β
0.169
n=45
C18:1 >= 48.315
C18:1 < 48.31
0.128
n=24
0.215
n=21
C16:0 < 4.22
C16:0 >= 4.22
0.091
n=8
0.147
n=16

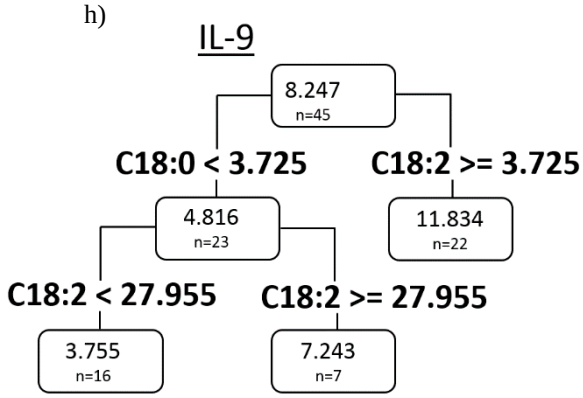
f)


IL-6
255.23
n=45
C18:2 >= 4.015
C18:2 < 4.015
215.48
n=37
439.05
n=8
C18:2 < 47.155
C18:2 >= 47.155
192.76
n=29
297.82
n=8

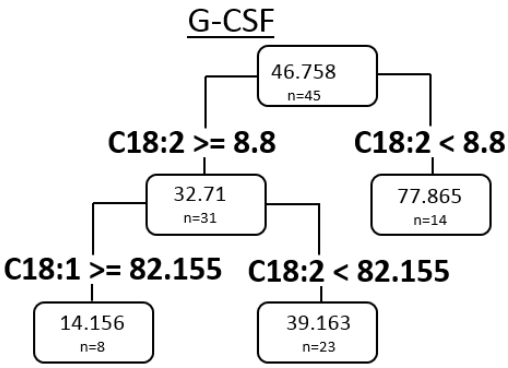
g)


IL-8
1095.8
n=45
C18:0 < 3.725
C18:0 >= 3.725
513.01
n=23
1705.0
n=22
C18:2 >= 4.015
C18:2 < 4.015
1482.4
n=14
2094.7
n=8

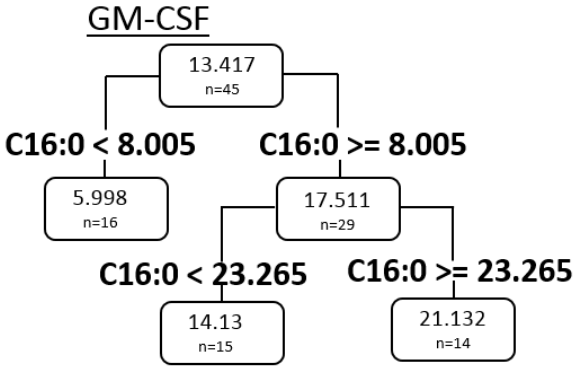
h)


IL-9
8.247
n=45
C18:0 < 3.725
C18:2 >= 3.725
4.816
n=23
11.834
n=22
C18:2 < 27.955
C18:2 >= 27.955
3.755
n=16
7.243
n=7

i)


G-CSF
46.758
n=45
C18:2 >= 8.8
C18:2 < 8.8
32.71
n=31
77.865
n=14
C18:1 >= 82.155
C18:2 < 82.155
14.156
n=8
39.163
n=23

j)


GM-CSF
13.417
n=45
C16:0 < 8.005
C16:0 >= 8.005
5.998
n=16
17.511
n=29
C16:0 < 23.265
C16:0 >= 23.265
14.13
n=15
21.132
n=14

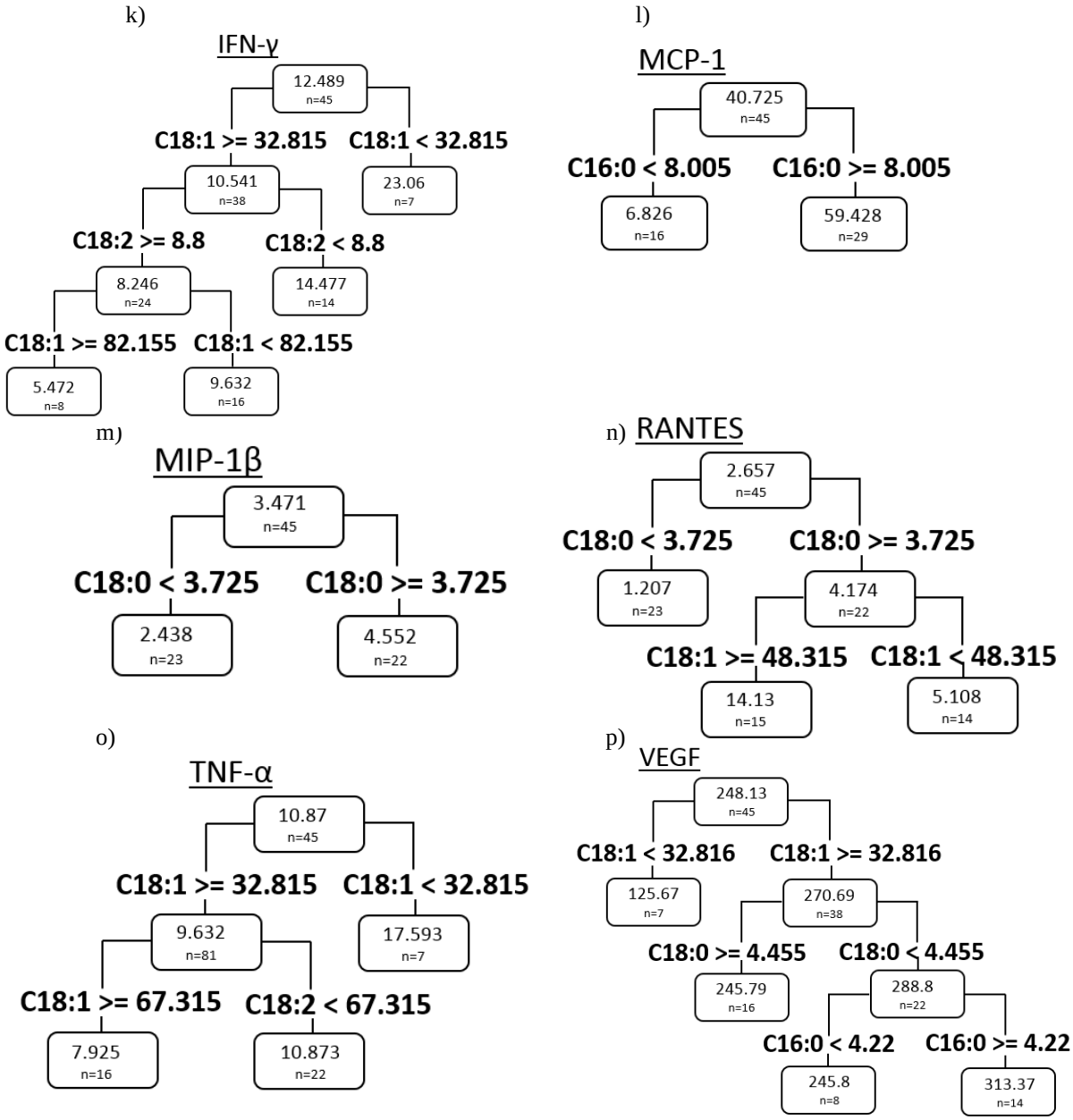


Figure 7.3a-p: Linear tree regression plots for all toxic outcomes. Data are split as per the most prominent FAME impacts. The numbers in the branches indicates the FAME where the split is the most prominent and the value is the percentage of that FAME where the data best splits. The number in each leaf indicates the mean of the measured toxic variable at that split in % for the cell viability and cell death data and µg/ml for the mediator release (n=sample size at that point).

7.3.4 Linear Modelling of FAME: The results of the linear regression models fits for each toxic outcome are shown in Table 7.3. All toxic outcome models showed were significant fit, except

for early apoptosis and IL-1β and thus these endpoints were excluded from further analysis. The linear regression model that was applied to each toxic outcome was:

$y=A + (B \times C16:0) + (C \times C18:0) + (D \times C18:1) + (E \times C18:2)$.

The intercept (A) and coefficient values (B, C, D and E) from the above model for each toxic outcome are displayed in Table 7.4. The predictions of each toxic outcome for new biodiesels can be estimated by applying the percentage of the whole FAME components of the new biodiesel to the above equation and the coefficients and intercept for the toxic outcome of interest (Table 7.4). If the results obtained by using the regression model exceed the significance threshold (Table 7.4), which is based on the lowest value for each toxic outcome for which significant differences compared to Air controls were found for the six tested biodiesels in Chapter 3, it can be considered to have a significantly toxic impact on that outcome. The total number of toxic impacts are then tallied and this tally can be compared to the six tested fuels in order to assess where the new fuel ranks in terms of toxicity (Table 7.5).

Table 7.3: The fits of separate linear regression models for each toxic outcome. Significant p values ($p<0.05$) are in bold. The R^2 values are a measure of the proportion of the variability for a toxic outcome explained by the FAME profile. The F value (arbitrary, with meaning only within model) is the probability that the null hypothesis is true and the P value is that probability translated to a non-arbitrary value.

Variable	R^2	F	P
Viability	0.430	1.982	**0.024**
Necrosis	0.617	4.822	**0.0016**
Early Apoptosis	0.362	1.702	0.089
Late Apoptosis	0.528	3.353	**0.0026**
IL-1β	0.409	2.073	0.052
IL-6	0.686	6.569	**0.0006**
IL-8	0.572	4.006	**0.0006**
IL-9	0.626	5.026	**0.0006**
G-CSF	0.534	3.432	**0.0056**
GM-CSF	0.607	4.635	**0.0016**
INF-γ	0.594	4.394	**0.006**
MCP-1	0.612	4.729	**0.0006**
MIP-1β	0.694	6.809	**0.0006**
RANTES	0.508	2.540	**0.0086**
TNF-α	0.466	2.163	**0.0216**
VEGF	0.407	2.059	**0.0416**

Table 7.4: Coefficients and intercepts for the linear regression models of each toxic outcome. If the results obtained by using the regression model exceeded the significance threshold (last column), which was based on the significant results ($p<0.05$, Chapter 3) for the 6 tested biodiesels, it can be considered to have a significantly toxic impact on that outcome.

Variable	Intercept (A)	C16:0 (B)	C18:0 (C)	C18:1 (D)	C18:2 (E)	Significance Threshold
Viability (%)	7163.10581	-70.7464	-78.0632	-70.5467	-69.9883	<95.6
IL-6 (µg/ml)	-90813.3289	910.6771	1017.333	909.1063	902.7131	>297.82
IL-8 (µg/ml)	-1693878.62	16979.08	18718.96	16934.98	16796.3	>1373.26
IL-9 (µg/ml)	-8490.4076	85.21329	93.75524	84.89772	84.22396	>9.77
G-CSF (µg/ml)	-53079.252	533.0373	585.2129	530.7095	526.3247	>47.22
GM-CSF (µg/ml)	-627.65827	6.539907	7.605787	6.346767	6.324105	>13.77
INF-γ (µg/ml)	25223.6057	-252.194	-277.276	-252.138	-249.826	>15.11
MCP-1 (µg/ml)	-22664.0987	227.8166	252.2044	226.4629	225.3314	>8.25
MIP-1β (µg/ml)	-3487.55972	34.98934	38.46523	34.88486	34.5936	>3.92
RANTES (µg/ml)	-7484.67657	75.06548	82.28514	74.83171	74.19792	>2.53
TNF-α (µg/ml)	13439.1223	-134.351	-147.831	-134.264	-133.041	>9.60
VEGF (µg/ml)	-353456.381	3542.598	3888.263	3536.205	3504.383	<125.67

Table 7.5: Number of significant toxic outcomes for each of the six tested biodiesel fuels. This includes only the endpoints that met the inclusion criteria for the final linear regression models.

Fuel	Canola	WCO	Cotton-seed	ULSD	Palm	Soy	Tallow
Number of Significant Toxic Outcomes	0	1	4	3	5	5	6

7.3.5 Testing the Linear Models: In order to assess the accuracy of the linear models, both Tallow and Canola biodiesel had their FAME profiles run through the model generated for each toxic outcome and predicted results were compared to the actual results obtained for each fuel (Table 7.6). A greater than 99.9% match was obtained for each of the 12 models for both of the fuels tested. The remaining fuel, WCO which was not used to generate the model for validation purposes, was also run through and predictions assessed against actual results. Viability and IL-6 were closest in terms of measurements matching, however toxic threshold (the lowest value at which significant difference was measured when compared to Air controls) was accurately predicted for 8 of the 12 toxicological variables (Table 7.7). Those that were not accurately assessed (G-CSF, MIP-1β, RANTES and TNF-α) have been highlighted and removed from further validation.

In addition to WCO, the FAME profile of Bran biodiesel was run through the models, although there are no biological outcomes for this biodiesel. Bran biodiesel has the following profile: 0% C14:0, 18.0% C16:0, 0% C16:1, 4.45% C18:0, 63.63% C18:1 and 1.30% C18:2. The predicted results for Bran biodiesel are shown (Table 7.8). A total of six variables met the toxic threshold, putting Bran biodiesel at the same toxicity level as Soy and Palm biodiesel. This shows that new biodiesel types can be successfully inputted into and assessed by the generated models.

The FAME profile for *F. persica* was also tested (Table 7.8) (Jafarihaghighi et al., 2020). As the oils tested in the Jafarihaghighi et al., 2020 study had a wider range for FAME types than tested here, additional FAMES longer than C18 were added to the C18 percentages (the closest carbon chain number) based on the number of double bonds in the chain. Thus: C16:0= 10.03% C16:0, C18:0 = 3.30% C18:0 + 1.33% C20:0 + 0.28% C22:0, C18:1= 19.32% C18:1 + 0.13 C20:1, C18:2= 56.17% C18:2 + 9.11% C18:3.

After running this profile through the regression model, a total of one variable met the toxic threshold, putting *F. persica* equal to WCO for toxicity ranking. This shows that FAME profiles outside the ones measured in our studies can also be successfully assessed by the model. That said, another FAME profile in the same study, *V. odorata*, containing chain length FAMEs between eight carbons to 22 carbons, was predicted to lead to a cellular viability of 41.22% after exposure which, although biologically possible, is far beyond the values measured in our studies and thus should be taken with caution.

Table 7.6: Predicted vs measured values for Canola and Tallow, where the predicted similarity was calculated as predicted/measured x 100.

Variable	Canola prediction	Canola actual	Prediction Similarity	Tallow Prediction	Tallow actual	Prediction Similarity
Viability (%)	104.83	104.83	100.00	96.15	96.15	100.00
IL-6 (µg/ml)	166.49	166.49	100.00	439.05	439.05	100.00
IL-8 (µg/ml)	388.21	388.30	100.02	2094.77	2094.67	100.00
IL-9 (µg/ml)	3.60	3.60	100.01	12.57	12.57	100.00
G-CSF (µg/ml)	15.64	15.64	99.99	66.89	66.89	100.00
GM-CSF (µg/ml)	8.85	8.85	100.00	23.07	23.07	100.00
INF-γ (µg/ml)	4.79	4.78	99.92	14.00	14.00	99.97
MCP-1 (µg/ml)	6.05	6.05	100.00	69.56	69.56	100.00
MIP-1β (µg/ml)	2.35	2.35	100.00	4.63	4.63	100.00
RANTES (µg/ml)	1.15	1.15	99.99	2.54	2.54	100.00
TNF-α (µg/ml)	9.60	9.60	100.02	10.25	10.25	100.01
VEGF (µg/ml)	254.59	254.62	100.01	234.95	234.97	100.01

Table 7.7: Predicted vs measured values for WCO, where the predicted similarity was compared between toxic threshold values. Highlighted variables indicate mismatches between predicted and actual results based on the toxic threshold in Table 7.4.

Variable	Predicted WCO Results	Predicted Results Meet Toxic Threshold	Actual WCO Results	Actual Results Meet Toxic Threshold
Viability (%)	100.72	No	101.82	No
IL-6 (µg/ml)	218.90	No	185.20	No
IL-8 (µg/ml)	1360.44	No	542.57	No
IL-9 (µg/ml)	8.77	No	4.22	No
G-CSF (µg/ml)	49.25	Yes	31.77	No
GM-CSF (µg/ml)	10.09	No	4.11	No
INF-γ (µg/ml)	-7.21	No	10.09	No
MCP-1 (µg/ml)	21.53	Yes	8.25	Yes
MIP-1β (µg/ml)	4.37	Yes	2.42	No
RANTES (µg/ml)	5.46	Yes	1.05	No
TNF-α (µg/ml)	2.93	No	7.23	Yes
VEGF (µg/ml)	443.30	No	284.76	No

Table 7.8: Results for Bran and *F. persica* biodiesel predictions and whether they meet toxic threshold (Table 7.4) for variables that were similar for WCO.

Variable	Prediction Bran	Meets toxic Threshold	Variable	Prediction *F. persica*	Meets toxic Threshold
Viability (%)	79.16	Yes	Viability (%)	106.68	No
IL-6 (µg/ml)	518.19	Yes	IL-6 (µg/ml)	218.01	No
IL-8 (µg/ml)	6421.47	Yes	IL-8 (µg/ml)	-401.34	No
IL-9 (µg/ml)	35.08	Yes	IL-9 (µg/ml)	1.19	No
GM-CSF (µg/ml)	15.78	Yes	GM-CSF (µg/ml)	-8.89	No
INF-γ (µg/ml)	-70.88	No	INF-γ (µg/ml)	13.59	No
MCP-1 (µg/ml)	105.36	Yes	MCP-1 (µg/ml)	39.24	Yes
VEGF (µg/ml)	1402.89	No	VEGF (µg/ml)	46.03	No

7.4 Discussion:

Using several different statistical models it was possible to first assess the suitability of exhaust gases and biodiesel FAME properties for their ability to predict the toxicological outcomes of exhaust exposure. Then a linear regression model was developed that can be used to predict the outcomes of new fuels in comparison to known data. The FAME profile of biodiesels explained the greatest variability observed when compared with the exhaust outputs, with the FAME profile explaining 50.95% compared to the exhaust profiles 40.82%. This is unsurprising as the exhaust used to obtain exposure outcomes was only partially characterised; it was intended for concentrations of polycyclic aromatic hydrocarbons (PAHs), heavy metals, volatile organic compounds, aldehydes and ketones to be characterised for each biodiesel exhaust however the exhaust concentrations used in the study were so dilute that it was not possible to collect enough particles for anything other than PAH analysis, which was found to be very limited and not worth adding to the model since the concentration of many of the tested PAHs was below detectable limits (Chapter 5). As such, the FAME analysis was found to be much more comprehensive and capable of explaining more than half the variability inherent within the biological data. It was also theorised that FAME profiles are more likely to be universal between studies, unlike exhaust components which are greatly impacted by a number of additional uncontrolled variables, which include but are not limited to engine type, exhaust after-treatment usage and the temperature at which the engine was started (Bass et al., 2015; Douki et al., 2018; Fontaras et al., 2009; Olfert et al., 2007).

Despite using FAME profile to generate the model, an important outcome for the exhaust RDA was identified. Submerged cultures were previously used for exposure experiments, which initially received criticism and forced the use of linear modelling to show that all measured exhaust components likely impacted exhaust toxicity (Chapter 2, (Landwehr et al., 2019)). In this study, it was again found that both the exhaust gases and exhaust particles were vital in contributing to the toxic results. The RDA allowed identification of the most likely characteristics contributing to exhaust toxicity and the model's ability to work around redundant and correlated variables makes it very useful for this analysis as many of the different exhaust components are inherently related. For example both carbon monoxide (CO) and particle number between 80-100 nm (PN100) were found to be highly correlated in the RDA, which is unsurprising as both are inherently created from incomplete fuel combustion and have previously been found to have high correlations (Jaeger, Ruschulte, Heine, & Piepenbrock, 2000; R. Li, Han, Wang, Shang, & Chen, 2019; Wu, Xu, Wang, & Cheng, 2016). Thus our findings that SO_2, NO_x and CO_2 contribute to toxicity, as do particle number and particle size, has negative implications for the majority of biodiesel exhaust toxicology literature which generally focus more on particle mass (Møller et al., 2020). Since many previous studies have solely used exhaust particles (Larcombe et al., 2015; Møller et al., 2020), they are limited in their applicability for real world conditions as they neglect the effects of the gaseous components, which are also contributing to the differences between diesel and biodiesel exhaust health impacts. In addition, it is the smaller sized particles that are generally more toxic than the larger, as shown with particle number contributing more to toxicity than particle mass in the RDA analysis; 90% of diesel exhaust particles are under 30 nm in size and yet only account for 10% of

the mass (D. Kittelson et al., 2002; Ris, 2007). As exhaust particles readily agglomerate, collecting exhaust particulate matter on filters to add directly to exposure models (as many biodiesel exhaust toxicity studies do (Larcombe et al., 2015; Møller et al., 2020)) removes many of the smaller ultrafine particles (<100 nm) and generates artificial particle spectra (Morin et al., 2008). This brings into question the relevance of this method when interpreting the effects of exposure.

The RDA analysis for the FAME profile was similar to what was initially predicted when the data were first obtained (Chapter 3), in that it is the ratio of saturated and unsaturated FAMEs that contribute the most towards toxicity. The first three RDA axes that contribute the most towards the biological variability are respectively related to C18:0, C18:1 and C18:2 instead of separating based on carbon chain length. This is further supported by the linear regression models, where the most important FAME variable was found to be a saturated FAME, C18:0, and then an unsaturated FAME of the same carbon length, C18:1 (Table 7.2.) In addition, many of the linear tree plots (Figure 7.3) separate the toxic results by the FAMEs C18:0 and C18:2. Iodine values are a measure of double bonds in biodiesel fuel, with higher iodine values denoting higher numbers of double bonds and thus a more unstable fuel as double bonds are more chemically reactive (Kyriakidis & Katsiloulis, 2000; McCormick et al., 2001). Cetane number is a measure of how completely diesel fuel is combusted, with higher cetane values indicating more complete combustion and higher fuel performance (Bamgboye & Hansen, 2008). Iodine number is greatly affected by the percentage of unsaturated FAME (Kyriakidis & Katsiloulis, 2000), particularly ones such as methyl lineolate (C18:2) with more than one double bond per molecule, whereas cetane number is greatly impacted by the percentage of saturated fatty acids (Bamgboye & Hansen, 2008; Giakoumis & Sarakatsanis, 2018), with a greater percentage indicating higher cetane values. As these measurements are indications of fuel properties and thus how well a particular fuel type performs, biodiesel fuel legislation in many countries have a minimum limit on cetane number (ASTM, 2020a, 2020b; Pöttering & Necas, 2009; SHELL, 2018) although there are few standards with maximum iodine values (Atabani et al., 2013). As the biodiesels with the extremes of both (Soy biodiesel= highest predicted iodine number, Palm and Tallow biodiesel= highest cetane numbers (Landwehr et al., 2021)) were found to be the most toxic, it would be beneficial from an health effects perspective if more iodine limits should be added to fuel standards and a maximum cetane number introduced.

As for the linear models themselves, the majority of the models were significant, indicating good fits for the regression. We generated the fits for five of the six fuels, leaving the WCO results to assess how well the FAME variables predicted the various toxicological outcomes. Then to minimise effects caused by biological variability between different volunteers, coefficients on the averages obtained from all volunteers were generated. When two of the fuels used to create the model were ran back through the generated equation, all predicted outcomes were within 0.1% of the actual measurements. Running the results of WCO back through the model accurately predicted toxic outcomes for 8 of the 12 biological variables, although the exact measurements were not as well fitted as hoped. It was also possible to successfully predict outcomes for other biodiesels not tested within the initial data set, including those generated by a separate study. These outcomes were within biological limits, lending support to the model.

That said, this model has some limitations. Results from 5 different biodiesel were used to generate it, which limits the robustness due to the small selection of fuels. However, this is a limitation universal to all mathematical model types, in that more data will always strengthen the generated model further. Although the model was highly accurate when data used to generate it were retested through the equation, it only accurately predicted toxic results in 8 of the 12 biological variables for WCO, the fuel not used to build the model. Furthermore, there are no published studies that assess toxicity of biodiesel exhaust using similar toxic outcomes as in this study and which also provided the FAME profile of the tested biodiesel. This limits the extent to which the generated models can be tested. In addition, the two successful tests were conducted on biodiesels with FAME profiles closely matching the ones used in this study. The test on a third biodiesel type, which had a FAME profile containing FAME molecules with carbon chains both longer and shorter than those used to generate the model had a predicted viability of 41%. This is not unrealistic, but it is well outside the range tested within the current study. It thus shows that the model has limitations in its ability to predict biological outcomes, with only biodiesels with FAME profiles close to the six used in this study being the most suitable for assessment. Thus biodiesels with too dissimilar FAME profiles should be applied to our model with caution. As the model was built using data generated by testing the exhaust of the more commonly used biodiesels, this can be remedied by doing testing on biodiesels with more extreme FAME profiles which would also strengthen the model robustness by increasing the number of fuels it is built upon. Ultimately more work is needed to build the model further and make it more robust and accurate for new biodiesel fuels with wider ranging FAME profiles.

7.5 Conclusion:

In conclusion, this study demonstrates that whole exhaust is a necessity when testing the extent of diesel and biodiesel exhaust exposure health impacts and that the level of saturated and unsaturated FAMEs within a biodiesel greatly impacts the resulting toxicological outcomes. A model was generated that can be used to estimate the biological outcomes of exhaust exposure based on the toxicology results from 6 tested biodiesels and this model was successful when rerunning two of those same biodiesels back through it. However, this model needs more testing using new biodiesels with more extreme FAME profiles. That said, this is the first study that has even attempted to make a screening tool for biodiesel exhaust exposure health impacts and it can be successfully used on biodiesels with similar FAME profiles to the ones used to generate it, giving an *in silico* option with which to test the health effects of exhaust exposure for new biodiesel fuels without the need for expensive toxicological testing.

Chapter 8: Final Discussion.

8.1 Discussion and Future Directions:

There are three overarching findings for my thesis that have been addressed across several chapters. The first is that lower doses of exhaust are equally as toxic, or potentially even more so, than higher exhaust concentrations. This is shown in Chapter 2, where one hour of exhaust exposure was more toxic than both two and four hours of exhaust exposures, and Chapter 6, where mice exposed to exhaust once, for two hours showed similar responses in some end point measurements as mice exposed for two hours per day for eight consecutive days. The 3D lung model exposure studies described in Chapter 5 also have concerning health implications for diesel and biodiesel exhaust exposures considering the exhaust concentration used was within safe work Australia standards. Previous studies have found that exposure to lower exhaust doses, both *in vivo* and *in vitro*, results in more severe health effects than higher doses (de Brito et al., 2018; Seriani et al., 2015) and the process of agglomeration is believed to be the cause. Ultrafine particles cause more severe health effects than other sizes (Breitner et al., 2011; Oberdörster et al., 1995) and they also readily agglomerate in the presence of other particles (Morin et al., 2008), thus, it would be logical to assume that a threshold exposure concentration would be reached where the more damaging smaller particles are agglomerated to less damaging larger size particles before contributing towards severe health impacts.

The second finding made in this thesis was that the biodiesel feedstock source directly affects toxicity, likely via the impact of different fatty acid methyl ester (FAME) profiles on fuel properties including cetane number and iodine number (Chapter 3) (Bamgboye & Hansen, 2008; Giakoumis & Sarakatsanis, 2018; Graboski et al., 2003; G. Knothe & Steidley, 2005; Kyriakidis & Katsiloulis, 2000; McCormick et al., 2001). As observed in Chapters 3, 4, 5 and 6, exhaust generated by the combustion of Tallow biodiesel was found to be the most toxic. This was the case for both pure Tallow biodiesel and blended Tallow biodiesel. Exposure to exhaust generated by the combustion of Canola biodiesel was consistently found to be the least toxic, with the other three tested feedstocks being similar to mineral diesel in terms of toxicity. Previous studies assessing biodiesel exhaust toxicity have been inconsistent, with some finding biodiesel exhaust to be more toxic than mineral diesel exhaust, and others less, as described in detail in Chapter 1.1. This is likely because the majority of previous studies focus on Soy and Canola biodiesel which were found to be at separate ends of the toxicity scale in this study (Chapters 3 and 4). This has been confounded further by the fact that there has been little attention paid to other types despite their common usage (Chapter 1.1). Literature reviews attempting to combine study findings to assess whether biodiesel exhaust is overall more or less toxic than mineral diesel exhaust have thus far been mostly unsuccessful and have highlighted the inability to group and compare fuels under one overarching biodiesel label (Larcombe et al., 2015; Madden, 2016; Møller et al., 2020; K. J. Swanson et al., 2007). Likely due to this issue, many of the reviews ultimately state that more research is needed. This conclusion is not incorrect, more research is always needed, but future reviews of biodiesel exhaust toxicity literature should always take feedstock type into account.

The number of double bonds is likely to be a part of the FAME profile that contributes directly to toxicity, as shown by the results of the multiple biodiesel exhaust exposures in Chapter 3 and the statistical model generated in Chapter 7. Double bonds, particularly FAME molecules that are saturated (i.e. no double bonds) and FAME molecules that are highly unsaturated (i.e. with two or more double bonds) (Bamgboye & Hansen, 2008; Giakoumis & Sarakatsanis, 2018; Kyriakidis & Katsiloulis, 2000), greatly impact iodine and cetane number; measures of fuel reactivity and how completely a particular fuel combusts (Bamgboye & Hansen, 2008; Giakoumis & Sarakatsanis, 2018; G. Knothe & Steidley, 2005; Kyriakidis & Katsiloulis, 2000; McCormick et al., 2001). As particular engines have been designed and fine-tuned for over a century to run on mineral diesel with as few emissions as possible, any deviation away from diesel fuel, such as high cetane and iodine numbers, would likely negatively impact the internal combustion process. Higher iodine numbers indicate a highly chemically unstable fuel (McCormick et al., 2001; Miller & Bowman, 1989) and higher cetane numbers increase combustion temperature so that more unburnt, or worse partially burnt (Fontaras et al., 2009; McCormick et al., 2001), fuel would make it through to the exhaust. This would increase the severity of health effects due to exposure. As fuel feedstock greatly determines exhaust toxicity, it means than biodiesel cannot be treated as one "type" when assessing health effects and that fuel feedstock type, and preferably even a FAME profile, should always be disclosed. This is not the case for all published literature where the exact feedstock type used is not always stated, and the FAME profile rarely mentioned (Ackland et al., 2007; Hawley et al., 2014).

Finally, the third overarching finding was that exhaust gases play a crucial role in exhaust toxicity and should always be included when assessing health impacts. This was shown in Chapters 2, 3 and 4 where exhaust gases were significantly correlated with several toxic outcomes. The redundancy analysis model in Chapter 7 also showed that the exhaust gas components are crucial for assessing exhaust toxicity and plays an equal role to the particle components in exhaust health effect contribution. Despite this, the majority of literature studying the effects of diesel or biodiesel exhaust exposure in *in vivo* and *in vitro* models only assesses the toxic effects of the particulate matter (Chapter 1, Chapter 1.1) (Larcombe et al., 2015; Møller et al., 2020), often in ways that also remove the ultrafine particles which are arguably the most toxic components of the exhaust particles (Breitner et al., 2011; Morin et al., 2008; Oberdörster et al., 1995). This limits the feasibility of their results for real world exposures and future experiments assessing diesel and biodiesel toxicity should attempt to use whole exhaust in their exposures.

Future directions of work in this field should extend toxicity studies outside of healthy models used in this thesis. All research conducted as part of this thesis used healthy exposure models - cells derived from healthy non-atopic volunteers and healthy mice. Results generated, namely the permeability results in Chapter 5, response to methacholine in Chapter 6 and all immunological data in Chapters 2-6 suggest that the most at risk from diesel and biodiesel exhaust exposure will be those suffering other pulmonary/accompanying conditions, including asthma, allergy and respiratory infections. Indeed, several previous studies have found diesel exhaust exposure to exacerbate asthmatic symptoms (Strand, Rak, Svartengren, & Bylin, 1997; J. J. Zhang et al., 2009). Other studies co-exposing diesel exhaust particles and respiratory infections, such as

influenza, non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae*, have found that diesel exhaust increases severity of disease (Gowdy et al., 2010; Larcombe et al., 2012; Shears et al., 2020; Maria C. Zarcone et al., 2017) and studies co-exposing subjects with allergy and allergic rhinitis to diesel exhaust show that diesel exhaust exacerbated allergic symptoms (Pawlak et al., 2016; Xin Zhang et al., 2016). A previous study has also successfully generated an animal model of airway hyperresponsiveness using only diesel exhaust and soy protein (Alvarez-Simón et al., 2017). Although both diesel and biodiesel exhaust exposures resulted in toxic effects in the healthy models used in this thesis, the findings that exposure to more than half of the tested biodiesels resulted in more widespread and severe health impacts than mineral diesel exhaust, suggest it is highly likely that exposure to biodiesel may also worsen the aforementioned conditions. It is likely that exposure to biodiesel exhaust will also exacerbate asthma (Chapter 6), increase the time needed for recovery from infection (by possibly increasing disease severity and causing immune dysregulation) and exacerbating and even inducing allergies (Chapter 5).

A minor limitation of the series of studies presented in this thesis is that the biodiesel types chosen for research were those currently in use (ARENA, 2018; EIA, 2020b; Flach et al., 2019; Toldrá-Reig et al., 2020), not what may be used in future. Most feedstocks used in these studies will compete with the food industry which may drive up prices and threaten food security. Non-edible oils such as *Jatropha spp*. or even sewerage waste (de Brito et al., 2018; Silitonga et al., 2017) are likely to be the future of biodiesel fuel, though they do come with their own complications- namely how to mass produce the oil and remove free floating fatty acids (Elgharbawy et al., 2021). In addition, the food oils chosen for biodiesel testing in this study are not the most environmentally friendly. Land clearance will be a concern for many of the tested types and this will decrease the sustainability of biodiesel usage (Beer et al., 2007). Of the all the feedstocks tested, two oils are considered waste products of already existing industries and thus are more environmentally friendly; Tallow and Waste Cooking Oil. As Tallow was found to be the most toxic fuel, it is not a desirable option, however the Waste Cooking Oil used in these studies was comprised mostly of Canola oil and also had similar exhaust toxicity profiles. This suggests that Waste Oil could be a viable source for biodiesel feedstock, so long as the type of oil used in the cooking process is controlled.

More work also needs to be performed on the screening tool generated in Chapter 7. Although this tool is as strong as it can be with the toxicological data generated from the small fuel numbers tested, further toxicological data in biodiesel exhausts with more extreme FAME profiles are needed to strengthen it. As is, the generated screening model is best implemented for new biodiesels with similar FAME profiles as the ones used to create it. The idea behind it is that the majority of biodiesel literature, and even diesel literature, only focuses on the exhaust profile and impact on the engine (Chapter 1.1) (Landwehr, Larcombe, Reid, & Mullins, 2020; Larcombe et al., 2015) instead of the health effects of the biodiesel and diesel exhausts. This is presumably because the biological testing needed is time consuming, expensive and spans multiple fields of research requiring experts from a wide range of disciplines that are rarely found together. With the generation of the screening model the health impacts of new biodiesel fuels can now be tested *in silico* and graded against the ones already assessed so that the healthiest types can be

chosen for future investment and legislation, although biodiesels with wider ranges of FAME molecules in their profiles should be tested with caution.

The studies within this thesis do have some limitations, however they are the first to test multiple health impacts of whole exhaust exposure from several different biodiesel fuels using multiple different advanced exposure models so as to comparatively assess the impact of feedstock on exhaust toxicity. In addition, this is the first study to generate a screening model so that new fuel feedstocks can be assessed without the need for time consuming and often expensive biological testing.

References:

Abe, S., Takizawa, H., Sugawara, I., & Kudoh, S. (2000). Diesel Exhaust (DE)–Induced Cytokine Expression in Human Bronchial Epithelial Cells. *American Journal of Respiratory Cell and Molecular Biology, 22*(3), 296-303. doi:10.1165/ajrcmb.22.3.3711

ACCC, Australian Competition & Consumer Commission (2021). Biofuels. Retrieved from https://www.accc.gov.au/consumers/petrol-diesel-lpg/biofuels

Ackland, M. L., Zou, L., Freestone, D., Van De Waasenburg, S., & Michalczyk, A. A. (2007). Diesel exhaust particulate matter induces multinucleate cells and zinc transporter-dependent apoptosis in human airway cells. *Immunology and cell biology, 85*(8), 617-622.

Adenuga, A. A., Wright, M. E., & Atkinson, D. B. (2016). Evaluation of the reactivity of exhaust from various biodiesel blends as a measure of possible oxidative effects: A concern for human exposure. *Chemosphere, 147*(Supplement C), 396-403. doi:doi:10.1016/j.chemosphere.2015.12.074

Agarwal, A. K., Singh, A. P., Gupta, T., Agarwal, R. A., Sharma, N., Rajput, P., . . . Ateeq, B. (2018). Mutagenicity and Cytotoxicity of Particulate Matter Emitted from Biodiesel-Fueled Engines. *Environmental Science & Technology, 52*(24), 14496-14507. doi:10.1021/acs.est.8b03345

AIOH, Australian Institute of Occupational Hygienists (2017). *DIESEL PARTICULATE MATTER AND OCCUPATIONAL HEALTH ISSUES- Position Paper*. Retrieved from

Allen, J., Trenga, C. A., Peretz, A., Sullivan, J. H., Carlsten, C. C., & Kaufman, J. D. (2009). Effect of diesel exhaust inhalation on antioxidant and oxidative stress responses in adults with metabolic syndrome. *Inhal Toxicol, 21*(13), 1061-1067. doi:10.3109/08958370902721424

Allen, J. G., MacNaughton, P., Cedeno-Laurent, J. G., Cao, X., Flanigan, S., Vallarino, J., . . . Spengler, J. D. (2018). Airplane pilot flight performance on 21 maneuvers in a flight simulator under varying carbon dioxide concentrations. *Journal of Exposure Science & Environmental Epidemiology*. doi:10.1038/s41370-018-0055-8

Alvarez-Simón, D., Muñoz, X., Gómez-Ollés, S., de Homdedeu, M., Untoria, M.-D., & Cruz, M.-J. (2017). Effects of diesel exhaust particle exposure on a murine model of asthma due to soybean. *PLoS ONE, 12*(6), e0179569-e0179569. doi:10.1371/journal.pone.0179569

Amanatidis, S., Ntziachristos, L., Giechaskiel, B., Bergmann, A., & Samaras, Z. (2014). Impact of Selective Catalytic Reduction on Exhaust Particle Formation over Excess Ammonia Events. *Environmental Science & Technology, 48*(19), 11527-11534. doi:10.1021/es502895v

Amjad, S., Neelakrishnan, S., & Rudramoorthy, R. (2010). Review of design considerations and technological challenges for successful development and deployment of plug-in hybrid electric vehicles. *Renewable and Sustainable Energy Reviews, 14*(3), 1104-1110. doi:doi:10.1016/j.rser.2009.11.001

Andersen, Z. J., Pedersen, M., Weinmayr, G., Stafoggia, M., Galassi, C., Jørgensen, J. T., . . . Raaschou-Nielsen, O. (2018). Long-term exposure to ambient air pollution and incidence of brain tumor: the European Study of Cohorts for Air Pollution Effects (ESCAPE). *Neuro-oncology, 20*(3), 420-432. doi:10.1093/neuonc/nox163

André, E., Stoeger, T., Takenaka, S., Bahnweg, M., Ritter, B., Karg, E., . . . Wjst, M. (2006). Inhalation of ultrafine carbon particles triggers biphasic pro-inflammatory response in the mouse lung. *European Respiratory Journal, 28*(2), 275-285. doi:10.1183/09031936.06.00071205

André, V., Barraud, C., Capron, D., Preterre, D., Keravec, V., Vendeville, C., . . . Sichel, F. (2015). Comparative mutagenicity and genotoxicity of particles and aerosols emitted by the combustion of standard vs. rapeseed methyl ester supplemented bio-diesel fuels: Impact of after treatment devices: Oxidation catalyst and particulate filter. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 777*, 33-42. doi:https://doi.org/10.1016/j.mrgentox.2014.11.007

ARENA (Australian Renewable Energy Agency), Clean Energy Finance Corporation (2018). *Biofuels and Transport: An Australian opportunity*. Retrieved from

ASTM International (2020a). D975-20c, Standard Specification for Diesel Fuel.

ASTM International (2020b). D7467-20a, Standard Specification for Diesel Fuel Oil, Biodiesel Blend (B6 to B20).

Atabani, A. E., Silitonga, A. S., Ong, H. C., Mahlia, T. M. I., Masjuki, H. H., Badruddin, I. A., & Fayaz, H. (2013). Non-edible vegetable oils: A critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production. *Renewable and Sustainable Energy Reviews, 18*, 211-245. doi:https://doi.org/10.1016/j.rser.2012.10.013

Attfield, M. D., Schleiff, P. L., Lubin, J. H., Blair, A., Stewart, P. A., Vermeulen, R., . . . Silverman, D. T. (2012). The Diesel Exhaust in Miners study: a cohort mortality study with emphasis on lung cancer. *Journal of the National Cancer Institute, 104*(11), 869-883. doi:10.1093/jnci/djs035

Bai, N., Kido, T., Suzuki, H., Yang, G., Kavanagh, T. J., Kaufman, J. D., . . . Eeden, S. F. v. (2011). Changes in atherosclerotic plaques induced by inhalation of diesel exhaust. *Atherosclerosis, 216*(2), 299-306. doi:10.1016/j.atherosclerosis.2011.02.019

Balti, E. V., Echouffo-Tcheugui, J. B., Yako, Y. Y., & Kengne, A. P. (2014). Air pollution and risk of type 2 diabetes mellitus: a systematic review and meta-analysis. *Diabetes Res Clin Pract, 106*(2), 161-172. doi:10.1016/j.diabres.2014.08.010

Bamgboye, A., & Hansen, A. C. (2008). Prediction of cetane number of biodiesel fuel from the fatty acid methyl ester (FAME) composition. *International Agrophysics, 22*(1), 21.

Barath, S., Mills, N. L., Lundbäck, M., Törnqvist, H., Lucking, A. J., Langrish, J. P., . . . Blomberg, A. (2010). Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions. *Part Fibre Toxicol, 7*, 19-19. doi:10.1186/1743-8977-7-19

Barnes, P. J. (2008). The cytokine network in asthma and chronic obstructive pulmonary disease. *The Journal of clinical investigation, 118*(11), 3546-3556. doi:10.1172/JCI36130

Barraud, C., Corbière, C., Pottier, I., Estace, E., Blanchard, K., Logie, C., . . . Sichel, F. (2017). Impact of after-treatment devices and biofuels on diesel exhausts genotoxicity in A549 cells exposed at air-liquid interface. *Toxicology in Vitro, 45*, 426-433. doi:https://doi.org/10.1016/j.tiv.2017.04.025

Barros, S. (2020). *Biofuels Annual- Brazil*. Retrieved from

Bass, V. L., Schladweiler, M. C., Nyska, A., Thomas, R. F., Miller, D. B., Krantz, T., . . . Kodavanti, U. P. (2015). Comparative cardiopulmonary toxicity of exhausts from soy-based biofuels and diesel in healthy and hypertensive rats. *Inhalation Toxicology, 27*(11), 545-556. doi:doi:10.3109/08958378.2015.1060279

Bassig, B. A., Dai, Y., Vermeulen, R., Ren, D., Hu, W., Duan, H., . . . Lan, Q. (2017). Occupational exposure to diesel engine exhaust and alterations in immune/inflammatory markers: a cross-sectional molecular epidemiology study in China. *Carcinogenesis, 38*(11), 1104-1111. doi:10.1093/carcin/bgx081

Beer, T., Grant, T. F., & Campbell, P. K. (2007). *The greenhouse and air quality emissions of biodiesel blends in Australia : report for Caltex Australia Limited* (KS54C/1/F2.29). Retrieved from http://www.cmar.csiro.au/e-print/open/2007/beert_b.pdf

Behndig, A. F., Larsson, N., Brown, J. L., Stenfors, N., Helleday, R., Duggan, S. T., . . . Blomberg, A. (2011). Proinflammatory doses of diesel exhaust in healthy subjects fail to elicit equivalent or augmented airway inflammation in subjects with asthma. *Thorax, 66*(1), 12-19. doi:10.1136/thx.2010.140053

Behndig, A. F., Shanmuganathan, K., Whitmarsh, L., Stenfors, N., Brown, J. L., Frew, A. J., . . . Wilson, S. J. (2015). Effects of controlled diesel exhaust exposure on apoptosis and proliferation markers in bronchial epithelium - an in vivo bronchoscopy study on asthmatics, rhinitics and healthy subjects. *BMC pulmonary medicine, 15*, 99-99. doi:10.1186/s12890-015-0096-x

Bemis, J. C., Torous, D. K., & Dertinger, S. D. (2015). Part 2. Assessment of micronucleus formation in rats after chronic exposure to new-technology diesel exhaust in the ACES bioassay. *Res Rep Health Eff Inst*(184), 69-82; discussion 141-171.

Benbrahim-Tallaa, L., Baan, R. A., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., . . . Straif, K. (2012). Carcinogenicity of diesel-engine and gasoline-engine exhausts and some nitroarenes. *The Lancet Oncology, 13*(7), 663-664. doi:doi:10.1016/S1470-2045(12)70280-2

Bendtsen, K. M., Gren, L., Malmborg, V. B., Shukla, P. C., Tunér, M., Essig, Y. J., . . . Vogel, U. B. (2020). Particle characterization and toxicity in C57BL/6 mice following instillation of five different diesel exhaust particles designed to differ in physicochemical properties. *Particle and Fibre Toxicology, 17*(1), 38. doi:10.1186/s12989-020-00369-9

Bernhard, W. (2016). Lung surfactant: Function and composition in the context of development and respiratory physiology. *Ann Anat, 208*, 146-150. doi:10.1016/j.aanat.2016.08.003

Betha, R., Pavagadhi, S., Sethu, S., Hande, M. P., & Balasubramanian, R. (2012). Comparative in vitro cytotoxicity assessment of airborne particulate matter emitted from stationary engine fuelled with diesel and waste cooking oil-derived biodiesel. *Atmospheric Environment, 61*, 23-29. doi:https://doi.org/10.1016/j.atmosenv.2012.06.086

Biodiesel International. (2019). Norwegian city rolls out biogas, biodiesel buses to reduce carbon footprint. Retrieved from https://biofuels-news.com/news/norwegian-city-rolls-out-biogas-biodiesel-buses-to-reduce-carbon-footprint/

Boland, S., Baeza-Squiban, A., Fournier, T., Houcine, O., Gendron, M.-C., Chévrier, M., . . . Marano, F. (1999). Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *American Journal of Physiology - Lung Cellular and Molecular Physiology, 276*(4), L604-L613.

Bonassi, S., El-Zein, R., Bolognesi, C., & Fenech, M. (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis, 26*(1), 93-100. doi:10.1093/mutage/geq075

Boussat, S., Eddahibi, S., Coste, A., Fataccioli, V., Gouge, M., Housset, B., . . . Maitre, B. (2000). Expression and regulation of vascular endothelial growth factor in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 279*(2), L371-378. doi:10.1152/ajplung.2000.279.2.L371

Boylen, C. E., Sly, P. D., Zosky, G. R., & Larcombe, A. N. (2011). Physiological and inflammatory responses in an anthropomorphically relevant model of acute diesel exhaust particle exposure are sex and dose-dependent. *Inhalation Toxicology, 23*(14), 906-917. doi:doi:10.3109/08958378.2011.625454

BR&Di, Biomass Research & Development Initiative. (2011). Increasing feedstock production for biofuels: economic drivers, environmental implications, & the role of research. *Washington, United States: Biomass Research & Development Initiative*.

Brandt, E. B., Biagini Myers, J. M., Acciani, T. H., Ryan, P. H., Sivaprasad, U., Ruff, B., . . . Khurana Hershey, G. K. (2015). Exposure to allergen and diesel exhaust particles potentiates secondary allergen-specific memory responses, promoting asthma susceptibility. *J Allergy Clin Immunol, 136*(2), 295-303.e297. doi:10.1016/j.jaci.2014.11.043

Brandt, E. B., Kovacic, M. B., Lee, G. B., Gibson, A. M., Acciani, T. H., Le Cras, T. D., . . . Khurana Hershey, G. K. (2013). Diesel exhaust particle induction of IL-17A contributes to severe asthma. *Journal of Allergy and Clinical Immunology, 132*(5), 1194-1204.e1192. doi:10.1016/j.jaci.2013.06.048

Breitner, S., Liu, L., Cyrys, J., Brüske, I., Franck, U., Schlink, U., . . . Peters, A. (2011). Sub-micrometer particulate air pollution and cardiovascular mortality in Beijing, China. *Science of The Total Environment, 409*(24), 5196-5204. doi:https://doi.org/10.1016/j.scitotenv.2011.08.023

Brito, J. M., Belotti, L., Toledo, A. C., Antonangelo, L., Silva, F. S., Alvim, D. S., . . . Rivero, D. H. R. F. (2010). Acute Cardiovascular and Inflammatory Toxicity Induced by Inhalation of Diesel and Biodiesel Exhaust Particles. *Toxicological Sciences, 116*(1), 67-78. doi:doi:10.1093/toxsci/kfq107

de Brito, J. M., Mauad, T., Cavalheiro, G. F., Yoshizaki, K., de André, P. A., Lichtenfels, A. J. F. C., . . . Saldiva, P. H. N. (2018). Acute exposure to diesel and sewage biodiesel exhaust causes pulmonary and systemic inflammation in mice. *Science of The Total Environment, 628-629*, 1223-1233. doi:https://doi.org/10.1016/j.scitotenv.2018.02.019

Brook, R. D., Rajagopalan, S., Pope, C. A., Brook, J. R., Bhatnagar, A., Diez-Roux, A. V., . . . Kaufman, J. D. (2010). Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement From the American Heart Association. *American Heart Association, 121*(21), 2331-2378. doi:doi:10.1161/CIR.0b013e3181dbece1

Bünger, J., Krahl, J., Baum, K., Schröder, O., Müller, M., Westphal, G., . . . Hallier, E. (2000). Cytotoxic and mutagenic effects, particle size and concentration analysis of diesel engine emissions using biodiesel and petrol diesel as fuel. *Archives of Toxicology, 74*(8), 490-498. doi:doi:10.1007/s002040000155

Bünger, J., Müller, M. M., Krahl, J., Baum, K., Weigel, A., Hallier, E., & Schulz, T. G. (2000). Mutagenicity of diesel exhaust particles from two fossil and two plant oil fuels. *Mutagenesis, 15*(5), 391-397.

Camuzeaux, J. R., Alvarez, R. A., Brooks, S. A., Browne, J. B., & Sterner, T. (2015). Influence of Methane Emissions and Vehicle Efficiency on the Climate Implications of Heavy-Duty Natural Gas Trucks. *Environmental Science & Technology, 49*(11), 6402-6410. doi:10.1021/acs.est.5b00412

Cardone, M., Prati, M. V., Rocco, V., Seggiani, M., Senatore, A., & Vitolo, S. (2002). *Brassica carinata* as an Alternative Oil Crop for the Production of Biodiesel in Italy: Engine Performance and Regulated and Unregulated Exhaust Emissions. *Environmental Science & Technology, 36*(21), 4656-4662. doi:doi:10.1021/es011078y

Carlsten, C., Kaufman, J. D., Trenga, C. A., Allen, J., Peretz, A., & Sullivan, J. H. (2008). Thrombotic markers in metabolic syndrome subjects exposed to diesel exhaust. *Inhal Toxicol, 20*(10), 917-921. doi:10.1080/08958370802074908

Carlsten, C., MacNutt, M. J., Zhang, Z., Sava, F., & Pui, M. M. (2014). Anti-Oxidant N-Acetylcysteine Diminishes Diesel Exhaust-Induced Increased Airway Responsiveness in Person with Airway Hyper-Reactivity. *Toxicological Sciences, 139*(2), 479-487. doi:10.1093/toxsci/kfu040

Carrara, M., & Niessner, R. (2011). Impact of a NO2-regenerated diesel particulate filter on PAH and NPAH emissions from an EURO IV heavy duty engine. *Journal of Environmental Monitoring, 13*(12), 3373-3379. doi:10.1039/C1EM10573F

Cauda, E. G., Ku, B. K., Miller, A. L., & Barone, T. L. (2012). Toward Developing a New Occupational Exposure Metric Approach for Characterization of Diesel Aerosols. *Aerosol science and technology : the journal of the American Association for Aerosol Research, 46*(12), 1370-1381. doi:10.1080/02786826.2012.715781

Celebi Sözener, Z., Cevhertas, L., Nadeau, K., Akdis, M., & Akdis, C. A. (2020). Environmental factors in epithelial barrier dysfunction. *J Allergy Clin Immunol, 145*(6), 1517-1528. doi:10.1016/j.jaci.2020.04.024

Cervena, T., Rossnerova, A., Sikorova, J., Beranek, V., Vojtisek-Lom, M., Ciganek, M., . . . Rossner, P. (2017). DNA Damage Potential of Engine Emissions Measured In Vitro by Micronucleus Test in Human Bronchial Epithelial Cells. *Basic & Clinical Pharmacology & Toxicology, 121*, 102-108. doi:doi:10.1111/bcpt.12693

Chakraborty, M., & Kotecha, S. (2013). Pulmonary surfactant in newborn infants and children. *Breathe, 9*(6), 476-488. doi:10.1183/20734735.006513

Chameides, W. L., Fehsenfeld, F., Rodgers, M. O., Cardelino, C., Martinez, J., Parrish, D., . . . Wang, T. (1992). Ozone Precursor Relationships in the Ambient Atmosphere. *Journal of Geophysical Research: Atmospheres, 97*(D5), 6037-6055. doi:doi:10.1029/91JD03014

ChemSpider. Naphthalene. Retrieved from http://www.chemspider.com/Chemical-Structure.906.html

Chen, K., Wolf, K., Breitner, S., Gasparrini, A., Stafoggia, M., Samoli, E., . . . Schneider, A. (2018). Two-way effect modifications of air pollution and air temperature on total natural and cardiovascular mortality in eight European urban areas. *Environment International, 116*, 186-196. doi:https://doi.org/10.1016/j.envint.2018.04.021

Chen, T.-M., Kuschner, W. G., Gokhale, J., & Shofer, S. (2007). Outdoor Air Pollution: Nitrogen Dioxide, Sulfur Dioxide, and Carbon Monoxide Health Effects. *The American Journal of the Medical Sciences, 333*(4), 249-256. doi:doi:10.1097/MAJ.0b013e31803b900f

Chen, Z., Salam, M. T., Eckel, S. P., Breton, C. V., & Gilliland, F. D. (2015). Chronic effects of air pollution on respiratory health in Southern California children: findings from the Southern California Children's Health Study. *Journal of Thoracic Disease, 7*(1), 46-58. doi:10.3978/j.issn.2072-1439.2014.12.20

Cheng, Y., Li, S. M., Liggio, J., Hayden, K., Han, Y., Stroud, C., . . . Poitras, M. J. (2017). The effects of biodiesels on semivolatile and nonvolatile particulate matter emissions from a light-duty diesel engine. *Environ Pollut, 230*, 72-80. doi:10.1016/j.envpol.2017.06.014

Chien, Y.-C., Lu, M., Chai, M., & Boreo, F. J. (2009). Characterization of Biodiesel and Biodiesel Particulate Matter by TG, TG−MS, and FTIR. *Energy & Fuels, 23*(1), 202-206. doi:doi:10.1021/ef800388m

Cho, C.-C., Hsieh, W.-Y., Tsai, C.-H., Chen, C.-Y., Chang, H.-F., & Lin, C.-S. (2018). In Vitro and In Vivo Experimental Studies of PM2.5 on Disease Progression. *International Journal of Environmental Research and Public Health, 15*(7), 1380.

Choi, H., Harrison, R., Komulainen, H., & Saborit, J. M. D. (2010). *Polycyclic aromantic hydrocarbons. In: WHO guidelines for indoor air quality: selected pollutants*.

Cliff, R., Curran, J., Hirota, J. A., Brauer, M., Feldman, H., & Carlsten, C. (2016). Effect of diesel exhaust inhalation on blood markers of inflammation and neurotoxicity: a controlled, blinded crossover study. *Inhal Toxicol, 28*(3), 145-153. doi:10.3109/08958378.2016.1145770

Clifford, R. L., Jones, M. J., MacIsaac, J. L., McEwen, L. M., Goodman, S. J., Mostafavi, S., . . . Carlsten, C. (2017). Inhalation of diesel exhaust and allergen alters human bronchial epithelium DNA methylation. *Journal of Allergy and Clinical Immunology, 139*(1), 112-121. doi:https://doi.org/10.1016/j.jaci.2016.03.046

Coburn, J. L., Cole, T. B., Dao, K. T., & Costa, L. G. (2018). Acute exposure to diesel exhaust impairs adult neurogenesis in mice: prominence in males and protective effect of pioglitazone. *Arch Toxicol, 92*(5), 1815-1829. doi:10.1007/s00204-018-2180-5

Collins, R. A., Gualano, R. C., Zosky, G. R., Atkins, C. L., Turner, D. J., Colasurdo, G. N., & Sly, P. D. (2005). Hyperresponsiveness to inhaled but not intravenous methacholine during acute respiratory syncytial virus infection in mice. *Respiratory Research, 6*(1), 142-142. doi:10.1186/1465-9921-6-142

Collyer, M. L., & Adams, D. C. (2021). RRPP: An r package for fitting linear models to high-dimensional data using residual randomization (version 1.0). *Methods in Ecology and Evolution, 9*(7), 1772-1779.

Conklin, D. J., & Kong, M. (2015). Part 4. Assessment of plasma markers and cardiovascular responses in rats after chronic exposure to new-technology diesel exhaust in the ACES bioassay. *Res Rep Health Eff Inst*(184), 111-139; discussion 141-171.

Cosselman, K. E., Krishnan, R. M., Oron, A. P., Jansen, K., Peretz, A., Sullivan, J. H., . . . Kaufman, J. D. (2012). Blood pressure response to controlled diesel exhaust exposure in human subjects. *Hypertension (Dallas, Tex. : 1979), 59*(5), 943-948. doi:10.1161/HYPERTENSIONAHA.111.186593

Cox, G., Gauldie, J., & Jordana, M. (1992). Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol, 7*(5), 507-513. doi:10.1165/ajrcmb/7.5.507

Crowley, G., Kwon, S., Caraher, E. J., Haider, S. H., Lam, R., Batra, P., . . . Nolan, A. (2019). Quantitative lung morphology: semi-automated measurement of mean linear intercept. *BMC Pulm Med, 19*(1), 206. doi:10.1186/s12890-019-0915-6

Curran, J., Cliff, R., Sinnen, N., Koehle, M., & Carlsten, C. (2018). Acute diesel exhaust exposure and postural stability: a controlled crossover experiment. *J Occup Med Toxicol, 13*, 2-2. doi:10.1186/s12995-017-0182-5

Dai, Y., Ren, D., Bassig, B. A., Vermeulen, R., Hu, W., Niu, Y., . . . Zheng, Y. (2018). Occupational exposure to diesel engine exhaust and serum cytokine levels. *Environ Mol Mutagen, 59*(2), 144-150. doi:10.1002/em.22142

Dallmann, T., & Menon, A. (2016). Technology pathways for diesel engines used in non-road vehicles and equipment. *International Council on Clean Transportation (ICCT): Washington, DC, USA*.

Daun, J., & Hougen, F. (1976). Sulfur content of rapeseed oils. *Journal of the American Oil Chemists' Society, 53*(5), 169-171.

Dayer, J.-M., Oliviero, F., & Punzi, L. (2017). A Brief History of IL-1 and IL-1 Ra in Rheumatology. *Frontiers in Pharmacology, 8*(293). doi:10.3389/fphar.2017.00293

Diaz-Sanchez, D., Jyrala, M., Ng, D., Nel, A., & Saxon, A. (2000). In Vivo Nasal Challenge with Diesel Exhaust Particles Enhances Expression of the CC Chemokines Rantes, MIP-1α, and MCP-3 in Humans. *Clinical Immunology, 97*(2), 140-145. doi:https://doi.org/10.1006/clim.2000.4921

Douki, T., Corbière, C., Preterre, D., Martin, P. J., Lecureur, V., André, V., . . . Sichel, F. (2018). Comparative study of diesel and biodiesel exhausts on lung oxidative stress and genotoxicity in rats. *Environmental Pollution, 235*, 514-524. doi:https://doi.org/10.1016/j.envpol.2017.12.077

Drummer, C., Friedel, V., Borger, A., Stormer, I., Wolter, S., Zittermann, A., . . . Heer, M. (1998). Effects of elevated carbon dioxide environment on calcium metabolism in humans. *Aviat Space Environ Med, 69*(3), 291-298.

Duffy, A. M., Bouchier-Hayes, D. J., & Harmey, J. H. (2013). Vascular endothelial growth factor (VEGF) and its role in non-endothelial cells: autocrine signalling by VEGF *Madame Curie Bioscience Database [Internet]*: Landes Bioscience.

Dziendzikowska, K., Gajewska, M., Wilczak, J., Mruk, R., Oczkowski, M., Żyła, E., . . . Gromadzka-Ostrowska, J. (2018). The effects of 1st and 2nd generation biodiesel exhaust exposure on hematological and biochemical blood indices of Fisher344 male rats – The FuelHealth project. *Environmental Toxicology and Pharmacology, 63*, 34-47. doi:https://doi.org/10.1016/j.etap.2018.08.001

Eea, European Environment Agency (2013). Bioenergy potential from a resource-efficiency perspective. *European Environment Agency. EEA Report*(6), 2013.

EERE, Energy Efficiency & Renewable Energy (2020). *Alternate Fuels Data Centre*. Retrieved from https://afdc.energy.gov/fuels/biodiesel.html.

EIA, United States Energy and Information Administration (2020a). International energy statistics. Retrieved from https://www.eia.gov/international/overview/world

EIA, United States Energy and Information Administration (2020b). *Monthy Biodiesel Production Report*.

Elgharbawy, A. S., Sadik, W. A., Sadek, O. M., & Kasaby, M. A. (2021). A REVIEW ON BIODIESEL FEEDSTOCKS AND PRODUCTION TECHNOLOGIES. *Journal of the Chilean Chemical Society, 66*, 5098-5109.

ElKassar, N., & Gress, R. E. (2010). An overview of IL-7 biology and its use in immunotherapy. *Journal of immunotoxicology, 7*(1), 1-7. doi:10.3109/15476910903453296

Elliott, A. R., Prisk, G. K., Schollmann, C., & Hoffmann, U. (1998). Hypercapnic ventilatory response in humans before, during, and after 23 days of low level CO2 exposure. *Aviat Space Environ Med, 69*(4), 391-396.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology, 35*(4), 495-516. doi:10.1080/01926230701320337

EU-Commission. (2011). Commission Regulation (EU) No 582/2011 of 25 May 2011. *Off J Eur Union L, 167*(1).

EU-OSHA, European Agency for Safety and Health at Work (2013). Retrieved from https://osha.europa.eu/en/themes/dangerous-substances/practical-tools-dangerous-substances/workplace-exposure-standards

EU, European Union. (2004). Directive 2004/37/EC of the European Parliament and of the Council of 29 April 2004 on the protection of workers from the risks related to exposure to carcinogens or mutagens at work. *Off. J. Eur. Union., 47*, 50-76.

EU, European Union (2009). Directive 2009/28/EC of the European Parliament and of the Council of 23 April 2009 on the promotion of the use of energy from renewable sources and amending and subsequently repealing Directives 2001/77/EC and 2003/30/EC. *Official Journal of the European Union, 5*, 2009.

EU, European Union (2016). Directive (EU) 2016/802 of the European Parliament and of the Council codification of 11 May 2016.

EU, European Union (2019). Directive (EU) 2019/130 of the European Parliament and of the Council of 16 January 2019 amending Directive 2004/37/EC on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Text with EEA relevance.). *Official Journal of the European Union*.

Evans, K. A., Halterman, J. S., Hopke, P. K., Fagnano, M., & Rich, D. Q. (2014). Increased ultrafine particles and carbon monoxide concentrations are associated with asthma exacerbation among urban children. *Environmental Research, 129*, 11-19. doi:doi:10.1016/j.envres.2013.12.001

Eze, I. C., Hemkens, L. G., Bucher, H. C., Hoffmann, B., Schindler, C., Künzli, N., . . . Probst-Hensch, N. M. (2015). Association between ambient air pollution and diabetes mellitus in Europe and North America: systematic review and meta-analysis. *Environ Health Perspect, 123*(5), 381-389. doi:10.1289/ehp.1307823

Faber, S. C., McNabb, N. A., Ariel, P., Aungst, E. R., & McCullough, S. D. (2020). Exposure Effects Beyond the Epithelial Barrier: Trans-Epithelial Induction of Oxidative Stress by Diesel Exhaust Particulates in Lung Fibroblasts in an Organotypic Human Airway Model. *Toxicological Sciences*. doi:10.1093/toxsci/kfaa085

Farraj, A. K., Haykal-Coates, N., Winsett, D. W., Gilmour, M. I., King, C., Krantz, Q. T., . . . Hazari, M. S. (2015). Comparative electrocardiographic, autonomic and systemic inflammatory responses to soy biodiesel and petroleum diesel emissions in rats. *Inhalation Toxicology, 27*(11), 564-575. doi:10.3109/08958378.2015.1057884

Filograna, R., Civiero, L., Ferrari, V., Codolo, G., Greggio, E., Bubacco, L., . . . Bisaglia, M. (2015). Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. *PLoS ONE, 10*(8), e0136769. doi:10.1371/journal.pone.0136769

Fisher, A. B., Hyde, R. W., Baue, A. E., Reif, J. S., & Kelly, D. F. (1969). Effect of carbon monoxide on function and structure of the lung. *Journal of Applied Physiology, 26*(1), 4-12. doi:doi:10.1152/jappl.1969.26.1.4

Flach, B., Lieberz, S., & Bolla, S. (2019). *EU Biofuels Annual 2019, Gain report NL9022*. Retrieved from

Fleisch, A. F., Kloog, I., Luttmann-Gibson, H., Gold, D. R., Oken, E., & Schwartz, J. D. (2016). Air pollution exposure and gestational diabetes mellitus among pregnant women in Massachusetts: a cohort study. *Environ Health, 15*, 40. doi:10.1186/s12940-016-0121-4

Floreth, T., Stern, E., Tu, Y., Stern, R., Garrity, E. R., Bhorade, S. M., & White, S. R. (2011). Differentiated transplant derived airway epithelial cell cytokine secretion is not regulated by cyclosporine. *Respiratory Research, 12*(1), 44. doi:10.1186/1465-9921-12-44

Fontaras, G., Karavalakis, G., Kousoulidou, M., Tzamkiozis, T., Ntziachristos, L., Bakeas, E., . . . Samaras, Z. (2009). Effects of biodiesel on passenger car fuel consumption, regulated and non-regulated pollutant emissions over legislated and real-world driving cycles. *Fuel, 88*(9), 1608-1617. doi:doi:10.1016/j.fuel.2009.02.011

Foong, R. E., Sly, P. D., Larcombe, A. N., & Zosky, G. R. (2010). No role for neutrophil elastase in influenza-induced cellular recruitment, cytokine production or airway hyperresponsiveness in mice. *Respir Physiol Neurobiol, 173*(2), 164-170. doi:10.1016/j.resp.2010.08.003

Fukagawa, N. K., Li, M., Poynter, M. E., Palmer, B. C., Parker, E., Kasumba, J., & Holmén, B. A. (2013). Soy biodiesel and petrodiesel emissions differ in size, chemical composition and stimulation of inflammatory responses in cells and animals. *Environmental Science & Technology, 47*(21), 12496-12504. doi:10.1021/es403146c

Fukuoka, A., Matsushita, K., Morikawa, T., Takano, H., & Yoshimoto, T. (2016). Diesel exhaust particles exacerbate allergic rhinitis in mice by disrupting the nasal epithelial barrier. *Clinical & Experimental Allergy, 46*(1), 142-152. doi:https://doi.org/10.1111/cea.12597

Ganguly, K., Ettehadieh, D., Upadhyay, S., Takenaka, S., Adler, T., Karg, E., . . . Stoeger, T. (2017). Early pulmonary response is critical for extra-pulmonary carbon nanoparticle mediated effects: comparison of

inhalation versus intra-arterial infusion exposures in mice. *Particle and Fibre Toxicology, 14*(1), 19. doi:10.1186/s12989-017-0200-x
Gao, H., Guo, C., Xing, J., Zhao, J., & Liu, H. (2010). Extraction and oxidative desulfurization of diesel fuel catalyzed by a Brønsted acidic ionic liquid at room temperature. *Green Chemistry, 12*(7), 1220-1224. doi:doi: 10.1039/c002108c
Garofalo, R. P., & Haeberle, H. (2000). Epithelial Regulation of Innate Immunity to Respiratory Syncytial Virus. *American Journal of Respiratory Cell and Molecular Biology, 23*(5), 581-585. doi:10.1165/ajrcmb.23.5.f204
Gauderman, W. J., Urman, R., Avol, E., Berhane, K., McConnell, R., Rappaport, E., . . . Gilliland, F. (2015). Association of Improved Air Quality with Lung Development in Children. *New England Journal of Medicine, 372*(10), 905-913. doi:10.1056/NEJMoa1414123
Gauderman, W. J., Vora, H., McConnell, R., Berhane, K., Gilliland, F., Thomas, D., . . . Peters, J. (2007). Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. *The Lancet, 369*(9561), 571-577. doi:https://doi.org/10.1016/S0140-6736(07)60037-3
Gavett, S. H., Wood, C. E., Williams, M. A., Cyphert, J. M., Boykin, E. H., Daniels, M. J., . . . Gilmour, M. I. (2015). Soy biodiesel emissions have reduced inflammatory effects compared to diesel emissions in healthy and allergic mice. *Inhalation Toxicology, 27*(11), 533-544. doi:10.3109/08958378.2015.1054966
Gerlofs-Nijland, M. E., Totlandsdal, A. I., Tzamkiozis, T., Leseman, D. L. A. C., Samaras, Z., Låg, M., . . . Cassee, F. R. (2013). Cell Toxicity and Oxidative Potential of Engine Exhaust Particles: Impact of Using Particulate Filter or Biodiesel Fuel Blend. *Environ Sci Technol, 47*(11), 5931-5938. doi:10.1021/es305330y
Gerlofs-Nijland, M. E., van Berlo, D., Cassee, F. R., Schins, R. P. F., Wang, K., & Campbell, A. (2010). Effect of prolonged exposure to diesel engine exhaust on proinflammatory markers in different regions of the rat brain. *Part Fibre Toxicol, 7*, 12-12. doi:10.1186/1743-8977-7-12
Ghio, A. J., Stonehuerner, J. G., Dailey, L. A., Richards, J. H., Madden, M. D., Deng, Z., . . . Piantadosi, C. A. (2008). Carbon monoxide reversibly alters iron homeostasis and respiratory epithelial cell function. *American Journal of Respiratory Cell and Molecular Biology, 38*(6), 715-723.
Ghogare, R., Chen, S., & Xiong, X. (2020). Metabolic Engineering of Oleaginous Yeast Yarrowia lipolytica for Overproduction of Fatty Acids. *Front Microbiol, 11*, 1717. doi:10.3389/fmicb.2020.01717
Giakoumis, E. G., Rakopoulos, C. D., Dimaratos, A. M., & Rakopoulos, D. C. (2012). Exhaust emissions of diesel engines operating under transient conditions with biodiesel fuel blends. *Progress in Energy and Combustion Science, 38*(5), 691-715. doi:https://doi.org/10.1016/j.pecs.2012.05.002
Giakoumis, E. G., & Sarakatsanis, C. K. (2018). Estimation of biodiesel cetane number, density, kinematic viscosity and heating values from its fatty acid weight composition. *Fuel, 222*, 574-585. doi:https://doi.org/10.1016/j.fuel.2018.02.187
Giles, L. V., Carlsten, C., & Koehle, M. S. (2018). The pulmonary and autonomic effects of high-intensity and low-intensity exercise in diesel exhaust. *Environ Health, 17*(1), 87-87. doi:10.1186/s12940-018-0434-6
Giles, L. V., Tebbutt, S. J., Carlsten, C., & Koehle, M. S. (2018). The effect of low and high-intensity cycling in diesel exhaust on flow-mediated dilation, circulating NOx, endothelin-1 and blood pressure. *PLoS One, 13*(2), e0192419. doi:10.1371/journal.pone.0192419
Ginsberg, G. L., Perkovich Foos, B., & Firestone, M. P. (2005). Review and Analysis of Inhalation Dosimetry Methods for Application to Children's Risk Assessment. *Journal of Toxicology and Environmental Health, Part A, 68*(8), 573-615. doi:10.1080/15287390590921793
Ginzburg, H., Liu, X., Baker, M., Shreeve, R., Jayanty, R. K. M., Campbell, D., & Zielinska, B. (2015). Monitoring study of the near-road PM2.5 concentrations in Maryland. *Journal Of The Air & Waste Management Association, 65*(9), 1062-1071. doi:10.1080/10962247.2015.1056887
Gioda, A., Rodríguez-Cotto, R. I., Amaral, B. S., Encarnación-Medina, J., Ortiz-Martínez, M. G., & Jiménez-Vélez, B. D. (2016). Biodiesel from Soybean Promotes Cell Proliferation in Vitro. *Toxicology in Vitro, 34*, 283-288. doi:doi:10.1016/j.tiv.2016.05.004
Goodson, J. M., Weldy, C. S., MacDonald, J. W., Liu, Y., Bammler, T. K., Chien, W.-M., & Chin, M. T. (2017). *In utero* exposure to diesel exhaust particulates is associated with an altered cardiac transcriptional response to transverse aortic constriction and altered DNA methylation. *The FASEB Journal*. doi:doi:10.1096/fj.201700032R
Gowdy, K. M., Krantz, Q. T., King, C., Boykin, E., Jaspers, I., Linak, W. P., & Gilmour, M. I. (2010). Role of oxidative stress on diesel-enhanced influenza infection in mice. *Part Fibre Toxicol, 7*, 34-34. doi:10.1186/1743-8977-7-34

Graboski, M. S., McCormick, R. L., Alleman, T. L., & Herring, A. M. (2003). *The Effect of Biodiesel Composition on Engine Emissions from a DDC Series 60 Diesel Engine*. Retrieved from Colorado Institute for Fuels and Engine Research, Colorado School of Mines, Golden, CO.: https://www.nrel.gov/docs/fy03osti/31461.pdf

Graver, B. M., Frey, H. C., & Hu, J. (2016). Effect of Biodiesel Fuels on Real-World Emissions of Passenger Locomotives. *Environmental Science & Technology, 50*(21), 12030-12039. doi:doi:10.1021/acs.est.6b03567

Hallberg, L. M., Ward, J. B., Hernandez, C., Ameredes, B. T., & Wickliffe, J. K. (2015). Part 3. Assessment of genotoxicity and oxidative damage in rats after chronic exposure to new-technology diesel exhaust in the ACES bioassay. *Res Rep Health Eff Inst*(184), 87-105; discussion 141-171.

Hamje, H. D., Hass, H., Lonza, L., Maas, H., Reid, A., Rose, K., & Venderbosch, T. (2014). EU renewable energy targets in 2020: Revised analysis of scenarios for transport fuels. *Sci. Policy Rep. Jt. Res. Cent. Eur. Comm*, 27.

Hannon, M., Gimpel, J., Tran, M., Rasala, B., & Mayfield, S. (2010). Biofuels from algae: challenges and potential. *Biofuels, 1*(5), 763-784. doi:10.4155/bfs.10.44

Hashimoto, A. H., Amanuma, K., Hiyoshi, K., Sugawara, Y., Goto, S., Yanagisawa, R., . . . Aoki, Y. (2007). Mutations in the lungs of gpt delta transgenic mice following inhalation of diesel exhaust. *Environ Mol Mutagen, 48*(8), 682-693. doi:10.1002/em.20335

Hawley, B., L'Orange, C., Olsen, D. B., Marchese, A. J., & Volckens, J. (2014). Oxidative stress and aromatic hydrocarbon response of human bronchial epithelial cells exposed to petro- or biodiesel exhaust treated with a diesel particulate filter. *Toxicol Sci, 141*(2), 505-514. doi:10.1093/toxsci/kfu147

Hazari, M. S., Haykal-Coates, N., Winsett, D. W., King, C., Krantz, Q. T., Gilmour, M. I., & Farraj, A. K. (2015). The effects of B0, B20, and B100 soy biodiesel exhaust on aconitine-induced cardiac arrhythmia in spontaneously hypertensive rats. *Inhal Toxicol, 27*(11), 557-563. doi:10.3109/08958378.2015.1054967

He, B. B., Van Gerpen, J. H., & Thompson, J. C. (2009). Sulfur content in selected oils and fats and their corresponding methyl esters. *Applied engineering in agriculture, 25*(2), 223-226. doi:10.13031/2013.26319

Hedmer, M., Tinnerberg, H., Li, H., Albin, M., Broberg, K., & Wierzbicka, A. (2017). Diesel Exhaust Exposure Assessment Among Tunnel Construction Workers—Correlations Between Nitrogen Dioxide, Respirable Elemental Carbon, and Particle Number. *Ann Work Expo Health, 61*(5), 539-553. doi:10.1093/annweh/wxx024

Heidari Nejad, S., Takechi, R., Mullins, B. J., Giles, C., Larcombe, A. N., Bertolatti, D., . . . Mamo, J. (2015). The effect of diesel exhaust exposure on blood–brain barrier integrity and function in a murine model. *Journal of Applied Toxicology, 35*(1), 41-47. doi:10.1002/jat.2985

Heinrich, J., & Slama, R. (2007). Fine particles, a major threat to children. *International Journal of Hygiene and Environmental Health, 210*(5), 617-622. doi:https://doi.org/10.1016/j.ijheh.2007.07.012

Hemmingsen, J. G., Møller, P., Nøjgaard, J. K., Roursgaard, M., & Loft, S. (2011). Oxidative Stress, Genotoxicity, And Vascular Cell Adhesion Molecule Expression in Cells Exposed to Particulate Matter from Combustion of Conventional Diesel and Methyl Ester Biodiesel Blends. *Environmental Science & Technology, 45*(19), 8545-8551. doi:doi:10.1021/es200956p

Hesterberg, T. W., Long, C. M., Sax, S. N., Lapin, C. A., McClellan, R. O., Bunn, W. B., & Valberg, P. A. (2011). Particulate Matter in New Technology Diesel Exhaust (NTDE) is Quantitatively and Qualitatively Very Different from that Found in Traditional Diesel Exhaust (TDE). *Journal of the Air & Waste Management Association, 61*(9), 894-913. doi:10.1080/10473289.2011.599277

Hiraiwa, K., & van Eeden, S. F. (2013). Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants. *Mediators of inflammation, 2013*, 619523-619523. doi:10.1155/2013/619523

Holdsworth, S. R., & Gan, P.-Y. (2015). Cytokines: Names and Numbers You Should Care About. *Clinical journal of the American Society of Nephrology : CJASN, 10*(12), 2243-2254. doi:10.2215/CJN.07590714

Hsia, C. C. W., Hyde, D. M., Ochs, M., Weibel, E. R., & Structure, o. b. o. t. A. E. J. T. F. o. t. Q. A. o. L. (2010). An Official Research Policy Statement of the American Thoracic Society/European Respiratory Society: Standards for Quantitative Assessment of Lung Structure. *American Journal of Respiratory and Critical Care Medicine, 181*(4), 394-418. doi:doi:10.1164/rccm.200809-1522ST

Hu, S., Herner, J. D., Robertson, W., Kobayashi, R., Chang, M. C., Huang, S. M., . . . Ayala, A. (2013). Emissions of polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs from heavy-duty diesel vehicles with DPF and SCR. *J Air Waste Manag Assoc, 63*(8), 984-996.

Hussain, S., Laumbach, R., Coleman, J., Youssef, H., Kelly-McNeil, K., Ohman-Strickland, P., . . . Kipen, H. (2012). Controlled exposure to diesel exhaust causes increased nitrite in exhaled breath condensate among subjects with asthma. *J Occup Environ Med, 54*(10), 1186-1191. doi:10.1097/JOM.0b013e31826bb64c

IARC, International Agency for Research on Cancer (2018). Agents Classified by the IARC Monographs, Volumes 1–120. Retrieved from http://monographs.iarc.fr/ENG/Classification/

Inomata, S., Fushimi, A., Sato, K., Fujitani, Y., & Yamada, H. (2015). 4-Nitrophenol, 1-nitropyrene, and 9-nitroanthracene emissions in exhaust particles from diesel vehicles with different exhaust gas treatments. *Atmospheric Environment, 110*, 93-102. doi:https://doi.org/10.1016/j.atmosenv.2015.03.043

Jaeger, K., Ruschulte, H., Heine, J., & Piepenbrock, S. (2000). [Carbon monoxide poisoning]. *Anaesthesiol Reanim, 25*(3), 74-77.

Jafarihaghighi, F., Ardjmand, M., Salar Hassani, M., Mirzajanzadeh, M., & Bahrami, H. (2020). Effect of Fatty Acid Profiles and Molecular Structures of Nine New Source of Biodiesel on Combustion and Emission. *ACS Omega, 5*(26), 16053-16063. doi:10.1021/acsomega.0c01526

Jaffe, L. S. (1968). Ambient Carbon Monoxide And Its Fate in the Atmosphere *Journal of the Air Pollution Control Association, 18*(8), 534-540. doi:10.1080/00022470.1968.10469168

Jalava, P. I., Aakko-Saksa, P., Murtonen, T., Happo, M. S., Markkanen, A., Yli-Pirilä, P., . . . Hirvonen, M.-R. (2012). Toxicological properties of emission particles from heavy duty engines powered by conventional and bio-based diesel fuels and compressed natural gas. *Particle and Fibre Toxicology, 9*(1), 37. doi:doi:10.1186/1743-8977-9-37

James, A. L., Hogg, J. C., Dunn, L. A., & Paré, P. D. (1988). The use of the internal perimeter to compare airway size and to calculate smooth muscle shortening. *Am Rev Respir Dis, 138*(1), 136-139. doi:10.1164/ajrccm/138.1.136

Jánosi, T. Z., Adamicza, Á., Zosky, G. R., Asztalos, T., Sly, P. D., & Hantos, Z. (2006). Plethysmographic estimation of thoracic gas volume in apneic mice. *Journal of Applied Physiology, 101*(2), 454-459. doi:doi:10.1152/japplphysiol.00011.2006

Jiang, R., Jones, M. J., Sava, F., Kobor, M. S., & Carlsten, C. (2014). Short-term diesel exhaust inhalation in a controlled human crossover study is associated with changes in DNA methylation of circulating mononuclear cells in asthmatics. *Part Fibre Toxicol, 11*, 71-71. doi:10.1186/s12989-014-0071-3

Kachuri, L., Villeneuve, P. J., Parent, M.-É., Johnson, K. C., Canadian Cancer Registries Epidemiology Research, G., & Harris, S. A. (2016). Workplace exposure to diesel and gasoline engine exhausts and the risk of colorectal cancer in Canadian men. *Environ Health, 15*, 4-4. doi:10.1186/s12940-016-0088-1

Kado, N., & Kuzmicky, P. (2003). Bioassay analyses of particulate matter from a diesel bus engine using various biodiesel feedstock fuels. *National Renewable Energy Laboratory*.

Karavalakis, G., Bakeas, E., Fontaras, G., & Stournas, S. (2011). Effect of biodiesel origin on regulated and particle-bound PAH (polycyclic aromatic hydrocarbon) emissions from a Euro 4 passenger car. *Energy, 36*(8), 5328-5337. doi:doi:10.1016/j.energy.2011.06.041

Karavalakis, G., Stournas, S., & Bakeas, E. (2009). Light vehicle regulated and unregulated emissions from different biodiesels. *Science of The Total Environment, 407*(10), 3338-3346. doi:https://doi.org/10.1016/j.scitotenv.2008.12.063

Kardas, G., Daszyńska-Kardas, A., Marynowski, M., Brząkalska, O., Kuna, P., & Panek, M. (2020). Role of Platelet-Derived Growth Factor (PDGF) in Asthma as an Immunoregulatory Factor Mediating Airway Remodeling and Possible Pharmacological Target. *Frontiers in Pharmacology, 11*(47). doi:10.3389/fphar.2020.00047

Karthikeyan, S., Thomson, E. M., Kumarathasan, P., Guénette, J., Rosenblatt, D., Chan, T., . . . Vincent, R. (2013). Nitrogen Dioxide and Ultrafine Particles Dominate the Biological Effects of Inhaled Diesel Exhaust Treated by a Catalyzed Diesel Particulate Filter. *Toxicological Sciences, 135*(2), 437-450. doi:10.1093/toxsci/kft162

Kavanagh, T. (2014). International Fuel Quality Standards and Their Implications for Australian Standards: Australian Government, Department of the Environment.

Khalek, I. A., Bougher, T. L., Merritt, P. M., & Zielinska, B. (2011). Regulated and Unregulated Emissions from Highway Heavy-Duty Diesel Engines Complying with U.S. Environmental Protection Agency 2007 Emissions Standards. *Journal of the Air & Waste Management Association, 61*(4), 427-442. doi:10.3155/1047-3289.61.4.427

Kicic, A., Sutanto, E. N., Stevens, P. T., Knight, D. A., & Stick, S. M. (2006). Intrinsic Biochemical and Functional Differences in Bronchial Epithelial Cells of Children with Asthma. *American Journal of Respiratory and Critical Care Medicine, 174*(10), 1110-1118. doi:10.1164/rccm.200603-392OC

Kisin, E. R., Shi, X. C., Keane, M. J., Bugarski, A. B., & Shvedova, A. A. (2013). Mutagenicity of biodiesel or diesel exhaust particles and the effect of engine operating conditions. *Journal of environmental engineering & ecological science, 2*(3), 10.7243/2050-1323-7242-7243. doi:doi:10.7243/2050-1323-2-3

Kittelson, D., Watts, W., & Johnson, J. (2002). Diesel Aerosol Sampling Methodology–CRC E-43. *Final report, Coordinating Research Council*.
Kittelson, D. B. (1998). ENGINES AND NANOPARTICLES: A REVIEW. *Journal of Aerosol Science, 29*(5), 575-588. doi:doi:10.1016/S0021-8502(97)10037-4
Knothe, G., de Castro, M. E. G., & Razon, L. F. (2015). Methyl Esters (Biodiesel) from and Fatty Acid Profile of Gliricidia sepium Seed Oil. *Journal of the American Oil Chemists' Society, 92*(5), 769-775. doi:10.1007/s11746-015-2634-3
Knothe, G., & Steidley, K. R. (2005). Kinematic viscosity of biodiesel fuel components and related compounds. Influence of compound structure and comparison to petrodiesel fuel components. *Fuel, 84*(9), 1059-1065. doi:doi:10.1016/j.fuel.2005.01.016
Ko, A., Woo, Y., Jang, J., Jung, Y., Pyo, Y., Jo, H., . . . Lee, Y. J. (2019). Complementary effects between NO oxidation of DPF and NO2 decomposition of SCR in light-duty diesel engine. *Journal of Industrial and Engineering Chemistry, 80*, 160-170. doi:https://doi.org/10.1016/j.jiec.2019.07.045
Kooter, I. M., Alblas, M. J., Jedynska, A. D., Steenhof, M., Houtzager, M. M. G., & Ras, M. v. (2013). Alveolar epithelial cells (A549) exposed at the air–liquid interface to diesel exhaust: First study in TNO's powertrain test center. *Toxicology in Vitro, 27*(8), 2342-2349. doi:https://doi.org/10.1016/j.tiv.2013.10.007
Kowalska, M., Wegierek-Ciuk, A., Brzoska, K., Wojewodzka, M., Meczynska-Wielgosz, S., Gromadzka-Ostrowska, J., . . . Lankoff, A. (2017). Genotoxic potential of diesel exhaust particles from the combustion of first- and second-generation biodiesel fuels—the FuelHealth project. *Environmental Science and Pollution Research*. doi:10.1007/s11356-017-9995-0
Krahl, J., Munack, A., Ruschel, Y., Schröder, O., & Bünger, J. (2008). *Exhaust Gas Emissions and Mutagenic Effects of Diesel Fuel, Biodiesel and Biodiesel Blends*. https://doi.org/10.4271/2008-01-2508
Kyriakidis, N. B., & Katsiloulis, T. (2000). Calculation of iodine value from measurements of fatty acid methyl esters of some oils: Comparison with the relevant American Oil Chemists Society method. *Journal of the American Oil Chemists' Society, 77*(12), 1235-1238. doi:10.1007/s11746-000-0193-3
Lambert, A. L., Mangum, J. B., DeLorme, M. P., & Everitt, J. I. (2003). Ultrafine Carbon Black Particles Enhance Respiratory Syncytial Virus-Induced Airway Reactivity, Pulmonary Inflammation, and Chemokine Expression. *Toxicological Sciences, 72*(2), 339-346. doi:10.1093/toxsci/kfg032
Landwehr, K. R., Hillas, J., Mead-Hunter, R., Brooks, P., King, A., O'Leary, R. A., . . . Larcombe, A. N. (2021). Fuel feedstock determines biodiesel exhaust toxicity in a human airway epithelial cell exposure model. *Journal of Hazardous Materials, 420*, 126637. doi:https://doi.org/10.1016/j.jhazmat.2021.126637
Landwehr, K. R., Hillas, J., Mead-Hunter, R., O'Leary, R. A., Kicic, A., Mullins, B. J., & Larcombe, A. N. (2019). Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells. *Environmental Science & Technology, 53*(19), 11437-11446. doi:10.1021/acs.est.9b01671
Landwehr, K. R., Larcombe, A. N., Reid, A., & Mullins, B. J. (2020). Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants? *Exposure and Health*. doi:10.1007/s12403-020-00379-0
Lane, C., Burgess, S., Kicic, A., Knight, D., & Stick, S. (2005). The use of non-bronchoscopic brushings to study the paediatric airway. *Respiratory Research, 6*(1), 53-53. doi:10.1186/1465-9921-6-53
Lankoff, A., Brzoska, K., Czarnocka, J., Kowalska, M., Lisowska, H., Mruk, R., . . . Kruszewski, M. (2017). A comparative analysis of in vitro toxicity of diesel exhaust particles from combustion of 1st- and 2nd-generation biodiesel fuels in relation to their physicochemical properties—the FuelHealth project. *Environmental Science and Pollution Research, 24*(23), 19357-19374. doi:doi:10.1007/s11356-017-9561-9
Larcombe, A. N., Foong, R. E., Berry, L. J., Zosky, G. R., & Sly, P. D. (2011). *In utero* cigarette smoke exposure impairs somatic and lung growth in BALB/c mice. *European Respiratory Journal, 38*(4), 932-938. doi:doi:10.1183/09031936.00156910
Larcombe, A. N., Foong, R. E., Boylen, C. E., & Zosky, G. R. (2012). Acute diesel exhaust particle exposure increases viral titre and inflammation associated with existing influenza infection, but does not exacerbate deficits in lung function. *Influenza and Other Respiratory Viruses, 7*(5), 701-709. doi:doi:10.1111/irv.12012
Larcombe, A. N., Foong, R. E., Bozanich, E. M., Berry, L. J., Garratt, L. W., Gualano, R. C., . . . Sly, P. D. (2011). Sexual dimorphism in lung function responses to acute influenza A infection. *Influenza Other Respir Viruses, 5*(5), 334-342. doi:10.1111/j.1750-2659.2011.00236.x
Larcombe, A. N., Janka, M. A., Mullins, B. J., Berry, L. J., Bredin, A., & Franklin, P. J. (2017). The effects of electronic cigarette aerosol exposure on inflammation and lung function in mice. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 313*(1), L67-L79. doi:10.1152/ajplung.00203.2016

Larcombe, A. N., Kicic, A., Mullins, B. J., & Knothe, G. (2015). Biodiesel exhaust: The need for a systematic approach to health effects research. *Respirology, 20*(7), 1034-1045. doi:doi:10.1111/resp.12587
Larcombe, A. N., Papini, M. G., Chivers, E. K., Berry, L. J., Lucas, R. M., & Wyrwoll, C. S. (2021). Mouse Lung Structure and Function after Long-Term Exposure to an Atmospheric Carbon Dioxide Level Predicted by Climate Change Modeling. *Environmental Health Perspectives, 129*(1), 017001. doi:doi:10.1289/EHP7305
Larcombe, A. N., Phan, J. A., Kicic, A., Perks, K. L., Mead-Hunter, R., & Mullins, B. J. (2014). Route of exposure alters inflammation and lung function responses to diesel exhaust. *Inhalation Toxicology, 26*(7), 409-418. doi:doi:10.3109/08958378.2014.909910
Larcombe, A. N., Zosky, G. R., Bozanich, E. M., Turner, D. J., Hantos, Z., & Sly, P. D. (2008). Absence of cholinergic airway tone in normal BALB/c mice. *Respir Physiol Neurobiol, 161*(3), 223-229. doi:10.1016/j.resp.2008.01.009
Latifovic, L., Villeneuve, P. J., Parent, M.-É., Johnson, K. C., Kachuri, L., Canadian Cancer Registries Epidemiology, G., & Harris, S. A. (2015). Bladder cancer and occupational exposure to diesel and gasoline engine emissions among Canadian men. *Cancer medicine, 4*(12), 1948-1962. doi:10.1002/cam4.544
León-Mejía, G., Luna-Rodríguez, I., Trindade, C., Oliveros-Ortíz, L., Anaya-Romero, M., Luna-Carrascal, J., . . . Quintana-Sosa, M. (2019). Cytotoxic and genotoxic effects in mechanics occupationally exposed to diesel engine exhaust. *Ecotoxicol Environ Saf, 171*, 264-273. doi:https://doi.org/10.1016/j.ecoenv.2018.12.067
Levesque, S., Surace, M. J., McDonald, J. D., & Block, M. L. (2011). Air pollution & the brain: Subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. *Journal of Neuroinflammation, 8*, 105-105. doi:doi:10.1186/1742-2094-8-105
Levesque, S., Taetzsch, T., Lull, M. E., Kodavanti, U., Stadler, K., Wagner, A., . . . Block, M. L. (2011). Diesel exhaust activates and primes microglia: air pollution, neuroinflammation, and regulation of dopaminergic neurotoxicity. *Environ Health Perspect, 119*(8), 1149-1155. doi:10.1289/ehp.1002986
Li, F., Liu, Z., Ni, Z., & Wang, H. (2019). Effect of biodiesel components on its lubrication performance. *Journal of Materials Research and Technology, 8*(5), 3681-3687. doi:https://doi.org/10.1016/j.jmrt.2019.06.011
Li, R., Han, Y., Wang, L., Shang, Y., & Chen, Y. (2019). Differences in oxidative potential of black carbon from three combustion emission sources in China. *J Environ Manage, 240*, 57-65. doi:10.1016/j.jenvman.2019.03.070
Libalova, H., Rossner, P., Vrbova, K., Brzicova, T., Sikorova, J., Vojtisek-Lom, M., . . . Topinka, J. (2016). Comparative Analysis of Toxic Responses of Organic Extracts from Diesel and Selected Alternative Fuels Engine Emissions in Human Lung BEAS-2B Cells. *International Journal of Molecular Sciences, 17*(11), 1833. doi:doi:10.3390/ijms17111833
Lichtveld, K. M., Ebersviller, S. M., Sexton, K. G., Vizuete, W., Jaspers, I., & Jeffries, H. E. (2012). In Vitro Exposures in Diesel Exhaust Atmospheres: Resuspension of PM from Filters versus Direct Deposition of PM from Air. *Environ Sci Technol, 46*(16), 9062-9070. doi:10.1021/es301431s
Limjunyawong, N., Fallica, J., Horton, M. R., & Mitzner, W. (2015). Measurement of the Pressure-volume Curve in Mouse Lungs. *JoVE*(95), e52376. doi:doi:10.3791/52376
Liotta, F. J., & Montalvo, D. M. (1993). *The Effect of Oxygenated Fuels on Emissions from a Modern Heavy-Duty Diesel Engine*. https://doi.org/10.4271/932734
Little, F. F., Cruikshank, W. W., & Center, D. M. (2001). Il-9 stimulates release of chemotactic factors from human bronchial epithelial cells. *Am J Respir Cell Mol Biol, 25*(3), 347-352. doi:10.1165/ajrcmb.25.3.4349
Lloyd, C. (2002). Chemokines in allergic lung inflammation. *Immunology, 105*(2), 144-154. doi:10.1046/j.1365-2567.2002.01344.x
Looi, K., Buckley, A. G., Rigby, P. J., Garratt, L. W., Iosifidis, T., Zosky, G. R., . . . Stick, S. M. (2018). Effects of human rhinovirus on epithelial barrier integrity and function in children with asthma. *Clinical & Experimental Allergy, 48*(5), 513-524. doi:https://doi.org/10.1111/cea.13097
Looi, K., Troy, N. M., Garratt, L. W., Iosifidis, T., Bosco, A., Buckley, A. G., . . . Stick, S. M. (2016). Effect of human rhinovirus infection on airway epithelium tight junction protein disassembly and transepithelial permeability. *Experimental Lung Research, 42*(7), 380-395. doi:10.1080/01902148.2016.1235237
Lucking, A. J., Lundback, M., Barath, S. L., Mills, N. L., Sidhu, M. K., Langrish, J. P., . . . Blomberg, A. (2011). Particle traps prevent adverse vascular and prothrombotic effects of diesel engine exhaust inhalation in men. *Circulation, 123*(16), 1721-1728. doi:10.1161/circulationaha.110.987263
Lundbäck, M., Mills, N. L., Lucking, A., Barath, S., Donaldson, K., Newby, D. E., . . . Blomberg, A. (2009). Experimental exposure to diesel exhaust increases arterial stiffness in man. *Part Fibre Toxicol, 6*(1), 7. doi:10.1186/1743-8977-6-7

Lung, S., Cassee, F. R., Gosens, I., & Campbell, A. (2014). Brain suppression of AP-1 by inhaled diesel exhaust and reversal by cerium oxide nanoparticles. *Inhal Toxicol, 26*(10), 636-641. doi:10.3109/08958378.2014.948651

Madden, M. C. (2016). A paler shade of green? The toxicology of biodiesel emissions: Recent findings from studies with this alternative fuel. *Biochimica et Biophysica Acta - General Subjects, 1860*(12), 2856-2862. doi:doi:10.1016/j.bbagen.2016.05.035

Magnusson, P., Dziendzikowska, K., Oczkowski, M., Øvrevik, J., Eide, D. M., Brunborg, G., . . . Myhre, O. (2019). Lung effects of 7- and 28-day inhalation exposure of rats to emissions from 1st and 2nd generation biodiesel fuels with and without particle filter – The FuelHealth project. *Environ Toxicol Pharmacol, 67*, 8-20. doi:https://doi.org/10.1016/j.etap.2019.01.005

Magnusson, P., Oczkowski, M., Øvrevik, J., Gajewska, M., Wilczak, J., Biedrzycki, J., . . . Myhre, O. (2017). No adverse lung effects of 7- and 28-day inhalation exposure of rats to emissions from petrodiesel fuel containing 20% rapeseed methyl esters (B20) with and without particulate filter – the FuelHealth project. *Inhalation Toxicology, 29*(5), 206-218. doi:10.1080/08958378.2017.1339149

Martinovich, K. M., Iosifidis, T., Buckley, A. G., Looi, K., Ling, K.-M., Sutanto, E. N., . . . Stick, S. M. (2017). Conditionally reprogrammed primary airway epithelial cells maintain morphology, lineage and disease specific functional characteristics. *Scientific Reports, 7*(1), 17971. doi:10.1038/s41598-017-17952-4

Matsumoto, A., Hiramatsu, K., Li, Y., Azuma, A., Kudoh, S., Takizawa, H., & Sugawara, I. (2006). Repeated exposure to low-dose diesel exhaust after allergen challenge exaggerates asthmatic responses in mice. *Clinical Immunology, 121*(2), 227-235. doi:https://doi.org/10.1016/j.clim.2006.08.003

Mazzon, E., & Cuzzocrea, S. (2007). Role of TNF-α in lung tight junction alteration in mouse model of acute lung inflammation. *Respiratory Research, 8*(1), 75. doi:10.1186/1465-9921-8-75

McCormick, R. L., Graboski, M. S., Alleman, T. L., Herring, A. M., & Tyson, K. S. (2001). Impact of Biodiesel Source Material and Chemical Structure on Emissions of Criteria Pollutants from a Heavy-Duty Engine. *Environmental Science & Technology, 35*(9), 1742-1747. doi:10.1021/es001636t

McDonald, J. D., Doyle-Eisele, M., Seagrave, J., Gigliotti, A. P., Chow, J., Zielinska, B., . . . Miller, R. A. (2015). Part 1. Assessment of carcinogenicity and biologic responses in rats after lifetime inhalation of new-technology diesel exhaust in the ACES bioassay. *Res Rep Health Eff Inst, 184*(9), 44.

Menten, P., Wuyts, A., & Van Damme, J. (2002). Macrophage inflammatory protein-1. *Cytokine & Growth Factor Reviews, 13*(6), 455-481. doi:https://doi.org/10.1016/S1359-6101(02)00045-X

Milborrow, S. (2020). Package 'rpart. plot'.

Miller, J. A., & Bowman, C. T. (1989). Mechanism and modeling of nitrogen chemistry in combustion. *Progress in Energy and Combustion Science, 15*(4), 287-338. doi:https://doi.org/10.1016/0360-1285(89)90017-8

Mills, N. L., Miller, M. R., Lucking, A. J., Beveridge, J., Flint, L., Boere, A. J. F., . . . Newby, D. E. (2011). Combustion-derived nanoparticulate induces the adverse vascular effects of diesel exhaust inhalation. *European heart journal, 32*(21), 2660-2671. doi:10.1093/eurheartj/ehr195

Mills , N. L., Törnqvist , H., Gonzalez , M. C., Vink , E., Robinson , S. D., Söderberg , S., . . . Newby , D. E. (2007). Ischemic and Thrombotic Effects of Dilute Diesel-Exhaust Inhalation in Men with Coronary Heart Disease. *New England Journal of Medicine, 357*(11), 1075-1082. doi:doi:10.1056/NEJMoa066314

Mitchell, C., Provost, K., Niu, N., Homer, R., & Cohn, L. (2011). IFN-γ acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease. *Journal of immunology (Baltimore, Md. : 1950), 187*(7), 3815-3820. doi:10.4049/jimmunol.1100436

Mitzner, W. (2008). Use of mean airspace chord length to assess emphysema. *J Appl Physiol (1985), 105*(6), 1980-1981. doi:10.1152/japplphysiol.90968.2008

Möhner, M., & Wendt, A. (2017). A critical review of the relationship between occupational exposure to diesel emissions and lung cancer risk. *Crit Rev Toxicol, 47*(3), 185-224. doi:10.1080/10408444.2016.1266598

Møller, P., Scholten, R. H., Roursgaard, M., & Krais, A. M. (2020). Inflammation, oxidative stress and genotoxicity responses to biodiesel emissions in cultured mammalian cells and animals. *Critical Reviews in Toxicology*, 1-19. doi:10.1080/10408444.2020.1762541

Morin, J.-P., Hasson, V., Fall, M., Papaioanou, E., Preterre, D., Gouriou, F., . . . Dionnet, F. (2008). Prevalidation of in vitro continuous flow exposure systems as alternatives to in vivo inhalation safety evaluation experimentations: Outcome from MAAPHRI-PCRD5 research program. *Experimental and Toxicologic Pathology, 60*(2), 195-205. doi:https://doi.org/10.1016/j.etp.2008.01.007

MSHA, Mine Safety and Health Administration. (2016). *Exposure of Underground Miners to Diesel Exhaust*.

Mullins, B. J., Kicic, A., Ling, K.-M., Mead-Hunter, R., & Larcombe, A. N. (2016). Biodiesel Exhaust–Induced Cytotoxicity and Proinflammatory Mediator Production in Human Airway Epithelial Cells. *Environmental Toxicology, 31*(1), 44-57. doi:doi:10.1002/tox.22020

Munack, A., Herbst, L., Kaufmann, A., Ruschel, Y., Schröder, O., Krahl, J., & Bünger, J. (2006). Comparison of shell middle distillate, premium diesel fuel and fossil diesel fuel with rapeseed oil methyl ester. *Research Project Final Report, Braunshweig*.

Mutlu, E., Warren, S. H., Matthews, P. P., Schmid, J. E., Kooter, I. M., Linak, W. P., . . . DeMarini, D. M. (2015). Health effects of soy-biodiesel emissions: bioassay-directed fractionation for mutagenicity. *Inhalation Toxicology, 27*(11), 597-612. doi:doi:10.3109/08958378.2015.1091054

Nejad, S. H., Takechi, R., Mullins, B. J., Giles, C., Larcombe, A. N., Bertolatti, D., . . . Mamo, J. (2015). The effect of diesel exhaust exposure on blood-brain barrier integrity and function in a murine model. *Journal of Applied Toxicology, 35*(1), 41-47. doi:doi:10.1002/jat.2985

Nemmar, A., Al-Salam, S., Zia, S., Marzouqi, F., Al-Dhaheri, A., Subramaniyan, D., . . . Kazzam, E. E. (2011). Contrasting actions of diesel exhaust particles on the pulmonary and cardiovascular systems and the effects of thymoquinone. *British journal of pharmacology, 164*(7), 1871-1882. doi:10.1111/j.1476-5381.2011.01442.x

Neophytou, M. A., Costello, M. S., Picciotto, D. S., Brown, H. D., Attfield, A. M., Blair, T. A., . . . Eisen, A. E. (2019). Diesel Exhaust, Respirable Dust, and Ischemic Heart Disease: An Application of the Parametric g-formula. *Epidemiology, 30*(2), 177-185. doi:10.1097/EDE.0000000000000954

Nikanjam, M. (1993). *Development of the First CARB Certified California Alternative Diesel Fuel*: SAE Technical Paper.

Niu, Y., Zhang, X., Meng, T., Wang, H., Bin, P., Shen, M., . . . Zheng, Y. (2018). Exposure characterization and estimation of benchmark dose for cancer biomarkers in an occupational cohort of diesel engine testers. *J Expo Sci Environ Epidemiol, 28*(6), 579-588. doi:10.1038/s41370-018-0061-x

Nordenhäll, C., Pourazar, J., Ledin, M.-C., Levin, J.-O., Sandström, T., & Ädelroth, E. (2001). Diesel exhaust enhances airway responsiveness in asthmatic subjects. *European Respiratory Journal, 17*(5), 909-915.

Oberdörster, G., Celein, R. M., Ferin, J., & Weiss, B. (1995). Association of Particulate Air Pollution and Acute Mortality: Involvement of Ultrafine Particles? *Inhalation Toxicology, 7*(1), 111-124. doi:doi:10.3109/08958379509014275

OECD/FAO, Organization for Economic Co-operation Development/ Food and Agriculture Organization. (2020). OECD-FAO Agricultural Outlook 2020-2029.

OECD/FAO, Organization for Economic Co-operation Development/ Food and Agriculture Organization (2015). OECD/FAO agricultural outlook 2015–2025 data. Retrieved from http://stats.oecd.org/index.aspx?queryid=48184#

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., . . . Wagner, H. (2020). The vegan package (versions 2.5-7). *Community ecology package, 10*(631-637), 719.

Okubo, T., Hosaka, M., & Nakae, D. (2015). In vitro effects induced by diesel exhaust at an air–liquid interface in a human lung alveolar carcinoma cell line A549. *Experimental and Toxicologic Pathology, 67*(7), 383-388. doi:https://doi.org/10.1016/j.etp.2015.03.004

Olfert, J. S., Symonds, J. P. R., & Collings, N. (2007). The effective density and fractal dimension of particles emitted from a light-duty diesel vehicle with a diesel oxidation catalyst. *Journal of Aerosol Science, 38*(1), 69-82. doi:https://doi.org/10.1016/j.jaerosci.2006.10.002

Olsson, A. C., Gustavsson, P., Kromhout, H., Peters, S., Vermeulen, R., Bruske, I., . . . Straif, K. (2011). Exposure to diesel motor exhaust and lung cancer risk in a pooled analysis from case-control studies in Europe and Canada. *Am J Respir Crit Care Med, 183*(7), 941-948. doi:10.1164/rccm.201006-0940OC

Olszewska-Pazdrak, B., Casola, A., Saito, T., Alam, R., Crowe, S. E., Mei, F., . . . Garofalo, R. P. (1998). Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *Journal of virology, 72*(6), 4756-4764. doi:10.1128/JVI.72.6.4756-4764.1998

Omidvarborna, H., Kumar, A., & Kim, D.-S. (2016). Variation of diesel soot characteristics by different types and blends of biodiesel in a laboratory combustion chamber. *Science of The Total Environment, 544*(Supplement C), 450-459. doi:doi:10.1016/j.scitotenv.2015.11.076

OSHA, US Occupational Safety and Health Administration (2021). Permissible Exposure Limits – Annotated Tables. Retrieved from https://www.osha.gov/annotated-pels/table-z-1

Øvrevik, J., Refsnes, M., Låg, M., Holme, J. A., & Schwarze, P. E. (2015). Activation of Proinflammatory Responses in Cells of the Airway Mucosa by Particulate Matter: Oxidant- and Non-Oxidant-Mediated Triggering Mechanisms. *Biomolecules, 5*(3), 1399-1440. doi:10.3390/biom5031399

Pawlak, E. A., Noah, T. L., Zhou, H., Chehrazi, C., Robinette, C., Diaz-Sanchez, D., . . . Jaspers, I. (2016). Diesel exposure suppresses natural killer cell function and resolution of eosinophil inflammation: a randomized controlled trial of exposure in allergic rhinitics. *Part Fibre Toxicol, 13*(1), 24-24. doi:10.1186/s12989-016-0135-7

Pechout, M., Kotek, M., Jindra, P., Macoun, D., Hart, J., & Vojtisek-Lom, M. (2019). Comparison of hydrogenated vegetable oil and biodiesel effects on combustion, unregulated and regulated gaseous pollutants and DPF regeneration procedure in a Euro6 car. *Science of The Total Environment, 696*, 133748. doi:https://doi.org/10.1016/j.scitotenv.2019.133748

Peng, D.-X. (2017). Biodiesel Improves Lubricity of Low-Sulfur Petro-Diesels. *Chemistry and Technology of Fuels and Oils, 52*(6), 699-703. doi:10.1007/s10553-017-0762-1

Peretz, A., Kaufman, J. D., Trenga, C. A., Allen, J., Carlsten, C., Aulet, M. R., . . . Sullivan, J. H. (2008). Effects of diesel exhaust inhalation on heart rate variability in human volunteers. *Environ Res, 107*(2), 178-184. doi:10.1016/j.envres.2008.01.012

Peretz, A., Peck, E. C., Bammler, T. K., Beyer, R. P., Sullivan, J. H., Trenga, C. A., . . . Kaufman, J. D. (2007). Diesel Exhaust Inhalation and Assessment of Peripheral Blood Mononuclear Cell Gene Transcription Effects: An Exploratory Study of Healthy Human Volunteers. *Inhal Toxicol, 19*(14), 1107-1119. doi:10.1080/08958370701665384

Peretz, A., Sullivan, J. H., Leotta, D. F., Trenga, C. A., Sands, F. N., Allen, J., . . . Kaufman, J. D. (2008). Diesel exhaust inhalation elicits acute vasoconstriction in vivo. *Environ Health Perspect, 116*(7), 937-942. doi:10.1289/ehp.11027

Peták, F., Hantos, Z., Adamicza, Á., Asztalos, T., & Sly, P. D. (1997). Methacholine-induced bronchoconstriction in rats: effects of intravenous vs. aerosol delivery. *Journal of Applied Physiology, 82*(5), 1479-1487. doi:10.1152/jappl.1997.82.5.1479

Peters, S., de Klerk, N., Reid, A., Fritschi, L., Musk, A., & Vermeulen, R. (2017). Estimation of quantitative levels of diesel exhaust exposure and the health impact in the contemporary Australian mining industry. *Occup Environ Med, 74*(4), 282-289. doi:10.1136/oemed-2016-103808

Pöttering, H., & Necas, P. (2009). Directive 2009/30/EC of the European parliament and of the Council of 23 April 2009 amending Directive 98/70/EC as regards the specification of petrol, diesel and gas-oil introducing a mechanism to monitor and reduce greenhouse gas emissions and amending Council Directive 1999/32/EC as regards the specification of fuel used by inland waterway vessels and repealing Directive 93/12/EC. *Off. J. Eur. Union, 140*, 88-112.

Price, M. (2019). Fuel Quality Standards (Automotive Diesel) Determination 2019.

Prokopowicz, A., Zaciera, M., Sobczak, A., Bielaczyc, P., & Woodburn, J. (2015). The Effects of Neat Biodiesel and Biodiesel and HVO Blends in Diesel Fuel on Exhaust Emissions from a Light Duty Vehicle with a Diesel Engine. *Environmental Science & Technology, 49*(12), 7473-7482. doi:doi:10.1021/acs.est.5b00648

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., . . . Tschaplinski, T. (2006). The Path Forward for Biofuels and Biomaterials. *Science, 311*(5760), 484-489.

Ramachandran, G., Paulsen, D., Watts, W., & Kittelson, D. (2005). Mass, surface area and number metrics in diesel occupational exposure assessment. *Journal of Environmental Monitoring, 7*(7), 728-735. doi:10.1039/B503854E

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., & Pérez, Á. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology, 100*(1), 261-268. doi:https://doi.org/10.1016/j.biortech.2008.06.039

Ravindra, K., Sokhi, R., & Van Grieken, R. (2008). Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. *Atmospheric Environment, 42*(13), 2895-2921. doi:doi:10.1016/j.atmosenv.2007.12.010

Reis, H., Reis, C., Sharip, A., Reis, W., Zhao, Y., Sinclair, R., & Beeson, L. (2018). Diesel exhaust exposure, its multi-system effects, and the effect of new technology diesel exhaust. *Environ Int, 114*, 252-265. doi:https://doi.org/10.1016/j.envint.2018.02.042

Rhead, M. M., & Hardy, S. A. (2003). The sources of polycyclic aromatic compounds in diesel engine emissions. *Fuel, 82*(4), 385-393. doi:doi:10.1016/S0016-2361(02)00314-9

Rice, E. K., Nikolic-Paterson, D. J., Hill, P. A., Metz, C. N., Bucala, R., Atkins, R. C., & Tesch, G. H. (2003). Interferon-gamma induces macrophage migration inhibitory factor synthesis and secretion by tubular epithelial cells. *Nephrology (Carlton), 8*(3), 156-161. doi:10.1046/j.1440-1797.2003.00152.x

Rider, C. F., Yamamoto, M., Günther, O. P., Hirota, J. A., Singh, A., Tebbutt, S. J., & Carlsten, C. (2016). Controlled diesel exhaust and allergen coexposure modulates microRNA and gene expression in humans: Effects on inflammatory lung markers. *Journal of Allergy and Clinical Immunology, 138*(6), 1690-1700. doi:https://doi.org/10.1016/j.jaci.2016.02.038

Riley, E. A., Carpenter, E. E., Ramsay, J., Zamzow, E., Pyke, C., Paulsen, M. H., . . . Simpson, C. D. (2018). Evaluation of 1-Nitropyrene as a Surrogate Measure for Diesel Exhaust. *Ann Work Expo Health, 62*(3), 339-350. doi:10.1093/annweh/wxx111

Ris, C. (2007). U.S. EPA Health Assessment for Diesel Engine Exhaust: A Review. *Inhalation Toxicology, 19*(sup1), 229-239. doi:doi:10.1080/08958370701497960

Rösler, B., & Herold, S. (2016). Lung epithelial GM-CSF improves host defense function and epithelial repair in influenza virus pneumonia—a new therapeutic strategy? *Molecular and Cellular Pediatrics, 3*(1), 29. doi:10.1186/s40348-016-0055-5

Rynning, I., Arlt, V. M., Vrbova, K., Neča, J., Rossner, P., Jr., Klema, J., . . . Mollerup, S. (2019). Bulky DNA adducts, microRNA profiles, and lipid biomarkers in Norwegian tunnel finishing workers occupationally exposed to diesel exhaust. *Occup Environ Med, 76*(1), 10-16. doi:10.1136/oemed-2018-105445

Saadeh, R., & Klaunig, J. (2014). Child's Development and Respiratory System Toxicity. *Journal of Environmental & Analytical Toxicology, 4*(5), 1.

Schirmer, W. N., Gauer, M. A., Tomaz, E., Rodrigues, P. R. P., de Souza, S. N. M., Chaves, L. I., . . . Cabral, A. R. (2016). Power generation and gaseous emissions performance of an internal combustion engine fed with blends of soybean and beef tallow biodiesel. *Environmental Technology, 37*(12), 1480-1489. doi:10.1080/09593330.2015.1119202

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Seaton, A., Godden, D., MacNee, W., & Donaldson, K. (1995). Particulate air pollution and acute health effects. *The Lancet, 345*(8943), 176-178. doi:doi:10.1016/S0140-6736(95)90173-6

Seriani, R., Junqueira, M. d. S., de Toledo, A. C., Martins, M. A., Seckler, M., Alencar, A. M., . . . Macchione, M. (2015). Diesel exhaust particulates affect cell signaling, mucin profiles, and apoptosis in trachea explants of Balb/C mice. *Environmental Toxicology, 30*(11), 1297-1308. doi:doi:10.1002/tox.22000

Shears, R. K., Jacques, L. C., Naylor, G., Miyashita, L., Khandaker, S., Lebre, F., . . . Kadioglu, A. (2020). Exposure to diesel exhaust particles increases susceptibility to invasive pneumococcal disease. *Journal of Allergy and Clinical Immunology, 145*(4), 1272-1284.e1276. doi:https://doi.org/10.1016/j.jaci.2019.11.039

Sheehan, J., Camobreco, V., Duffield, J., Graboski, M., & Shapouri, H. (1998). *Life cycle inventory of biodiesel and petroleum diesel for use in an urban bus*. Retrieved from https://www.nrel.gov/docs/legosti/fy98/24089.pdf

SHELL. (2018). SHELL (Australia) diesel extra, automotive diesel fuel (SDS).

Silitonga, A. S., Hassan, M. H., Ong, H. C., & Kusumo, F. (2017). Analysis of the performance, emission and combustion characteristics of a turbocharged diesel engine fuelled with Jatropha curcas biodiesel-diesel blends using kernel-based extreme learning machine. *Environ Sci Pollut Res Int, 24*(32), 25383-25405. doi:10.1007/s11356-017-0141-9

Silverman, D. T., Samanic, C. M., Lubin, J. H., Blair, A. E., Stewart, P. A., Vermeulen, R., . . . Attfield, M. D. (2012). The Diesel Exhaust in Miners study: a nested case-control study of lung cancer and diesel exhaust. *Journal of the National Cancer Institute, 104*(11), 855-868. doi:10.1093/jnci/djs034

Sirignano, M., & D'Anna, A. (2018). Filtration and coagulation efficiency of sub-10 nm combustion-generated particles. *Fuel, 221*, 298-302. doi:https://doi.org/10.1016/j.fuel.2018.02.107

Skuland, T. S., Refsnes, M., Magnusson, P., Oczkowski, M., Gromadzka-Ostrowska, J., Kruszewski, M., . . . Øvrevik, J. (2017). Proinflammatory effects of diesel exhaust particles from moderate blend concentrations of 1st and 2nd generation biodiesel in BEAS-2B bronchial epithelial cells—The FuelHealth project. *Environmental Toxicology and Pharmacology, 52*(Supplement C), 138-142. doi:https://doi.org/10.1016/j.etap.2017.04.004

Sly, P. D., Collins, R. A., Thamrin, C., Turner, D. J., & Hantos, Z. (2003). Volume dependence of airway and tissue impedances in mice. *Journal of Applied Physiology, 94*(4), 1460-1466. doi:10.1152/japplphysiol.00596.2002

Smith, B., Nitschke, M., Pilotto, L. S., Ruffin, R., Pisaniello, D., & Willson, K. J. (2000). *Health effects of daily indoor nitrogen dioxide exposure in people with asthma* (Vol. 16).

Sokol, C. L., & Luster, A. D. (2015). The chemokine system in innate immunity. *Cold Spring Harbor perspectives in biology, 7*(5), a016303. doi:10.1101/cshperspect.a016303
Steiner, S., Bisig, C., Petri-Fink, A., & Rothen-Rutishauser, B. (2016). Diesel exhaust: current knowledge of adverse effects and underlying cellular mechanisms. *Archives of Toxicology, 90*, 1541-1553. doi:10.1007/s00204-016-1736-5
Steiner, S., Czerwinski, J., Comte, P., Müller, L. L., Heeb, N. V., Mayer, A., . . . Rothen-Rutishauser, B. (2013). Reduction in (pro-)inflammatory responses of lung cells exposed in vitro to diesel exhaust treated with a non-catalyzed diesel particle filter. *Atmospheric Environment, 81*, 117-124. doi:https://doi.org/10.1016/j.atmosenv.2013.08.029
Steiner, S., Czerwinski, J., Comte, P., Popovicheva, O., Kireeva, E., Müller, L., . . . Rothen-Rutishauser, B. (2013). Comparison of the toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells in vitro. *Atmospheric Environment, 81*, 380-388. doi:https://doi.org/10.1016/j.atmosenv.2013.08.059
Stevens, T., Krantz, Q. T., Linak, W. P., Hester, S., & Gilmour, M. I. (2008). Increased Transcription of Immune and Metabolic Pathways in Naïve and Allergic Mice Exposed to Diesel Exhaust. *Toxicological Sciences, 102*(2), 359-370. doi:10.1093/toxsci/kfn006
Strand, V., Rak, S., Svartengren, M., & Bylin, M. (1997). Nitrogen dioxide exposure enhances asthmatic reaction to inhaled allergen in subjects with asthma. *American Journal of Respiratory and Critical Care Medicine, 155*(3), 881-887. doi:doi:10.1164/ajrccm.155.3.9117021
Suda, T., Sato, A., Sugiura, W., & Chida, K. (1995). Induction of MHC class II antigens on rat bronchial epithelial cells by interferon-gamma and its effect on antigen presentation. *Lung, 173*(2), 127. doi:10.1007/BF02981472
Suppes, G. J., & Storvick, T. S. (2016). Chapter 5 - The New Electric Vehicle Society. In G. J. Suppes & T. S. Storvick (Eds.), *Sustainable Power Technologies and Infrastructure* (pp. 161-190). Boston: Academic Press.
Surawski, N. C., Miljevic, B., Ayoko, G. A., Elbagir, S., Stevanovic, S., Fairfull-Smith, K. E., . . . Ristovski, Z. D. (2011). Physicochemical Characterization of Particulate Emissions from a Compression Ignition Engine: The Influence of Biodiesel Feedstock. *Environmental Science & Technology, 45*(24), 10337-10343. doi:10.1021/es2018797
Svanes, C., Omenaas, E., Jarvis, D., Chinn, S., Gulsvik, A., & Burney, P. (2004). Parental smoking in childhood and adult obstructive lung disease: results from the European Community Respiratory Health Survey. *Thorax, 59*(4), 295-302. doi:10.1136/thx.2003.009746
SWA, Safe Work Australia (2018). *Confined spaces Code of Practice*.
SWA, Safe Work Australia (2019). *WORKPLACE EXPOSURE STANDARDS FOR AIRBORNE CONTAMINANTS*.
Swanson, K. J., Kado, N. Y., Funk, W. E., Pleil, J. D., Madden, M. C., & Ghio, A. J. (2009). Release of the pro-inflammatory markers by BEAS-2B cells following in vitro exposure to biodiesel extracts. *Open Toxicology Journal, 3*, 8-15.
Swanson, K. J., Madden, M. l. C., & Ghio, A. J. (2007). Biodiesel Exhaust: The Need for Health Effects Research. *Environmental Health Perspectives, 115*(4), 496-499. doi:doi:10.1289/ehp.9631
Szikriszt, B., Póti, Á., Pipek, O., Krzystanek, M., Kanu, N., Molnár, J., . . . Szüts, D. (2016). A comprehensive survey of the mutagenic impact of common cancer cytotoxics. *Genome Biology, 17*, 99. doi:10.1186/s13059-016-0963-7
Tanaka, M., Aoki, Y., Takano, H., Fujitani, Y., Hirano, S., Nakamura, R., . . . Inoue, K.-i. (2013). Effects of exposure to nanoparticle-rich or -depleted diesel exhaust on allergic pathophysiology in the murine lung. *The Journal of Toxicological Sciences, 38*(1), 35-48. doi:10.2131/jts.38.35
Tanaka, M., Takano, H., Fujitani, Y., Hirano, S., Ichinose, T., Shimada, A., & Inoue, K.-I. (2013). Effects of exposure to nanoparticle-rich diesel exhaust on 8-OHdG synthesis in the mouse asthmatic lung. *Experimental and therapeutic medicine, 6*(3), 703-706. doi:10.3892/etm.2013.1198
Taxell, P., & Santonen, T. (2017). Diesel Engine Exhaust: Basis for Occupational Exposure Limit Value. *Toxicological Sciences, 158*(2), 243-251. doi:10.1093/toxsci/kfx110
R Team. (2018). R: A language and environment for statistical computing. . Retrieved from https://www.R-project.org/.
Therneau, T., Atkinson, B., Ripley, B., & Ripley, M. B. (2019). Package 'rpart' (version 4.1-15). *Available online: cran.ma.ic.ac.uk/web/packages/rpart/rpart.pdf (accessed on 04 august 2021)*.
Toldrá-Reig, F., Mora, L., & Toldrá, F. (2020). Trends in Biodiesel Production from Animal Fat Waste. *Applied Sciences, 10*(10), 3644.

Tong, H., Rappold, A. G., Caughey, M., Hinderliter, A. L., Graff, D. W., Berntsen, J. H., . . . Samet, J. M. (2014). Cardiovascular effects caused by increasing concentrations of diesel exhaust in middle-aged healthy GSTM1 null human volunteers. *Inhal Toxicol, 26*(6), 319-326. doi:10.3109/08958378.2014.889257

Totlandsdal, A. I., Cassee, F. R., Schwarze, P., Refsnes, M., & Låg, M. (2010). Diesel exhaust particles induce CYP1A1 and pro-inflammatory responses via differential pathways in human bronchial epithelial cells. *Particle and Fibre Toxicology, 7*, 41-41. doi:10.1186/1743-8977-7-41

Tsukue, N., Kato, A., Ito, T., Sugiyama, G., & Nakajima, T. (2010). Acute effects of diesel emission from the urea selective catalytic reduction engine system on male rats. *Inhal Toxicol, 22*(4), 309-320. doi:10.3109/08958370903307652

Unosson, J., Kabéle, M., Boman, C., Nyström, R., Sadiktsis, I., Westerholm, R., . . . Bosson, J. A. (2021). Acute cardiovascular effects of controlled exposure to dilute Petrodiesel and biodiesel exhaust in healthy volunteers: a crossover study. *Particle and Fibre Toxicology, 18*(1), 22-22. doi:10.1186/s12989-021-00412-3

US EPA, U.S. Environmental Protection Agency. (2002). *Health Assessment Document for Diesel Engine Exhaust.* . Washington, DC.

Valand, R., Magnusson, P., Dziendzikowska, K., Gajewska, M., Wilczak, J., Oczkowski, M., . . . Myhre, O. (2018). Gene expression changes in rat brain regions after 7- and 28 days inhalation exposure to exhaust emissions from 1st and 2nd generation biodiesel fuels - The FuelHealth project. *Inhal Toxicol, 30*(7-8), 299-312. doi:10.1080/08958378.2018.1520370

Vermeulen, R., Silverman, D. T., Garshick, E., Vlaanderen, J., Portengen, L., & Steenland, K. (2014). Exposure-response estimates for diesel engine exhaust and lung cancer mortality based on data from three occupational cohorts. *Environ Health Perspect, 122*(2), 172-177. doi:10.1289/ehp.1306880

Vieira, J. L., Guimaraes, G. V., de Andre, P. A., Cruz, F. D., Saldiva, P. H. N., & Bocchi, E. A. (2016). Respiratory Filter Reduces the Cardiovascular Effects Associated With Diesel Exhaust Exposure: A Randomized, Prospective, Double-Blind, Controlled Study of Heart Failure: The FILTER-HF Trial. *JACC: Heart Failure, 4*(1), 55-64. doi:https://doi.org/10.1016/j.jchf.2015.07.018

Vogel, C. F. A., Kado, S. Y., Kobayashi, R., Liu, X., Wong, P., Na, K., . . . Kado, N. Y. (2019). Inflammatory marker and aryl hydrocarbon receptor-dependent responses in human macrophages exposed to emissions from biodiesel fuels. *Chemosphere, 220*, 993-1002. doi:https://doi.org/10.1016/j.chemosphere.2018.12.178

Wade, W. R., White, J. E., & Florek, J. J. (1981). *Diesel Particulate Trap Regeneration Techniques*. https://doi.org/10.4271/810118

Wang, H., Cui, L., Yang, M., Zheng, Y., Leng, S., Duan, H., . . . Zhang, L. (2017). Local and Systemic Inflammation May Mediate Diesel Engine Exhaust–Induced Lung Function Impairment in a Chinese Occupational Cohort. *Toxicological Sciences, 162*(2), 372-382. doi:10.1093/toxsci/kfx259

Wang, J.-S., Tseng, C.-Y., & Chao, M.-W. (2017). Diesel Exhaust Particles Contribute to Endothelia Apoptosis via Autophagy Pathway. *Toxicological Sciences, 156*(1), 72-83. doi:10.1093/toxsci/kfw237

Wang, Kimberley C. W., Cras, T. D. L., Larcombe, Alexander N., Zosky, Graeme R., Elliot, John G., James, Alan L., & Noble, Peter B. (2018). Independent and combined effects of airway remodelling and allergy on airway responsiveness. *Clinical Science, 132*(3), 327-338. doi:10.1042/cs20171386

Weaver , L. K. (2009). Carbon Monoxide Poisoning. *New England Journal of Medicine, 360*(12), 1217-1225. doi:doi:10.1056/NEJMcp0808891

Westphal, G. A., Krahl, J., Munack, A., Rosenkranz, N., Schröder, O., Schaak, J., . . . Bünger, J. (2013). Combustion of Hydrotreated Vegetable Oil and Jatropha Methyl Ester in a Heavy Duty Engine: Emissions and Bacterial Mutagenicity. *Environmental Science & Technology, 47*(11), 6038-6046. doi:doi:10.1021/es400518d

Westphal, G. A., Krahl, J., Munack, A., Ruschel, Y., Schröder, O., Hallier, E., . . . Bünger, J. (2012). Mutagenicity of Diesel Engine Exhaust Is Eliminated in the Gas Phase by an Oxidation Catalyst but Only Slightly Reduced in the Particle Phase. *Environmental Science & Technology, 46*(11), 6417-6424. doi:doi:10.1021/es300399e

Wierzbicka, A., Nilsson, P. T., Rissler, J., Sallsten, G., Xu, Y., Pagels, J. H., . . . Gudmundsson, A. (2014). Detailed diesel exhaust characteristics including particle surface area and lung deposited dose for better understanding of health effects in human chamber exposure studies. *Atmospheric Environment, 86*, 212-219. doi:https://doi.org/10.1016/j.atmosenv.2013.11.025

Win-Shwe, T.-T., Fujimaki, H., Fujitani, Y., & Hirano, S. (2012). Novel object recognition ability in female mice following exposure to nanoparticle-rich diesel exhaust. *Toxicol Appl Pharmacol, 262*(3), 355-362. doi:https://doi.org/10.1016/j.taap.2012.05.015

Win-Shwe, T.-T., Yamamoto, S., Fujitani, Y., Hirano, S., & Fujimaki, H. (2008). Spatial learning and memory function-related gene expression in the hippocampus of mouse exposed to nanoparticle-rich diesel exhaust. *Neurotoxicology, 29*(6), 940-947. doi:https://doi.org/10.1016/j.neuro.2008.09.007

W.H.O., World Health Organization (2006). *Air quality guidelines: global update 2005: particulate matter, ozone, nitrogen dioxide, and sulfur dioxide*: World Health Organization.

Wu, J., Xu, C., Wang, Q., & Cheng, W. (2016). Potential Sources and Formations of the PM2.5 Pollution in Urban Hangzhou. *Atmosphere, 7*, 100. doi:10.3390/atmos7080100

Xu, S., Höglund, M., Håkansson, L., & Venge, P. (2000). Granulocyte colony-stimulating factor (G-CSF) induces the production of cytokines in vivo. *British Journal of Haematology, 108*(4), 848-853. doi:doi:10.1046/j.1365-2141.2000.01943.x

Yamagishi, N., Ito, Y., Ramdhan, D. H., Yanagiba, Y., Hayashi, Y., Wang, D., . . . Nakajima, T. (2012). Effect of nanoparticle-rich diesel exhaust on testicular and hippocampus steroidogenesis in male rats. *Inhal Toxicol, 24*(8), 459-467. doi:10.3109/08958378.2012.688225

Yamamoto, M., Singh, A., Sava, F., Pui, M., Tebbutt, S. J., & Carlsten, C. (2013). MicroRNA Expression in Response to Controlled Exposure to Diesel Exhaust: Attenuation by the Antioxidant N-Acetylcysteine in a Randomized Crossover Study. *Environ Health Perspect, 121*(6), 670-675. doi:doi:10.1289/ehp.1205963

Yanamala, N., Hatfield, M. K., Farcas, M. T., Schwegler-Berry, D., Hummer, J. A., Shurin, M. R., . . . Shvedova, A. A. (2013). Biodiesel versus diesel exposure: Enhanced pulmonary inflammation, oxidative stress, and differential morphological changes in the mouse lung. *Toxicology and applied pharmacology, 272*(2), 373-383. doi:doi:10.1016/j.taap.2013.07.006

Yang, P.-M., Wang, C.-C., Lin, Y.-C., Jhang, S.-R., Lin, L.-J., & Lin, Y.-C. (2017). Development of novel alternative biodiesel fuels for reducing PM emissions and PM-related genotoxicity. *Environmental Research, 156*, 512-518. doi:https://doi.org/10.1016/j.envres.2017.03.045

Yoza, B., Matsumoto, M., & Matsunaga, T. (2002). DNA extraction using modified bacterial magnetic particles in the presence of amino silane compound. *Journal of Biotechnology, 94*(3), 217-224. doi:https://doi.org/10.1016/S0168-1656(01)00427-8

Zarcone, M. C., Duistermaat, E., Alblas, M. J., van Schadewijk, A., Ninaber, D. K., Clarijs, V., . . . Kooter, I. M. (2018). Effect of diesel exhaust generated by a city bus engine on stress responses and innate immunity in primary bronchial epithelial cell cultures. *Toxicology in Vitro, 48*, 221-231. doi:https://doi.org/10.1016/j.tiv.2018.01.024

Zarcone, M. C., Duistermaat, E., Schadewijk, A. v., Jedynska, A., Hiemstra, P. S., & Kooter, I. M. (2016). Cellular response of mucociliary differentiated primary bronchial epithelial cells to diesel exhaust. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 311*(1), L111-L123. doi:10.1152/ajplung.00064.2016

Zarcone, M. C., van Schadewijk, A., Duistermaat, E., Hiemstra, P. S., & Kooter, I. M. (2017). Diesel exhaust alters the response of cultured primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD) to non-typeable Haemophilus influenzae. *Respir Res, 18*(1), 27-27. doi:10.1186/s12931-017-0510-4

Zhang, J. J., McCreanor, J. E., Cullinan, P., Chung, K. F., Ohman-Strickland, P., Han, I. K., . . . Nieuwenhuijsen, M. J. (2009). Health effects of real-world exposure to diesel exhaust in persons with asthma. *Res Rep Health Eff Inst*(138), 5-109; discussion 111-123.

Zhang, X., Duan, H., Gao, F., Li, Y., Huang, C., Niu, Y., . . . Zheng, Y. (2015). Increased Micronucleus, Nucleoplasmic Bridge, and Nuclear Bud Frequencies in the Peripheral Blood Lymphocytes of Diesel Engine Exhaust-Exposed Workers. *Toxicological Sciences, 143*(2), 408-417. doi:10.1093/toxsci/kfu239

Zhang, X., Hirota, J. A., Yang, C., & Carlsten, C. (2016). Effect of GST variants on lung function following diesel exhaust and allergen co-exposure in a controlled human crossover study. *Free Radical Biology and Medicine, 96*, 385-391. doi:https://doi.org/10.1016/j.freeradbiomed.2016.04.202

Zhang, X., Li, J., He, Z., Duan, H., Gao, W., Wang, H., . . . Zheng, Y. (2016). Associations between DNA methylation in DNA damage response-related genes and cytokinesis-block micronucleus cytome index in diesel engine exhaust-exposed workers. *Arch Toxicol, 90*(8), 1997-2008. doi:10.1007/s00204-015-1598-2

Zhou, Y., McLane, M., & Levitt, R. C. (2001). Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. *Respiratory Research, 2*(2), 80-84. doi:10.1186/rr42

Zhu, N., Li, H., Han, M., Guo, L., Chen, L., Yun, Y., . . . Sang, N. (2012). Environmental nitrogen dioxide (NO_2) exposure influences development and progression of ischemic stroke. *Toxicology Letters, 214*(2), 120-130. doi:doi:10.1016/j.toxlet.2012.08.021

Appendix

Permission to use published work in this thesis was obtained from each journal.
These can be found at the end of the appendix.

Attribution Statements

Chapter 1: Landwehr, K. R., Larcombe, A. N., Reid, A., & Mullins, B. J. (2020). Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants? *Exposure and Health*. doi:10.1007/s12403-020-00379-0

Chapter 2: Landwehr, K. R., Hillas, J., Mead-Hunter, R., O'Leary, R. A., Kicic, A., Mullins, B. J., & Larcombe, A. N. (2019). Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway Epithelial Cells. *Environmental Science & Technology, 53*(19), 11437-11446. doi:10.1021/acs.est.9b01671

Chapter 3: Landwehr, K. R., Hillas, J., Mead-Hunter, R., Brooks, P., King, A., O'Leary, R. A., . . . Larcombe, A. N. (2021). Fuel feedstock determines biodiesel exhaust toxicity in a human airway epithelial cell exposure model. *Journal of Hazardous Materials, 420*, 126637. doi:https://doi.org/10.1016/j.jhazmat.2021.126637

Chapter 5: Submitted to Particle and Fibre Toxicology

Katherine Landwehr

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design	x	x	x	x
Acquisition of data & methods	x	x	x	x
Data conditioning & manipulation	x	x	x	x
Analysis & statistical methods	x	x	x	x
Interpretation & discussion	x	x	x	x
Final approval	x	x	x	x

Alison Reid

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design	x			
Acquisition of data & methods				
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion	x			
Final approval	x			

Jessica Hillas

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design		x		
Acquisition of data & methods		x	x	x
Data conditioning & manipulation		x		
Analysis & statistical methods				
Interpretation & discussion				
Final approval		x	x	x

Ryan Mead-Hunter

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design		x	x	
Acquisition of data & methods		x	x	x
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion				
Final approval		x	x	x

Peter Brooks

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design				
Acquisition of data & methods			x	
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion				
Final approval			x	

Andrew King

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design			x	x
Acquisition of data & methods			x	
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion				
Final approval			x	x

Rebecca O'Leary

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design				
Acquisition of data & methods				
Data conditioning & manipulation				
Analysis & statistical methods		x	x	x
Interpretation & discussion		x	x	
Final approval		x	x	x

Anthony Kicic

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design		x	x	x
Acquisition of data & methods		x		x
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion		x	x	x
Final approval		x	x	x

Benjamin Mullins

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design	x	x	x	x
Acquisition of data & methods		x	x	
Data conditioning & manipulation				
Analysis & statistical methods				
Interpretation & discussion	x	x	x	x
Final approval	x	x	x	x

Alexander Larcombe

	Paper 1: Critical Review (Chapter 1)	Paper 2: Soy Biodiesel Time course (Chapter 2)	Paper 3: Multiple Biodiesels (Chapter 3)	Paper 4: Air Liquid Interface (Chapter 5)
Conception and design	x	x	x	x
Acquisition of data & methods		x	x	x
Data conditioning & manipulation				
Analysis & statistical methods		x		
Interpretation & discussion	x	x	x	x
Final approval	x	x	x	x

Supplementary Materials

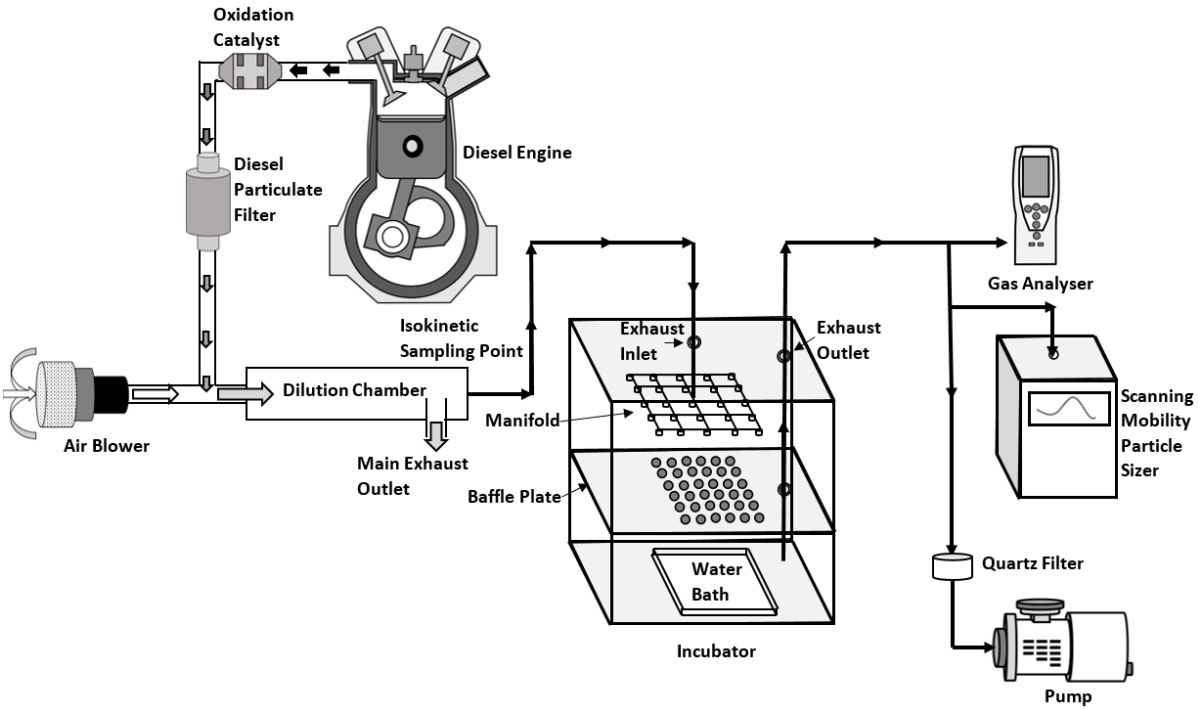


Figure S2.1: Experimental set up for exposures. Exhaust generated from the diesel engine is diluted 1:10 with HEPA filtered air into the dilution/mixing chamber before being extracted through an isokinetic sampling point and pumped into a sealed incubator at a rate of 10 L/min though a manifold spreader. Cells are situated in a baffle plate and exhaust is pumped over them before being extracted at the bottom of the incubator for analysis of physicochemical characteristic such as gases and particle sizes. Finally, particles are collected on a quartz filter.

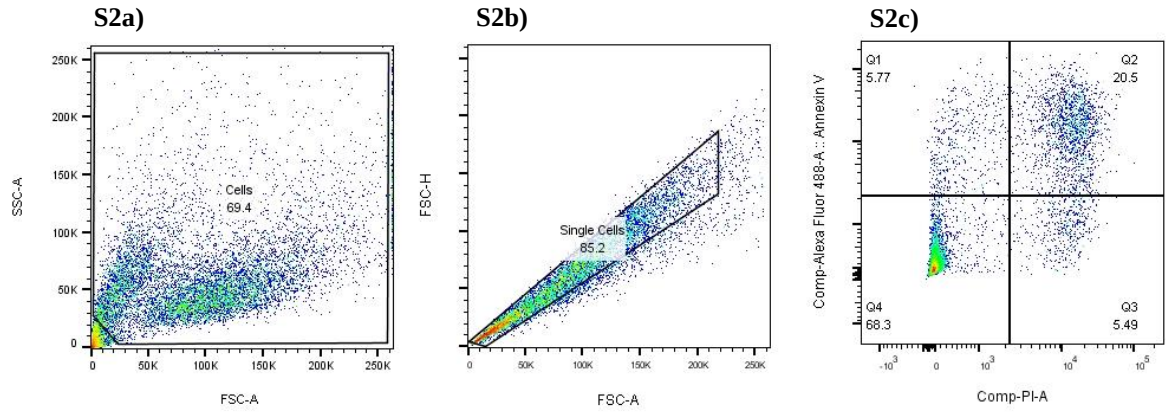


Figure S2.2: Gating for flow analysis. After compensation using FlowJo compensation analysis tools and single stained cell populations, cellular debris is excluded from the analysis using forward and side scatter area (S2a). Next single cells are selected using forward scattering characteristics (S2b). Finally, populations are separated into alive (S2c, Q3), Annexin V -ve, PI -

ve), necrotic (S2c, Q4), Annexin V -ve, PI +ve) or apoptotic populations (S2c, Q1&2), Annexin V +ve, PI +ve or -ve). Unstained and single stained controls using additional cells from the same experimental sample at the same fluorophore concentration were used to verify gating positions for the final gating step.

Table S2.1: Significance values (p-values) for the univariate linear regression analysis of exhaust particulate properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	Exhaust Particulate Property				
Biological Outcome	Particle Concentration	Median Particle Size	Total Particle Number	Particle Number >23 nm	Particle Number <23 nm
Raw Viability	6.44E-05	0.349	1.71E-05	4.86E-05	1.64E-05
Necrosis	7.64E-08	2.5E-06	4.05E-07	4.77E-07	4.4E-07
IL-1β	0.391	0.000998	0.162	0.653	0.136
IL-1RA	0.104	0.0177	0.133	0.0677	0.142
IL-6	0.00381	0.128	0.00125	0.00404	0.00114
IL-8	0.00539	0.344	0.00147	0.00308	0.00141
G-CSF	0.00642	0.415	0.00101	0.00663	0.000857
GM-CSF	0.000835	0.0447	9.49E-05	0.003025	6.69E-05
IP-10	0.163	0.00743	0.266	0.207	0.274
Mip-1β	7.24E-05	0.864	7.08E-06	4.28E-05	6.31E-06
RANTES	0.00539	0.138	0.01	0.00714	0.0106
VEGF	0.333	0.0627	0.275	0.407	0.265
TNF-α	0.484	0.987	0.598	0.522	0.607

Table S2.2: Significance values (p-values) for the univariate linear regression analysis of exhaust gas properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	Exhaust Gas Property					
Biological Outcome	Oxygen	Carbon Monoxide	Carbon Dioxide	Nitrogen Monoxide	Nitrogen Dioxide	Sulfur Dioxide
Raw Viability	0.419	8.82E-07	0.430	0.289	0.488	0.801
Necrosis	7.79E-10	0.00016	2.67E-09	1.79E-10	5.19E-09	1.2E-07
IL-1β	0.0644	0.654	0.108	0.135	0.0914	0.145
IL-1RA	0.00105	0.429	0.000322	0.005542	0.000254	0.000305
IL-6	0.0374	0.00215	0.044	0.494	0.0468	0.105
IL-8	0.803	0.000196	0.751	0.814	0.786	0.923
G-CSF	0.244	0.00109	0.259	0.332	0.265	0.510
GM-CSF	0.0463	0.000964	0.0766	0.0459	0.0817	0.223
IP-10	0.0033	0.678	0.00382	0.00331	0.00379	0.00339
Mip-1β	0.1467	2.44E-06	0.132	0.176	0.144	0.335
RANTES	0.0169	0.0317	0.0171	0.00975	0.0221	0.0511
VEGF	0.0838	0.117	0.0605	0.210	0.0431	0.0155
TNF-α	0.778	0.566	0.833	0.587	0.889	0.963

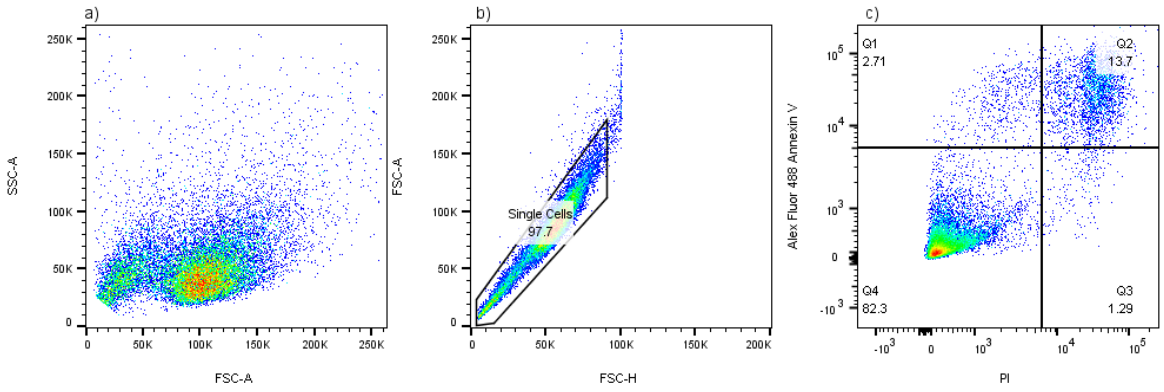


Figure S3.1: Gating strategy for analysis of flow cytometry results. First cells were selected using forward and side scatter area (S2a). Next single cells populations were selected using forward scattering characteristics (S2b). Finally cells are separated into Viable, Early Apoptotic, Late Apoptotic and Necrotic populations using the FIT-C channel to measure Alexa Fluor 488 Annexin V staining and the TEXAS Red channel to measure PI staining in order to minimise the need for compensation (S2c). 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. Unstained and single stained controls using additional cells from the same experimental sample were used to verify gating positions for the final gating step.

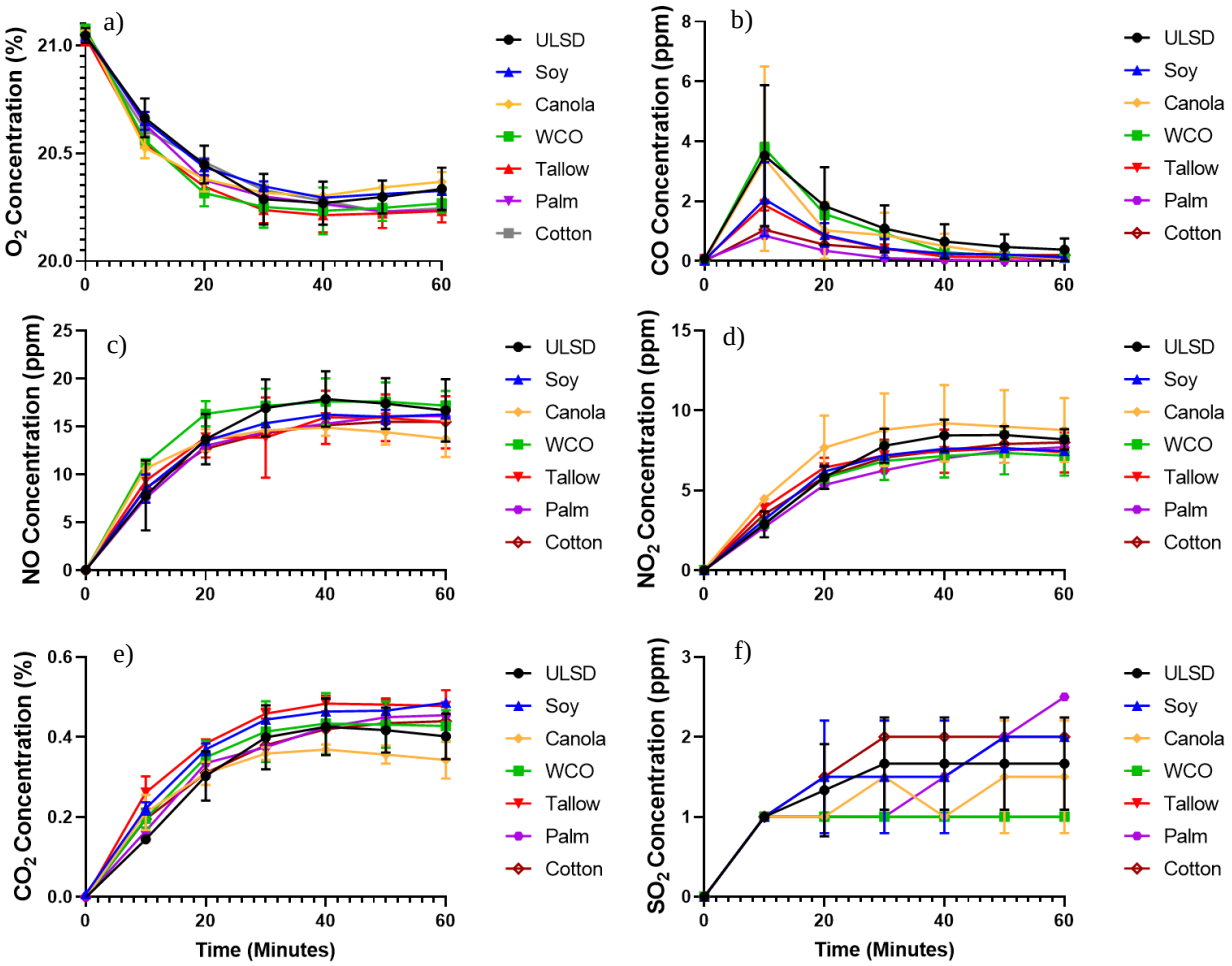


Figure S3.2: Exhaust gas concentrations over time. S2a-f in order: oxygen concentration, carbon monoxide concertation, nitrogen monoxide concentration, nitrogen dioxide concentration, carbon dioxide concentration and sulfur dioxide concentration.

Table S3.1: Mean (standard deviation) mediator release for the 14 cytokines released above the limits of detection. All values have been normalised by subtracting Air controls for each individual participant. Each fuel has been labelled with a corresponding letter to indicate significant differences between biodiesel fuels ($p<0.05$): a=Soy, b=Canola, c=WCO, d=Tallow, e=Palm and f=Cottonseed.

Mediator Concentration (pg/mL)	Fuel						
	ULSD	Soy (a)	Canola (b)	WCO (c)	Tallow (d)	Palm (e)	Cotton (f)
IL-1β	0.23 (0.65)	0.32 (0.19) b,d	0.15 (0.34) a,c,e,f	0.04 (0.11) b,d	-0.03 (0.09) a,c,e,f	0.07 (0.12) b,d	0.07 (0.27) b,d

IL-1RA	10.10 (42.45)	-56.77 (116.49) b,c,d,f	82.99 (159.72) a	73.61 (97.21) a	81.52 (87.52) a	12.42 (148.61) f	131.94 (173.85) a,e
IL-6	-17.04 (68.57)	81.88 (147.78) b,c,d,f	-66.18 (1.64) a,d	-30.75 (155.94) a,d	223.11 (368.83) a,b,c,e,f	-3.06 (67.00) d	-17.33 (222.79) a,d
IL-8	255.47 (705.55)	784.43 (1467.67) b,c,d,f	-215.15 (374.62) a,d,e	-35.93 (587.63) a,d,e	1516.17 (2908.79) a,b,c,e,f	1014.91 (1586.81) b,c,d,f	29.75 (622.18) a,d,e
IL-9	1.98 (4.01)	3.79 (10.63) b,c,d,f	-2.71 (4.24) a,d,e	-1.78 (5.07) a,d,e	6.57 (14.24) a,b,c,e,f	6.32 (9.55) b,c,d,f	0.62 (7.37)a,d,e
G-CSF	28.81 (62.43)	23.35 (33.59) b,c,d,e,f	-9.71 (24.66) a,d,e	7.90 (58.47) a,d,e	43.03 (69.50) a,b,c,f	66.16 (79.27) a,b,c,f	12.33 (46.12) a,d,e
GM-CSF	8.44 (14.36)	10.94 (17.06) b,c,d,e	5.05 (9.49) a,d	1.27 (1.36) a,d,f	20.23 (30.47) a,b,c,e,f	14.95 (13.76) a,d	11.32 (16.73) c,d
INF-γ	-1.95 (5.56)	1.93 (6.50) d,f	-0.50 (7.59) d,e,f	2.15 (4.29) d,f	7.34 (16.20) a,b,c	5.00 (10.08) b,f	13.31 (11.12) a,b,c,e
IP-10	-8.76 (200.80)	30.23 (133.54)	32.56 (195.74)	158.95 (170.03) d	-40.69 (166.35) c	16.66 (240.45)	54.66 (205.05)
MCP-1	24.88 (59.99)	57.43 (97.49) b,c,e,f	2.70 (3.98) a,d,e,f	6.15 (10.19) a,d,e,f	66.87 (175.49) b,c,e,f	53.93 (121.01) a,b,c,d,f	47.58 (124.80) a,b,c,d,e
MIP-1β	1.23 (1.61)	1.76 (3.19) b,c,f	-0.03 (1.06) a,d,e	0.25 (0.91) a,d,e	2.46 (4.14) b,c,e,f	2.64 (2.36) b,c,d,f	0.40 (2.20) a,d,e
RANTES	-0.29 (1.03)	2.56 (4.09) c,f	0.17 (2.04)	0.14 (2.37) a	1.66 (2.80)	6.02 (6.17) f	0.37 (0.75) a,e
TNF-α	5.16 (4.33)	6.85 (6.46) f	4.91 (8.61) f	3.51 (3.70) f	7.43 (6.78) f	7.52 (11.51)	13.48 (10.57) a,b,c,d
VEGF	93.64 (167.33)	7.28 (158.03) f	44.07 (161.51) f	51.05 (376.80) f	48.85 (217.44) f	43.16 (164.40) f	-131.50 (246.32) a,b,c,d,e

a= significantly different to Soy.
b= significantly different to Canola.
c= significantly different to WCO.
d= significantly different to Tallow.
e= significantly different to Palm.
f= significantly different to Cottonseed.

Table S3.2: Significance values (p-values) for the univariate linear regression analysis of biodiesel exhaust gas properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	O_2 (%)	CO (ppm)	CO_2 (%)	NO_x (ppm)	NO (ppm)	NO_2 (ppm)	SO_2 (ppm)
Viability	0.231	0.293	0.0295	0.496	0.834	0.293	0.379
Necrosis	0.255	0.858	0.445	0.943	0.784	0.51	0.316
Early Apoptosis	0.517	0.116	0.562	0.0404	0.102	0.914	0.288
Late Apoptosis	0.36	0.158	0.0223	0.262	0.682	0.178	0.583
IL-1β	0.829	0.395	0.882	0.500	0.736	0.175	0.317
IL-1RA	0.248	0.833	0.233	0.721	0.839	0.448	0.683
IL-6	0.292	0.512	0.0127	0.838	0.756	0.712	0.283
IL-8	0.317	0.237	0.0341	0.374	0.587	0.415	0.458
IL-9	0.485	0.251	0.0613	0.346	0.488	0.545	0.593
G-CSF	0.292	0.268	0.0420	0.454	0.766	0.0727	0.581
GM-CSF	0.638	0.0621	0.174	0.0959	0.175	0.592	0.813
INF-γ	0.558	0.0353	0.549	0.113	0.368	0.301	0.265
IP-10	0.88	0.247	0.587	0.202	0.102	0.624	0.816
MCP-1	0.965	0.169	0.234	0.248	0.493	0.453	0.620
MIP-1β	0.379	0.179	0.175	0.189	0.185	0.897	0.512
RANTES	0.693	0.0377	0.956	0.0644	0.128	0.754	0.072
TNF-α	0.823	0.0254	0.319	0.0150	0.308	0.0939	0.387
VEGF	0.495	0.343	0.801	0.413	0.520	0.896	0.159

Table S3.3: Significance values (p-values) for the univariate linear regression analysis of biodiesel exhaust particle properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	Particle Mass Concentration (µg/m3)	Median Particle Size (nm)	Total Particle Number (particles/cm^3)	Particle Concentration Between 80-100 nm (particles/cm^3)	Particle Concentration Between 20-35 nm (particles/cm^3)	Particle Proportion >23 nm (%)
Viability	0.221	0.311	0.455	0.269	0.276	0.251
Necrosis	0.847	0.253	0.891	0.82	0.633	0.28
Early Apoptosis	0.337	0.204	0.127	0.061	0.145	0.123
Late Apoptosis	0.391	0.139	0.33	0.143	0.189	0.0897
IL-1β	0.833	0.816	0.743	0.71	0.496	0.919
IL-1RA	0.199	0.0733	0.758	0.791	0.525	0.0423
IL-6	0.0868	0.769	0.347	0.413	0.543	0.684
IL-8	0.264	0.29	0.28	0.1811	0.241	0.218
IL-9	0.376	0.309	0.232	0.176	0.287	0.227
G-CSF	0.462	0.163	0.851	0.355	0.286	0.125
GM-CSF	0.196	0.827	0.0888	0.0449	0.0551	0.619
INF-γ	0.0151	0.0593	0.363	0.0984	0.0189	0.100
IP-10	0.696	0.738	0.0658	0.113	0.291	0.583
MCP-1	0.356	0.975	0.312	0.192	0.249	0.782
MIP-1β	0.467	0.217	0.0927	0.0744	0.134	0.158
RANTES	0.0934	0.0490	0.151	0.0685	0.0416	0.101
TNF-α	0.503	0.0163	0.176	0.0225	0.0625	0.00467
VEGF	0.409	0.118	0.571	0.448	0.447	0.17

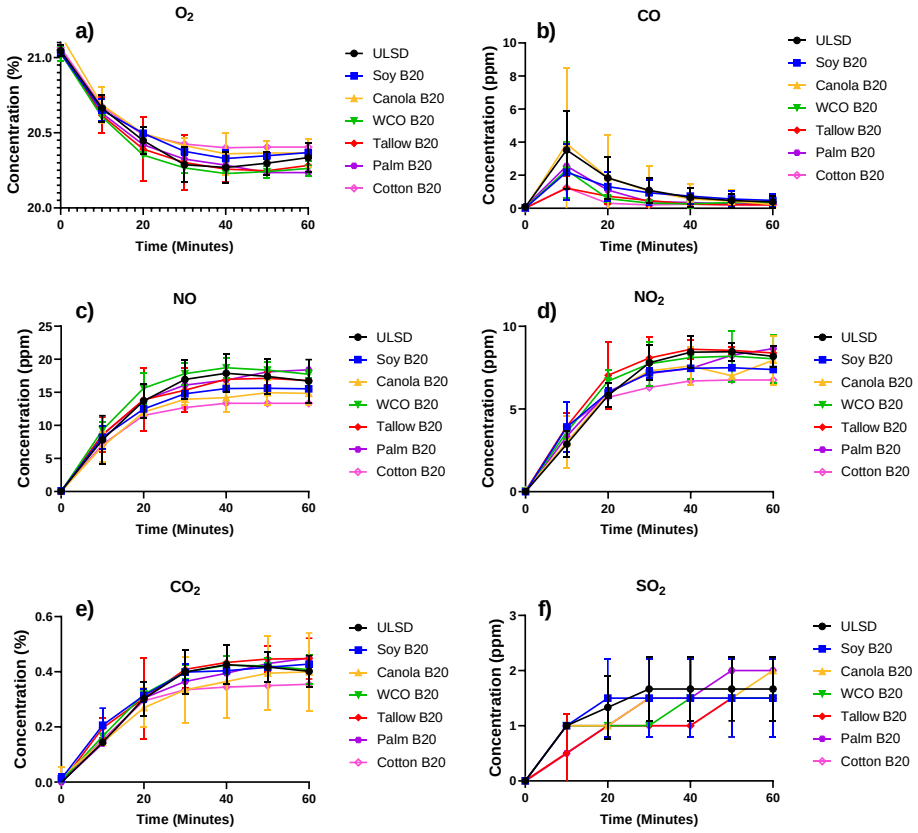


Figure S4.1: Exhaust gas concentrations over time. S1a-f in order: oxygen concentration, carbon monoxide concertation, nitrogen monoxide concentration, nitrogen dioxide concentration, carbon dioxide concentration and sulfur dioxide concentration.

Table S4.1: Mean (standard deviation) mediator release for the 14 cytokines released above the limits of detection. All values have been normalised by subtracting Air controls for each individual participant. Each fuel has been labelled with a corresponding letter to indicate significant differences between biodiesel fuels ($p<0.05$): a=Soy B20, b=Canola B20, c=WCO B20, d=Tallow B20, e=Palm B20 and f=Cottonseed B20.

Mediator Concentration (pg/mL)	Fuel						
	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cotton B20
IL-1β	0.23 (0.65)	0.17 (0.55) b,d,e,f	0.15 (0.34) a	0.09 (0.13)	-0.03 (0.13) a	0.05 (0.17) a	0.10 (0.35) a
IL-1RA	10.09 (42.45)	9.26 (296.96)	-26.56 (87.28) f	102.84 (213.82)	-13.29 (121.24)	35.06 (206.63)	143.00 (223.84) b
IL-6	-70.79 (117.13)	121.35 (238.44) c,e,f	-21.83 (110.25) d	19.84 (147.73) a,d	217.08 (297.55) b,c,e,f	27.43 (269.21) a,d	-0.11 (259.42) a,d

IL-8	255.47 (705.55)	2180.67 (3774.04) b,c,d,e,f	-29.93 (151.52) a,d,e	153.63 (430.81) a,d,e	3003.06 (5481.21) a,b,c,e,f,	920.25 (1307.33) a,b,c,d,f	-4.17 (513.87) a,d,e
IL-9	1.98 (4.01)	4.41 (9.52) b,c,e	1.71 (4.07) a	2.45 (6.42) a,d	3.68 (6.20) c	5.36 (6.58)	2.28 (7.21) a
G-CSF	28.81 (62.43)	59.17 (113.76) b,c,d,e,f	56.19 (69.32) a,d,e,f	34.79 (45.71) a,d	124.63 (196.73) a,b,c,e,f	49.90 (46.75) a,b,d	23.94 (64.45) a,b,d
GM-CSF	8.44 (14.36)	12.65 (19.46) c	7.22 (9.61) d	5.79 (8.22) b,d	19.94 (24.01) b,c,e	19.07 (23.64) d	16.97 (22.59)
IFN-γ	-1.95 (5.56)	1.83 (8.77)	1.25 (4.23) f	8.45 (13.40)	1.92 (7.07) e	6.73 (11.74)	16.05 (14.20) b,d
IP-10	-8.76 (200.80)	143.58 (204.32)	27.21 (157.15)	-22.08 (95.02)	32.37 (112.22)	18.30 (203.81)	68.62 (227.35)
MCP-1	24.88 (59.99)	62.12 (105.44)	3.14 (3.29)	6.66 (12.13)	53.42 (126.85)	57.38 (132.72)	41.73 (108.51)_
MIP-1β	1.23 (1.61)	0.38 (1.36) e	-0.22 (1.15) e	0.23 (2.00)	0.32 (1.05) e	2.42 (1.12) a,b,c,d,f	0.18 (1.72) e
RANTES	-0.30 (1.03)	3.14 (4.4) b,d	-0.07 (0.95) a,e	0.92 (3.23)	0.34 (0.39) a,e	6.34 (5.26) b,d,f	1.17 (0.81) e
TNF-α	5.16 (4.33)	7.08 (7.11)	5.07 (2.78) e,f	8.03 (10.39) f	4.71 (5.73) e,f	12.44 (16.74) b,d	15.64 (11.9) b,c,d
VEGF	93.64 (167.33)	-39.34 (166.74)	-1.80 (127.09) f	16.46 (59.67)	10.05 (237.84)	81.90 (201.20) a	-92.75 (197.12) b,e

a= significantly different to Soy B20.
b= significantly different to Canola B20.
c= significantly different to WCO B20.
d= significantly different to Tallow B20.
e= significantly different to Palm B20.
f= significantly different to Cottonseed B20.

Table S4.2: Significance values (p-values) for the univariate linear regression analysis of biodiesel exhaust gas properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	O_2 (%)	CO (ppm)	CO_2 (%)	NO_x (ppm)	NO (ppm)	NO_2 (ppm)	SO_2 (ppm)
Viability	0.595	0.0509	0.819	0.221	0.243	0.931	0.00246
Necrosis	0.795	0.0862	0.945	0.918	0.826	0.808	0.354
Early Apoptosis	0.417	0.158	0.718	0.466	0.365	0.969	0.906
Late Apoptosis	0.839	0.196	0.607	0.412	0.349	0.697	0.00451

IL-1β	0.557	0.386	0.569	0.736	0.817	0.402	0.220
IL-1RA	0.838	0.1896	0.490	0.480	0.822	0.392	0.404
IL-6	0.466	0.0997	0.0403	0.518	0.814	0.0793	0.0674
IL-8	0.410	0.200	0.0238	0.330	0.637	0.0724	0.253
IL-9	0.456	0.381	0.212	0.486	0.394	0.518	0.915
G-CSF	0.567	0.569	0.129	0.502	0.817	0.132	0.310
GM-CSF	0.967	0.2927	0.743	0.874	0.657	0.765	0.272
INF-γ	0.410	0.0358	0.153	0.0762	0.0272	0.1354	0.0505
IP-10	0.213	0.755	0.975	0.273	0.232	0.419	0.984
MCP-1	0.914	0.403	0.521	0.979	0.818	0.899	0.755
MIP-1β	0.0177	0.639	0.367	0.0244	0.0105	0.286	0.495
RANTES	0.279	0.236	0.114	0.0997	0.225	0.806	0.414
TNF-α	0.749	0.426	0.356	0.703	0.595	0.804	0.383
VEGF	0.106	0.382	0.419	0.0515	0.0725	0.236	0.241

Table S4.3: Significance values (p-values) for the univariate linear regression analysis of biodiesel exhaust particle properties and biological outcomes. Shaded values are less than <0.05 and are considered significant.

	Particle Mass Concentration (µg/m3)	Median Particle Size (nm)	Total Particle Number (particles/cm^3)	Particle Concentration Between 80-100 nm (particles/cm^3)	Particle Concentration Between 20-35 nm (particles/cm^3)	Particle Proportion >23 nm (%)
Viability	0.208	0.248	0.0177	0.713	0.546	0.0587
Necrosis	0.208	0.907	0.053	0.205	0.26	0.786
Early Apoptosis	0.292	0.456	0.0796	0.0777	0.669	0.532
Late Apoptosis	0.796	0.383	0.103	0.874	0.903	0.121
IL-1β	0.719	0.876	0.691	0.813	0.855	0.787
IL-1RA	0.668	0.613	0.316	0.304	0.708	0.955
IL-6	0.0441	0.343	0.149	0.751	0.1593	0.455
IL-8	0.0466	0.450	0.211	0.700	0.139	0.733
IL-9	0.198	0.655	0.631	0.984	0.316	0.769
G-CSF	0.2625	0.295	0.495	0.719	0.238	0.455
GM-CSF	0.713	0.289	0.608	0.120	0.517	0.327
INF-γ	0.783	0.275	0.234	0.0786	0.202	0.941
IP-10	0.419	0.676	0.631	0.442	0.818	0.630
MCP-1	0.413	0.392	0.601	0.177	0.85	0.326
MIP-1β	0.687	0.271	0.400	0.518	0.912	0.225
RANTES	0.710	0.0448	0.0730	0.0341	0.0509	0.208
TNF-α	0.710	0.00871	0.202	0.0375	0.194	0.00356
VEGF	0.451	0.892	0.245	0.582	0.774	0.564

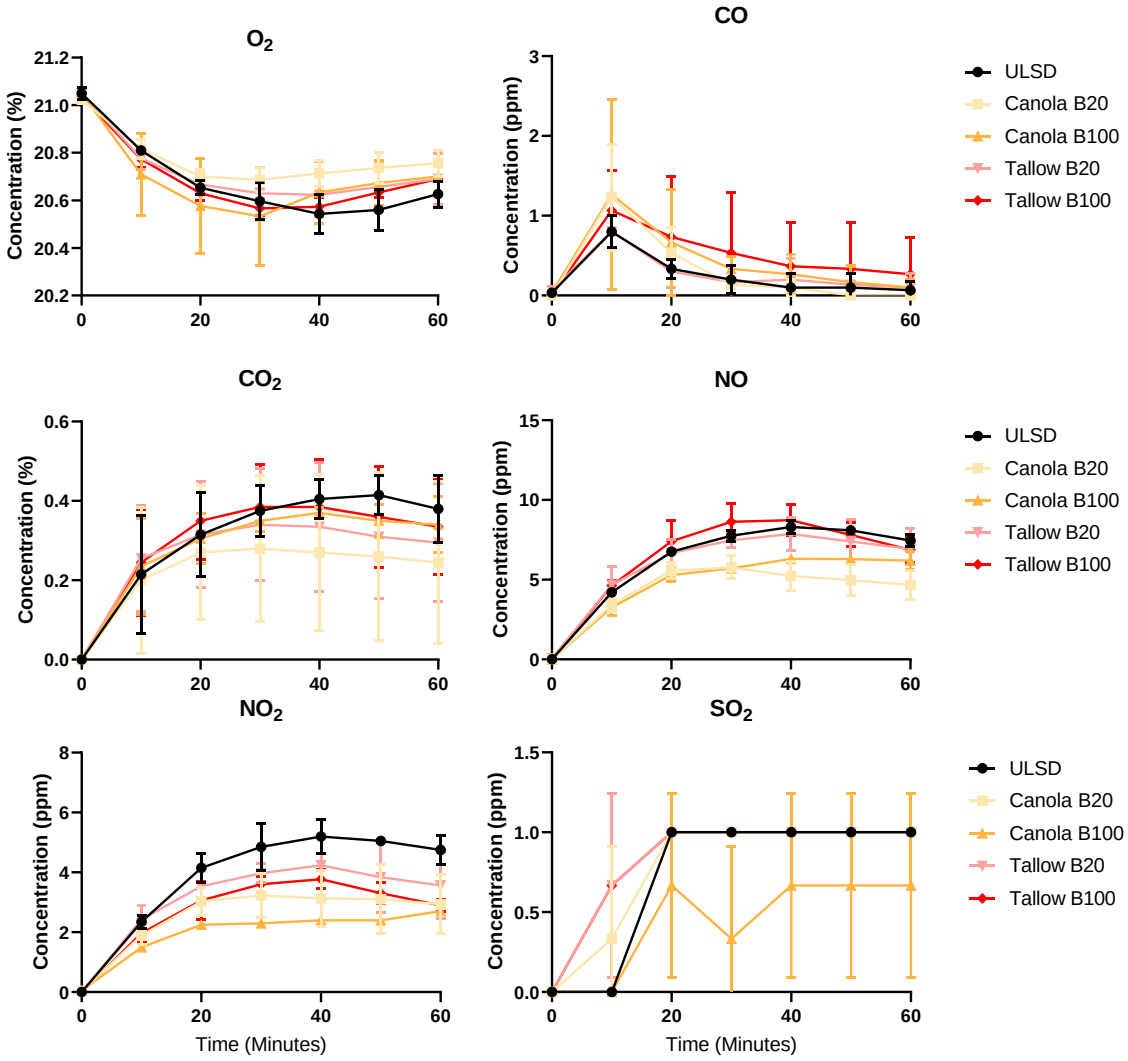


Figure S5.1: Trends in gas concentration over time for all exhausts.

Table S5.1: PAH analysis results for ULSD, Canola B100 and Tallow B100. The majority of PAHs tested were under the limits of detection.

PAH (ng/sample)	Limit of Reporting (ng/sample)	Fuel		
		ULSD	Canola B100	Tallow B100
Naphthalene	0.50	< 0.50	< 0.50	< 0.50
2-methylnapththalene	0.50	< 0.50	< 0.50	< 0.50
1-methylnapththalene	0.50	< 0.50	< 0.50	< 0.50
Biphenyl	0.50	< 0.50	< 0.50	< 0.50
2-ethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50
2,6-dimethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50
2,2'-dimethylbiphenyl	0.50	< 0.50	< 0.50	< 0.50
1,3-dimethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50

1,4-dimethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50
2-methoxynapthalene	0.50	< 0.50	< 0.50	< 0.50
Acenaphthylene	0.50	< 0.50	< 0.50	< 0.50
1,2-dimethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50
1,8-dimethylnaphthalene	0.50	< 0.50	< 0.50	< 0.50
Acenaphthene	0.50	< 0.50	< 0.50	< 0.50
3,3'-dimethylbiphenyl	0.50	< 0.50	< 0.50	< 0.50
Fluorene	0.50	< 0.50	< 0.50	< 0.50
4,4'-dimethylbiphenyl	0.50	< 0.50	< 0.50	< 0.50
1-methylfluorene	0.50	< 0.50	< 0.50	< 0.50
Phenanthrene	0.50	< 0.50	**0.54**	< 0.50
Anthracene	0.50	< 0.50	< 0.50	< 0.50
2-methylanthracene	0.50	< 0.50	< 0.50	< 0.50
9-methylanthracene	0.50	< 0.50	< 0.50	< 0.50
Fluoranthene	0.50	**0.87**	**1.1**	**1.1**
Pyrene	0.50	< 0.50	**0.54**	**0.70**
Chrysene	0.50	< 0.50	< 0.50	**0.59**
Indeno(1,2,3 cd)pyrene	10	< 10	< 10	< 10
Dibenz(a,h)anthracene	10	< 10	< 10	< 10
Benzo(ghi)perylene	10	< 10	< 10	< 10

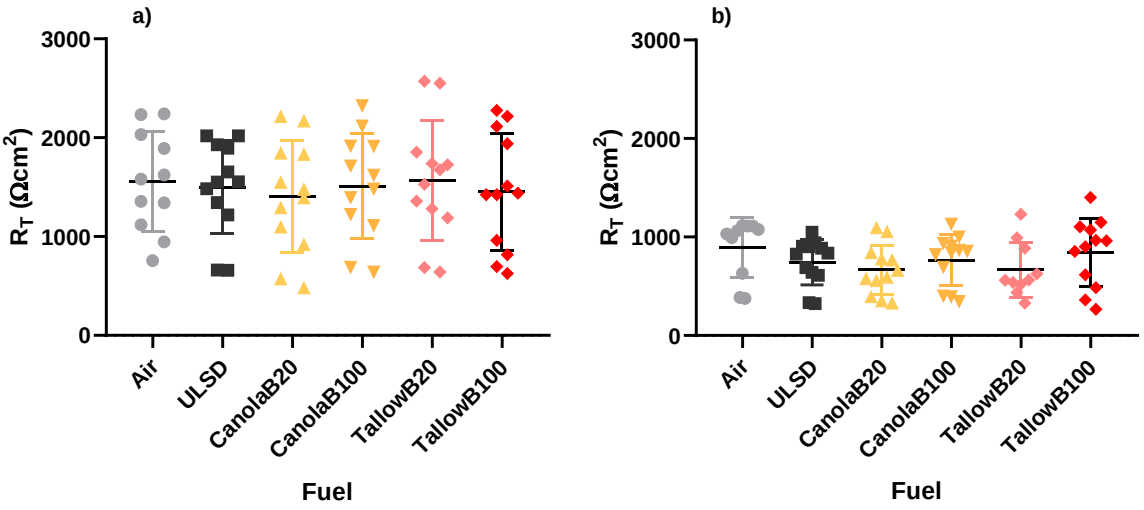


Figure S5.2: Transepithelial resistance for all inserts: a) in UNC-ALI media before exposures started, showing that all inserts are well differentiated and barrier function is active. b) in HBSS-HEPES buffered media after exposure but before permeability, showing that even after exposure barrier function is active.

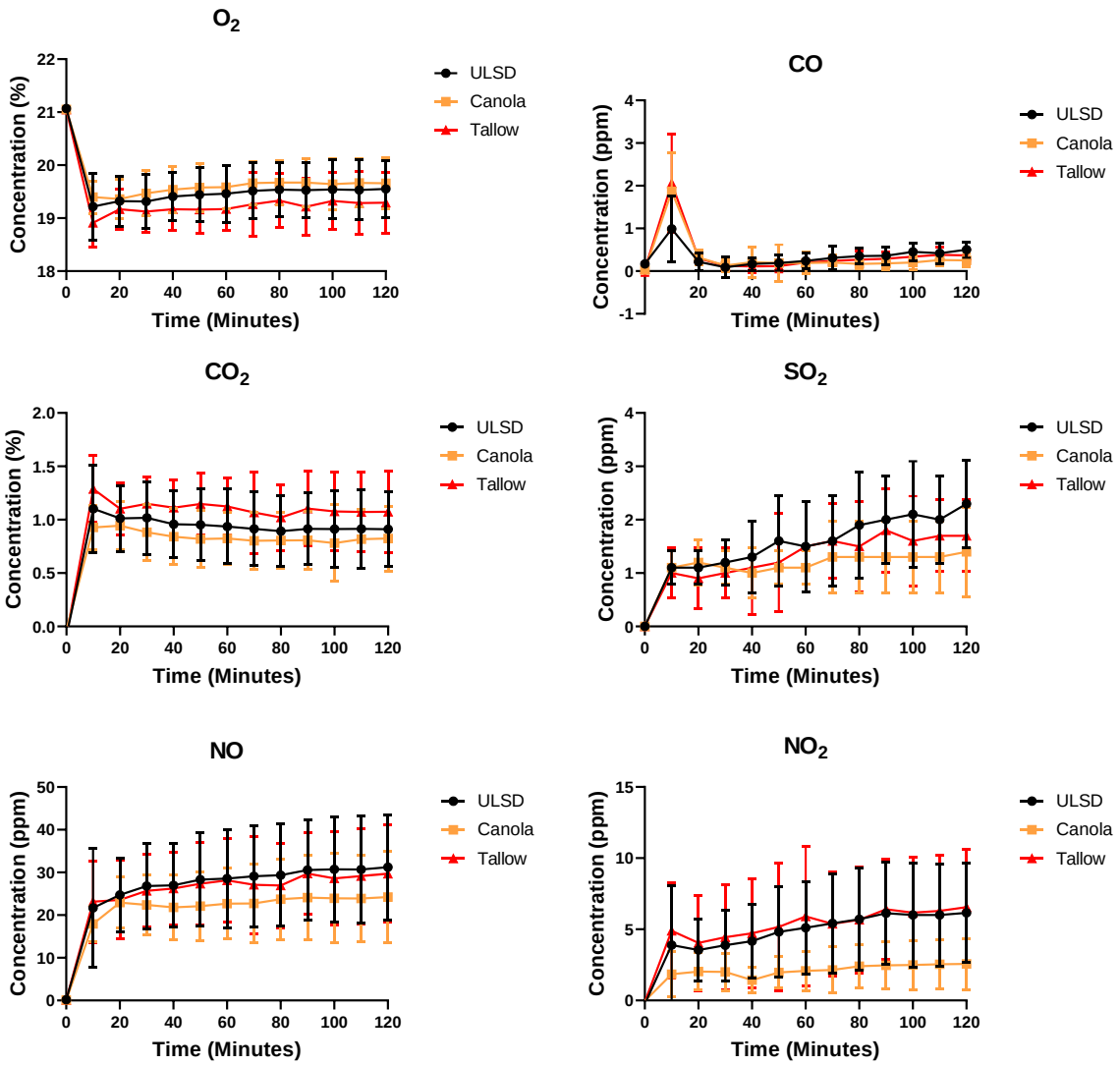


Figure S6.1: Changes in gas concentrations over time.

Table S7.1: Toxic outcomes used for all models. Obtained in Chapter 3 from cells from 8 individual volunteers.

Fuel	ID	Viability	Early Apop-tosis	Late Apop-tosis	Necr-osis	IL1B	IL1RA	IL6	IL8	IL9	G-CSF	GM-CSF	IFNY	IP10	MCP-1	MIP-1B	RANTES	TNF-A	VEGF
Canola	ERP6	89.91	8.09	12.49	2.22	0.08	454.72	16.56	163.72	0.79	2.27	0.14	10.98	648.22	0.16	0.50	0.31	0.80	175.23
Canola	ERP14	105.39	1.88	7.66	4.46	0.08	270.77	355.19	365.40	3.74	1.98	13.45	6.98	91.47	18.87	2.94	0.28	9.48	98.15
Canola	ERP15	111.98	2.68	6.25	0.38	0.08		33.64	168.77	1.77	28.70	0.15	4.02		5.66	0.60	0.74	0.86	139.77
Canola	ERP22	102.96	2.30	8.14	2.66	0.15	169.22	68.10	231.86	0.81	2.34	0.31	6.50	471.92	0.71	0.68	0.32	0.83	158.12
Canola	ERP23	104.85	1.09	7.12	3.18	0.08	155.50	378.74	512.59	5.42	32.88	35.37	1.75	118.44	4.68	4.13	0.31	33.20	257.13
Canola	ERP28	103.04	0.79	6.11	1.50	0.09	159.10	22.34	317.93	5.01	1.95	1.20	5.60	339.01	0.16	3.24	2.45	10.74	410.08
Canola	ERP38	103.71	0.70	5.85	2.96	0.08	91.73	39.57	419.78	5.49	2.04	10.95	1.61	195.30	1.96	3.81	0.29	11.59	444.54
Canola	ERP40	103.56	2.40	8.49	1.91	0.08	134.60	284.51	726.72	3.30	41.09	1.50	6.34	851.77	10.97	1.23	3.79	1.49	283.36
Cottonseed	ERP6	102.02	3.38	6.67	2.36	0.45	517.77	263.72	307.71	3.88	19.11	6.42	30.50	385.06	0.25	1.87	1.08	21.84	103.66
Cottonseed	ERP14	94.85	3.80	13.84	4.95	0.10	331.48	425.00	2414.25	22.71	52.91	57.83	42.53	122.84	339.58	8.80	3.87	1.11	250.55
Cottonseed	ERP22	103.79	1.91	6.96	3.54	0.68	259.77	154.59	222.33	3.34	13.03	6.00	18.18	241.73	0.65	1.41	0.41	28.44	130.14
Cottonseed	ERP23	105.09	3.66	6.47	1.08	0.10	347.10	72.51	134.21	1.59	17.22	1.71	21.71	474.71	0.16	0.90	1.53	16.48	33.57
Cottonseed	ERP28	103.04	1.30	5.03	2.06	0.09	144.82	233.59	742.86	9.55	101.47	9.48	11.56	169.32	5.47	3.07	1.41	13.47	74.87
Cottonseed	ERP38	102.90	2.65	6.58	0.97	0.13	216.13	41.06	200.55	1.97	13.48	5.02	17.22	411.65	0.50	1.31	0.40	16.73	124.19
Cottonseed	ERP40	107.01	1.65	5.09	3.16	0.10	293.28	365.19	530.08	7.66	51.66	15.28	19.74	375.08	4.47	2.18	2.20	25.08	162.71
Palm	ERP6	85.06	2.26	15.32	9.50	0.35	266.10	105.80	343.22	4.91	52.16	18.20	16.63	54.78	0.20	2.27	0.37	6.16	247.27
Palm	ERP14	101.72	0.44	15.46	6.06	0.08	219.86	503.01	4451.02	25.80	67.19	23.99	31.00	110.44	309.12	8.66	13.49	2.80	83.69
Palm	ERP22	94.24	1.55	13.61	4.64	0.08	211.14	150.70	318.83	4.70	111.66	32.47	12.37	43.61	0.15	1.97	0.29	33.53	363.18
Palm	ERP23	105.68	1.45	4.51	4.73	0.38	181.15	577.38	3906.79	31.81	296.65	35.72	12.31	515.60	29.21	11.10	16.69	18.04	150.57
Palm	ERP28	101.12	2.72	4.87	2.50	0.40	204.21	13.82	438.18	9.32	15.43	0.17	11.22	503.40	0.21	4.11	6.42	7.45	550.22
Palm	ERP38	105.31	1.66	5.15	1.29	0.09	100.04	3.55	309.19	5.06	11.87	0.75	7.17	519.45	1.39	3.54	6.45	4.66	714.20
Soy	ERP6	102.14	2.32	8.84	1.14	0.20	45.27	28.93	89.14	0.54	1.56	0.39	0.56	246.81	0.11	0.25	0.21	3.38	190.64
Soy	ERP14	98.04	2.27	10.71	7.01	0.05	277.25	961.25	4149.35	28.80	59.30	57.89	32.35	101.71	215.58	10.75	15.59	5.67	405.84
Soy	ERP15	100.12	3.29	14.50	1.11	0.07		104.11	950.55	3.37	16.79	0.96	5.39		221.89	1.26	0.43	0.56	136.43
Soy	ERP22	104.98	1.88	8.21	1.31	0.69	56.74	31.63	196.98	1.45	5.38	0.26	2.57	406.08	0.14	0.46	0.28	6.91	169.21
Soy	ERP23	106.39	1.14	5.48	3.50	0.11	122.86	743.64	4284.50	34.25	179.38	36.06	13.57	99.33	26.20	12.17	5.20	19.26	219.69
Soy	ERP28	95.05	2.57	12.49	0.44	0.14	107.25	62.53	183.39	4.58	20.53	2.47	5.52	435.86	0.21	2.94	3.85	19.10	354.94

Soy	ERP38	103.71	2.27	6.81	0.42	0.10	106.68	37.29	146.69	1.38	14.97	6.45	7.98	115.77	0.23	2.36	0.39	17.05	434.97
Soy	ERP40	86.10	5.14	20.19	2.17	0.11	199.14	413.22	985.46	3.85	79.81	5.74	5.48	530.57	11.21	1.25	1.85	12.66	141.10
Tallow	ERP6	85.95	4.22	19.84	2.15	0.11	307.73	150.76	74.44	1.09	3.14	0.20	7.66	213.75	0.22	0.11	0.43	3.56	27.68
Tallow	ERP14	95.22	2.70	11.02	8.56	0.10	435.35	1636.29	7864.34	48.73	142.55	92.73	66.91	157.85	511.78	15.67	10.92	11.33	257.39
Tallow	ERP15	91.11	3.28	22.67	0.25	0.08		37.45	175.53	1.02	2.94	0.19	1.55		2.67	0.31	0.40	1.04	1.62
Tallow	ERP22	91.71	3.15	16.95	2.50	0.08	186.70	46.85	159.57	0.84	2.42	0.15	4.12	204.85	0.17	0.35	0.33	4.85	78.72
Tallow	ERP23	104.62	0.93	5.33	5.35	0.14	144.90	894.41	6829.69	33.20	271.01	61.62	11.03	129.11	34.98	11.80	1.86	23.54	176.58
Tallow	ERP28	100.34	1.99	7.28	1.54	0.11	138.03	57.61	303.47	6.09	24.57	4.51	10.79	593.45	0.52	3.69	5.75	18.03	426.17
Tallow	ERP38	103.36	1.56	7.12	1.11	0.09	85.84	41.82	278.68	5.44	7.77	9.44	4.82	222.24	1.48	3.24	0.28	17.18	460.19
Tallow	ERP40	96.91	2.24	13.02	3.13	0.08	106.27	647.23	1071.62	4.12	80.73	15.73	5.11	169.93	4.67	1.87	0.33	2.47	451.40
WCO	ERP6	97.59	5.68	9.69	0.84	0.09	127.39	22.75	57.13	0.88	2.54	0.16	1.38	230.14	0.18	0.26	0.35	3.38	211.76
WCO	ERP14	94.98	4.02	10.81	7.66	0.07	371.43	686.43	1756.98	19.96	80.19	30.34	22.95	125.59	24.62	7.77	2.16	12.61	125.50
WCO	ERP15	91.98	5.56	18.59	1.36	0.07		141.11	2041.58	5.50	25.57	0.80	2.11		174.10	2.01	1.35	0.74	93.82
WCO	ERP22	97.75	3.16	12.57	1.77	0.08	102.04	35.20	148.65	0.76	2.17	0.14	1.08	465.23	0.47	0.40	0.30	7.39	164.42
WCO	ERP23	100.12	1.87	7.59	5.92	0.36	269.54	423.27	1632.03	19.80	262.56	48.06	14.28	146.38	11.36	8.33	0.44	23.15	661.40
WCO	ERP28	104.72	1.40	3.37	2.12	2.08	153.52	22.62	222.96	1.65	33.98	8.07	8.74	140.57	0.28	2.57	0.47	6.18	361.65
WCO	ERP38	98.89	1.94	7.15	4.60	0.14	136.30	26.76	521.41	13.58	12.13	1.92	10.50	86.88	5.13	5.22	1.13	9.78	899.86
WCO	ERP40	95.96	6.91	11.25	1.04	0.13	208.05	233.10	290.94	1.72	2.29	0.77	4.42	252.12	1.15	0.65	0.31	7.80	143.65
ULSD	ERP6	104.23	3.48	6.21	0.81	0.08	265.05	15.83	228.04	1.79	2.46	0.16	8.50	433.26	0.17	0.71	0.34	0.87	166.99
ULSD	ERP14	99.02	3.53	11.72	3.94	0.12	559.24	481.44	736.32	7.92	16.64	12.50	21.54	176.76	19.05	5.05	0.35	7.89	77.41
ULSD	ERP15	96.91	4.83	15.89	0.79	0.09		42.43	182.15	1.24	3.56	0.23	0.87		5.72	0.45	0.49	1.26	54.61
ULSD	ERP22	102.84	2.99	7.85	2.35	0.46	149.43	148.36	471.32	3.68	19.68	3.05	4.35	235.56	1.18	1.12	0.36	9.70	835.89
ULSD	ERP23	104.38	2.39	8.12	1.29	0.10	200.89	199.74	558.98	9.48	28.37	8.79	7.10	166.30	7.57	4.83	0.33	11.98	235.82
ULSD	ERP28	103.37	1.91	5.49	0.70	0.49	202.16	26.44	129.50	1.41	13.91	1.13	13.37	259.40	0.24	2.30	0.40	14.61	260.67
ULSD	ERP38	103.48	1.90	6.53	1.27	0.12	209.53	19.69	148.11	1.03	2.07	2.27	11.45	146.11	1.47	2.01	0.29	8.80	234.78
ULSD	ERP40	100.36	2.42	7.35	5.74	0.09	221.03	547.63	1886.11	7.21	167.46	4.78	13.53	907.59	30.63	2.92	5.83	2.70	411.95

Table S7.2: Exhaust gas and particle characteristics obtained in Chapter 3 for all the fuels used in the redundancy analysis.

Fuel	O_2 (%)	CO (ppm)	CO_2 (%)	NO_x (ppm)	NO (ppm)	NO_2 (ppm)	SO_2 (ppm)	Particle Mass ($\mu g/m^3$)	Particle Size (PS) (nm)	Particle Number (PN) (n/m^3)	Particle Number 20-35 nm (PN30) (n/m^3)	Particle Number 80-100 nm (PN100) (n/m^3)	Particle Number <23 nm (PN23) (n/m^3)
Canola	20.37	3.42	0.32	21.43	13.65	7.99	1.3	28.53	26	120184	37167	5815	52437
Cottonseed	20.36	1.05	0.36	20.25	13.63	6.63	1.8	11.69	38	43797	12082	3295	11902
Palm	20.34	0.85	0.37	19.75	13.75	6.08	1.5	24.47	18	49111	8642	2290	28880
Soy	20.39	2.07	3.42	3.77	1.87	0.85	1.05	22.4	35	99040	33695	4593	48012
Tallow	20.3	1.87	0.42	20.97	14.07	6.67	1	10.69	26	58586	16789	3817	26096
WCO	20.38	3.53	0.34	21.94	15.07	6.69	1.5	12.85	44	25993	3187	3453	9093
ULSD	20.31	3.77	0.38	22.4	16.19	6.21	1	19.82	26	113176	38129	8330	47462

Table S7.3: FAME profiles for all the biodiesel fuels, used in all FAME models.

Fuel	C:14 (%)	C16:1 (%)	C16:0 (%)	C18:2 (%)	C18:1 (%)	C18:0 (%)
Canola	0	0	2.29	13	83.43	1.29
Cottonseed	0	0	21.99	42.91	31.73	3.37
Palm	0.35	0.03	46.33	6.33	42.88	4.08
Soy	0	0	9.86	51.4	33.9	4.83
Tallow	1.03	0.79	24.54	1.7	53.75	18.19
WCO	0	0	6.15	11.27	80.88	1.7

St John of God Human Ethics Confirmation

9 December 2015

Clinical Prof. Francis Lannigan
School of Surgery, M704,
University of Western Australia
35 Stirling Highway
CRAWLEY WA 6009

Dear Professor Lannigan,

Re: WA Epithelial Research Program for Childhood Respiratory Diseases
(Our ref No: 901)

Thank you for your reply of the 4 December 2015, addressing the queries raised by the Scientific Review Sub-Committee (SRC). The SRC has reviewed your reply out of session and your proposed research program was tabled at the recent St John of God Health Care (SJGHC) Human Research Ethics Committee (HREC) meeting on 9 December 2015.

I am pleased to advise that your research program has been granted ethical approval as satisfying the ethical requirements set out in the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research (NHMRC, 2007) ("the National Statement"), in particular Section 3.2 "Databanks" and Section 3.4.3 "Prospective collection of human biospecimens for research." The Committee notes that for all future individual research studies conducted within this research program, these will undergo separate HREC review principally to ensure that the studies are a satisfactory use of the biospecimens collected as part of the research program.

This ethical approval is inclusive of the following research program documentation:

1. Original Study Protocol submitted to the SJGHC HREC on 30 October 2015
2. ERP Kids Information Brochure Version 1.1 St John of God Hospital dated October 2015.
3. The three Participant Consent Forms (ie Medical Research Consent on behalf of Child, Medical Research Assent, and Medical Research Consent) - recorded on the Surface Pro as part of the paper light system of recruiting.
4. WA Epithelial Research Program For Childhood Lung Disease Version 1.1 St John of God Subiaco Hospital dated October 2015.
5. EPR Audit Sheet Version 1.1 St John of God Hospital dated October 2015.

This study approval is granted for a time frame from the date of this approval letter to 22 January 2028. Should an extension of this timeframe be required, then you must seek continued approval from the Committee *before* the expiry
of this time period. …/2 You are reminded that this letter constitutes ***ethical approval only***. You must not commence this project at SJGHC until separate authorisation from SJGHC has been obtained.

The Committee is a HREC that is constituted and operates in accordance with the National Statement. In line with the National Statement requirements, researchers need to keep the Committee and the institution (specifically, St John of God Subiaco Hospital) promptly and regularly informed on the progress of their approved research including:

1. any adverse events or unexpected outcomes that may affect continued ethical approval of the research program.
2. any proposed changes in the research protocol.
3. when the research program is completed or abandoned.

The Committee would also appreciate receiving *at a minimum* an *annual* progress report, as well as a final report on the research program results and/or any subsequent publications.

I wish you well with your research program.

Yours sincerely

Clinical Professor Dr Simon Dimmitt
Chairman
St John of God Health Care Human Research Ethics Committee

Enc.

cc. Ms Liz Starcevich, Telethon Kids Institute (via email) Dr Anthony Kicic, Telethon Kids Institute (via email)
Dr Luke Garratt, Telethon Kids Institute (via email)
Adjunct A/Professor Nik Zeps, Research Director, SJG Subiaco Hospital (via email)

ST JOHN OF GOD HEALTH CARE HUMAN RESEARCH ETHICS COMMITTEE MEMBERSHIP

NAME	CORE MEMBER	SEX	APPOINTMENT	POSITION
Clinical Professor Simon Dimmitt	Core	M	Chair (with suitable experience whose other responsibilities will not impair the HREC's capacity to carry out its obligations under the National Statement).	Consultant Physician, General & Cardiovascular Medicine (accredited to St John of God Health Care)
Ms Tracey Piani	Core	F	Member with knowledge of and current experience in the professional care, counselling or treatment of humans (ie medical practitioner, clinical psychologist, social worker, nurse as appropriate)	Deputy Director of Nursing, St John of God Midland Public & Private Hospitals
Fr Joe Parkinson	Core	M	Member who performs a pastoral care role in a community for example an Aboriginal Elder, a minister of religion	Minister of Religion; Bioethicist, Director L. J. Goody Bioethics Centre
Mr Eric Heenan	Core	M	Member who is a lawyer, and where possible who is not engaged to advise the institution	Retired Supreme Court Judge, WA

Dr Janie Brown	Core	F	Member with current research experience that is relevant to research proposals to be considered at the meetings.	Nursing and Midwifery Research Coordinator, St John of God Subiaco Hospital
Sr Leonie O'Brien	Core	F	Laywoman who has no affiliation with the institution and does not currently engage in medical, scientific, legal or academic work.	Mercy Sister
Professor Sally Sandover	Core	F	Member with current research experience that is relevant to research proposals to be considered at the meetings.	Academic Co-ordinator Carrick Support Initiative, UWA Co-ordinator Regional Programs & PBL Consultant, University of WA
Mr Hamish Milne	Core	M	Layman who has no affiliation with the institution and does not currently engage in medical, scientific, legal or academic work	Self-employed Consultant
Mr Patrick O'Connor		M	Community member with expert knowledge in clinical psychology	Senior Clinical Psychologist, Health Dept WA (mental health services) *and* Clinical Psychologist, Hillarys Medical Centre
Mr Jeffrey Williams		M	Hospital Representative. Expert knowledge in Quality and Risk Management, public hospital management.	Director of Nursing, St John of God Midland Public & Private Hospitals
Mr Colin Keogh		M	Hospital Representative. Expert knowledge in Mission and culture.	Director of Mission, St John of God Murdoch Hospital
Ms Mary Rigby		F	Hospital Representative. Expert knowledge in nursing, particularly in palliative care & oncology.	Ward Nurse Manager, St John of God Subiaco Hospital

The St John of God Health Care Human Research Ethics Committee is a Human Research Ethics Committee that is constituted and operates in accordance with the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research (2007).

Date of Ethics Committee Meeting: _9 December 2015_Chairman's Signature:

Office of Research and Development

GPO Box U1987
Perth Western Australia 6845

Telephone +61 8 9266 7863
Facsimile +61 8 9266 3793
Web research.curtin.edu.au

09-May-2018

Name: Alexander Larcombe
Department/School: School of Public Health
Email: Alexander.Larcombe@curtin.edu.au

Dear Alexander Larcombe

RE: Reciprocal ethics approval
Approval number: HRE2018-0227

Thank you for your application submitted to the Human Research Ethics Office for the project Development of a screening tool to identify safer biodiesels.

Your application has been approved by the Curtin University Human Research Ethics Committee (HREC) through a reciprocal approval process with the lead HREC.

The lead HREC for this project has been identified as St John of God Health Care Human Research Ethics Committee.

Approval number from the lead HREC is noted as 901.

The Curtin University Human Research Ethics Office approval number for this project is **HRE2018-0227**. Please use this number in all correspondence with the Curtin University Ethics Office regarding this project.

Approval is granted for a period of one year from **09-May-2018** to **08-May-2019**. Continuation of approval will be granted on an annual basis following submission of an annual report.

Personnel authorised to work on this project:

Name	Role
Larcombe, Alexander	CI
Kicic, Anthony	Co-Inv
Mullins, Ben	Co-Inv
Landwehr, Katherine	Student

You must comply with the lead HREC's reporting requirements and conditions of approval. You must also:

- Keep the Curtin University Ethics Office informed of submissions to the lead HREC, and of the review outcomes for those submissions

Conduct your research according to the approved proposal

- Report to the lead HREC anything that might warrant review of the ethics approval for the project
- Submit an annual progress report to the Curtin University Ethics Office on or before the anniversary of approval, and a completion report on completion of the project. These can be the same reports submitted to the lead HREC.
- Personnel working on this project must be adequately qualified by education, training and experience for their role, or supervised
- Personnel must disclose any actual or potential conflicts of interest, including any financial or other interest or affiliation, that bears on this project
- Data and primary materials must be managed in accordance with the Western Australian University Sector Disposal Authority (WAUSDA) and the Curtin University Research Data and Primary Materials policy

Where practicable, results of the research should be made available to the research participants in a timely and clear manner The Curtin University Ethics Office may conduct audits on a portion of approved projects.

This letter constitutes ethical approval only. This project may not proceed until you have met all of the Curtin University research governance requirements.

Should you have any queries regarding consideration of your project, please contact the Ethics Support Officer for your faculty or the Ethics Office at hrec@curtin.edu.au or on 9266 2784.

Yours sincerely

Amy Bowater
Acting Manager, Research Integrity

Research Office at Curtin

GPO Box U1987
Perth Western Australia 6845

Telephone +61 8 9266 7863
Facsimile +61 8 9266 3793
Web research.curtin.edu.au

07-Aug-2020

Name: Alexander Larcombe
Department/School: School of Public Health
Email: Alexander.Larcombe@curtin.edu.au

Dear Alexander Larcombe

RE: Animal ethics approval Approval number: ARE2020-16

Thank you for submitting your application to the Animal Ethics Office for the project **Development of a screening tool to identify safer biodiesels in vivo study.**.

Your application was reviewed by the Curtin University Animal Ethics Committee at their meeting on **23-Jul-2020**.

The review outcome is: **Approved**.

Approval is granted for a period of one year from **07-Aug-2020** to **06-Aug-2021**. Continuation of approval will be granted on an annual basis following submission of an annual report.

Personnel authorised to work on this project:

Name	Role
Larcombe, Alexander	CI
Landwehr, Katherine	Student

Approved species:

Common name	Species	Total number approved
Mouse	Mouse	

Standard conditions of approval

- An Annual Progress Report must be submitted to the Ethics Office annually, on the anniversary of approval.
- An Annual Animal Use Report that captures the relevant details regarding the number of animals used in the preceding year i.e. 1 January to 31 December must be submitted before 31 January of the following year.
- Any amendments to the approved protocol must be submitted to the Ethics Office.
- A Completion Report must be submitted to the Ethics Office on completion of the project.
- Should any animal(s) experience an adverse or unexpected outcome resulting from the experimentation, the AEC is to be notified in writing immediately.
- Please ensure that you quote the Animal Ethics Committee approval number whenever you order animals for this project.
- Note also that an AEC approval number must be displayed on the cage(s)/aquaria etc used to house/maintain animals during an approved activity.

If the results of this research will be published, citations should state: "All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes".

Special Conditions of Approval

conditions of approval:

1. Building 205 will need to be inspected and approved by the Animal Welfare Officers prior to work commencing on this project.

2. Please organise for the Animal Welfare Officers to be present for the first inhaling procedure to monitor for distress in the animals. If possible,please provide a video to the AEC of this procedure taking place within 4 weeks of commencing.

Special Condition of Approval:
It is the responsibility of the Chief Investigator to ensure that any activity undertaken under this project adheres to the latest available advice from the Government or the University regarding COVID-19.

This letter constitutes ethical approval only. This project may not proceed until you have met all of the Curtin University research governance requirements.

Should you have any queries regarding consideration of your project, please contact the Ethics Officer at aec@curtin.edu.au or on 9266 2784.

Yours sincerely

Mr Peter Metcalfe
Chair, Animal Ethics Committee

Chapter 1 Permission:

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS
Sep 03, 2021

This Agreement between Ms. Katherine Landwehr ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	5141420990951
License date	Sep 03, 2021
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Exposure and Health
Licensed Content Title	Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants?
Licensed Content Author	Katherine R. Landwehr et al
Licensed Content Date	Nov 18, 2020
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	full article/chapter
Will you be translating?	no
Circulation/distribution	30 - 99
Author of this Springer Nature content	yes
Title	Developing a Screening Tool to identify Safer Biodiesels
Institution name	Curtin University
Expected presentation date	Sep 2021
Order reference number	0000001
Requestor Location	Ms. Katherine Landwehr Northern Entrance, Perth Children's Hosp Perth, Nedlands, Western Australia 6009 Australia Attn: Ms. Katherine Landwehr
Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking

'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. **Grant of License**

    1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

    2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

    3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. **Scope of Licence**

    1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

    2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

    3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

    4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

    5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

- **Duration of Licence**

    1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

- **Acknowledgement**

1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

- **Restrictions on use**

1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

2. You must not use any Licensed Material as part of any design or trademark.

3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

- **Ownership of Rights**

1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

- **Warranty**

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- **Limitations**

1. ***BOOKS ONLY:***Where '**reuse in a dissertation/thesis**' has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

2. For content reuse requests that qualify for permission under the STM Permissions Guidelines, which may be updated from time to time, the STM Permissions Guidelines supersede the terms and conditions contained in this licence.

- **Termination and Cancellation**

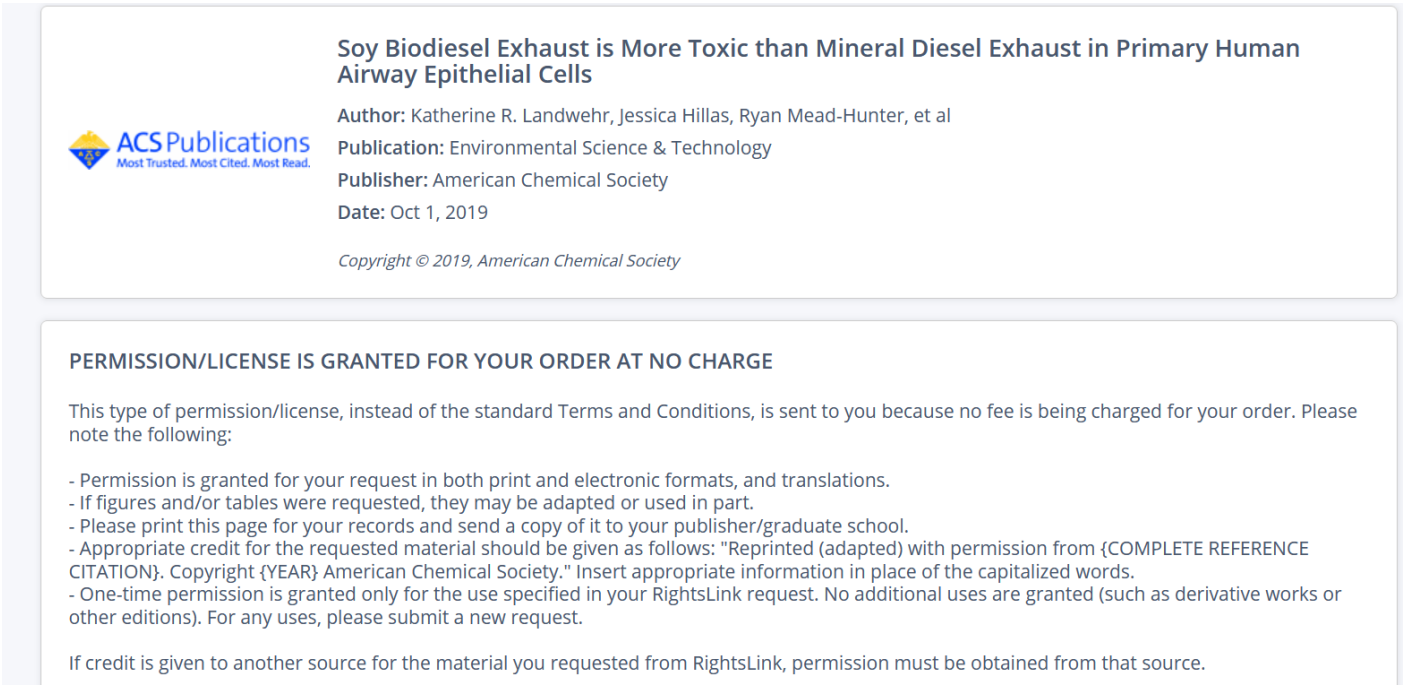
1. Licences will expire after the period shown in Clause 3 (above).

2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)
For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)
For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)
Note: For any republication from the British Journal of Cancer, the following credit line style applies:
Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT]** (year of publication)
For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])
For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Chapter 2 Permission:



ACS Publications
Most Trusted. Most Cited. Most Read.

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

Author: Katherine R. Landwehr, Jessica Hillas, Ryan Mead-Hunter, et al
Publication: Environmental Science & Technology
Publisher: American Chemical Society
Date: Oct 1, 2019

Copyright © 2019, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms and Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from {COMPLETE REFERENCE CITATION}. Copyright {YEAR} American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your RightsLink request. No additional uses are granted (such as derivative works or other editions). For any uses, please submit a new request.

If credit is given to another source for the material you requested from RightsLink, permission must be obtained from that source.

Chapters 3 Permissions:

Author rights

The below table explains the rights that authors have when they publish with Elsevier, for authors who choose to publish either open access or subscription. These apply to the corresponding author and all co-authors.

Author rights in Elsevier's proprietary journals	Published open access	Published subscription
Retain patent and trademark rights	√	√
Retain the rights to use their research data freely without any restriction	√	√
Receive proper attribution and credit for their published work	√	√
Re-use their own material in new works without permission or payment (with full acknowledgement of the original article): 1. Extend an article to book length 2. Include an article in a subsequent compilation of their own work 3. Re-use portions, excerpts, and their own figures or tables in other works.	√	√
Use and share their works for scholarly purposes (with full acknowledgement of the original article): 1. In their own classroom teaching. Electronic and physical distribution of copies is permitted 2. If an author is speaking at a conference, they can present the article and distribute copies to the attendees 3. Distribute the article, including by email, to their students and to research colleagues who they know for their personal use 4. Share and publicize the article via Share Links, which offers 50 days' free access for anyone, without signup or registration 5. Include in a thesis or dissertation (provided this is not published commercially) 6. Share copies of their article privately as part of an invitation-only work group on commercial sites with which the publisher has a hosting agreement	√	√
Publicly share the preprint on any website or repository at any time.	√	√