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# The respiratory health effects of acute *in vivo* diesel and biodiesel exhaust in a mouse model

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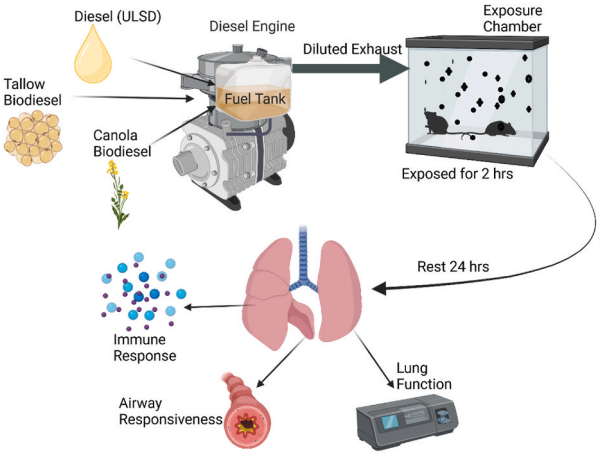
## HIGHLIGHTS

- Exhaust characteristics changed significantly between different biodiesel feedstock types.
- Different exposure types caused different biological effects.
- A single exposure to both ULSD and Tallow biodiesel exhaust induced airway hyperreactivity.
- Indications of immune dysregulation were observed after a single exposure to Tallow biodiesel exhaust.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* Biodiesel, a renewable diesel fuel that can be created from almost any natural fat or oil, is promoted as a greener and healthier alternative to commercial mineral diesel without the supporting experimental data to back these claims. The aim of this research was to assess the health effects of acute exposure to two types of biodiesel exhaust, or mineral diesel exhaust or air as a control in mice. Male BALB/c mice were exposed for 2-hrs to diluted exhaust obtained from a diesel engine running on mineral diesel, Tallow biodiesel or Canola biodiesel. A room air exposure group was used as a control. Twenty-four hours after exposure, a variety of respiratory related end point measurements were assessed, including lung function, responsiveness to methacholine and airway and systemic immune responses.

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*Results:* Tallow biodiesel exhaust exposure resulted in the greatest number of significant effects compared to Air controls, including increased airway hyperresponsiveness (178.1 ± 31.3% increase from saline for Tallow biodiesel exhaust exposed mice compared to 155.8 ± 19.1 for Air control), increased airway inflammation (63463 ± 13497 cells/mL in the bronchoalveolar lavage of Tallow biodiesel exhaust exposed mice compared to 40561 ± 11800 for Air exposed controls) and indications of immune dysregulation. In contrast, exposure to Canola biodiesel exhaust resulted in fewer significant effects compared to Air controls with a slight increase in airway resistance at functional residual capacity and indications of immune dysregulation. Exposure to mineral diesel exhaust resulted in significant effects between that of the two biodiesels with increased airway hyperresponsiveness and indications of immune dysregulation.
*Conclusion:* These data show that a single, brief exposure to biodiesel exhaust can result in negative health impacts in a mouse model, and that the biological effects of exposure change depending on the feedstock used to make the biodiesel.

## 1. Introduction

Despite the rapid global increase in biodiesel usage (over 50 fold increase since 2000 (EIA, 2021)), the potential health effects of biodiesel exhaust exposure have so far been under examined (Larcombe et al., 2015; Møller et al., 2020). As both mineral diesel and biodiesel exhaust share many of the same physico-chemical characteristics (Fontaras et al., 2009; Graver et al., 2016), the health effects of breathing biodiesel exhaust are theorised to be similar with likely toxic impacts to the cardiovascular, nervous and respiratory systems (Lloyd and Cackette, 2001; Silverman et al., 2012; Andersen et al., 2018; Unosson et al., 2021). Biodiesel can be made from many different types of natural fats and oils (Møller et al., 2020; Landwehr et al., 2021b), with the use of different base oils changing both the fuel properties and the physico-chemical characteristics the exhaust produced (Graboski et al., 2003; Landwehr et al., 2021b; Gülüm, 2023). Previous studies into engine and exhaust characteristics found that, compared to mineral diesel, many types of biodiesel contain greater concentrations of nitrogen oxides ($NO_x$), polycyclic aromantic hydrocarbons (PAH) and smaller median particle sizes resulting in a greater number of ultrafine particles, although less overall particulate matter by mass (Fontaras et al., 2009; Westphal et al., 2013; Gioda et al., 2016; Graver et al., 2016; Mullins et al., 2016; Gülüm, 2022). This is of concern as $NO_x$ and PAHs are linked to toxic health outcomes (Chen et al., 2007; Zhang et al., 2020), and ultrafine particles are linked more often to the negative outcomes of air pollution compared with larger sized particles (Oberdörster et al., 1995; Breitner et al., 2011). The exact physico-chemical differences between the exhaust from the biodiesel of choice and reference mineral diesel depends mainly on what that biodiesel is made from (Graboski et al., 2003; Landwehr et al., 2021b, 2023a), and it is thus expected that health effects of exhaust exposure will change as well.

Despite this, different biodiesel types are often treated as interchangeable in exhaust toxicology literature, with broad overarching statements about its toxicity being made based on the study of only one type of exhaust (Hawley et al., 2014). Attempts to compare toxic effects between different studies using different biodiesel types (Swanson et al., 2007; Madden et al., 2011; Larcombe et al., 2015; Møller et al., 2020) are hampered by the different methodologies used for exposure, different health outcomes measured, different engine types and after-treatment technology and different percentages of biodiesel blended into the fuel of interest. Until methodology becomes more consistent, directly comparing health effects between different biodiesels within a single study where these variables are controlled for is the only way to accurately compare toxicity (Hemmingsen et al., 2011; Landwehr et al., 2021b, 2022a), although it does increase the body of work that needs to be performed substantially.

One of the most accepted and widely used methods for assessing the impacts of inhaled toxicants is *in vivo* animal exposure models (Gad and Gad, 2016). This is due to the advantage of having an intact biological system which allows for more in-depth understanding of how different exposures impact both the lungs and other body systems (Boverhof et al., 2011; Heidari Nejad et al., 2015). For exhaust toxicology experiments, exposure methodology is usually done in one of two ways: nasal/-tracheal instillation of diesel exhaust particles suspended in solution, or inhalation studies using whole exhaust (Larcombe et al., 2014; Magnusson et al., 2019; Bendtsen et al., 2020). Instillation allows for more precise control of dosage, making comparisons between different exposures easier (Yanamala et al., 2013), however this method ignores the exhaust gases which are known to cause their own health effects (Wooding et al., 2019), and creates an unrealistic size spectrum of particles. Inhalation exposure does not involve these limitations which often means a lower and more "real-world" relevant exhaust dosage can be used (Lichtveld et al., 2012). Unfortunately, exact exhaust dosages can only be estimated and this method requires complex and costly equipment set ups (de Brito et al., 2018). Thus, instillation studies are more common for both diesel and biodiesel exhaust exposures (Landwehr et al., 2021; Møller et al., 2020).

In our previous studies (Landwehr et al., 2021b, 2023a), using primary human airway epithelial cells to directly compare exhaust toxicity of up to 6 different blended and/or pure biodiesel fuels, we found a broad spectrum of toxic outcomes with some types being more toxic than mineral diesel fuel and some less toxic. When we performed a *in vivo* inhalation exposure study, exposing mice for 2 h per day for 8 days to diluted exhaust from an engine running on either Tallow or Canola biodiesel with ultra-low sulfur diesel (ULSD) used as a control, we found a suite of health effects that changed depending on the biodiesel feedstock type (Landwehr et al., 2023b) including both increased and decreased airway hyperresponsiveness, increased airway inflammation and immune dysregulation. As these impacts were found after only 8 days of part-time exposure, the next logical step was to assess if a single exposure would be sufficient to elicit measurable health effects in our mouse model. This has important implications in multiple scenarios (such as for people with existing lung disease receiving a discrete exposure), but specifically would suggest that continuous low-level exposures are not required for negative impacts of biodiesel exhaust exposure to be seen. Thus, for this study, we exposed mice for 2 h to diluted exhaust from an engine running on mineral diesel, Canola biodiesel or Tallow biodiesel, with air as a negative control. The main hypotheses were that (i) a single 2-h exposure to biodiesel exhaust diluted to reflect real world concentrations would be enough to induce airway hyperreactivity and pulmonary inflammation that are measurable 24-h post exposure and that (ii) exposure would result in a spectrum of health impacts with Tallow being the most severe. No study has previously assessed health effects of mice exposed to biodiesel exhaust for such a short period of time at occupationally relevant concentrations.

## 2. Results

### 2.1. Exhaust gas characteristics

Mean and standard deviation for each exhaust and gas type are shown (Table 1), with the exception of CO which shows only the highest

**Table 1**
**Mean (standard deviation) combustion gas concentrations and particle characteristics for the three measured exhausts.** Gas measurements are shown as the mean concentrations for all of the exposures except for CO which is shown as the mean peak measurement. Particle data are shown as mean concentrations with the exception of median particle size. Data in square brackets are a ratio in comparison to ULSD, particle data in parentheses are a percentage of the total within the fuel.

| Exhaust Component | ULSD | Canola | Tallow |
|---|---|---|---|
| $O_2$ (%) | 19.17 (0.56) | 19.64 (0.46)*** ### | 18.95 (0.31) ### |
| CO (ppm) | 0.99 (0.93) | 1.87 (1.07) | 2.06 (1.55) |
| $CO_2$ (%) | 1.14 (0.37) | 0.85 (0.28)*** ### | 1.28 (0.24) ### |
| $NO_x$ (ppm) | 32.84 (12.26) | 21.78 (4.31) *** ### | 33.12 (12.37) ### |
| NO (ppm) | 28.47 (10.32) | 20.08 (4.50) *** ### | 29.67 (8.81) ### |
| $NO_2$ (ppm) | 4.38 (2.15) | 1.70 (0.83) *** ### | 4.17 (2.67) ### |
| $SO_2$ (ppm) | 1.53 (0.70) | 1.03 (0.17)*** | 1.25 (0.73)* |
| Particle Mass Concentration ($\mu g/m^3$) | 73.96 | 43.15 [0.58] | 46.97 [0.64] |
| Median Particle Size (nm) | 17 | 21 | 22 |
| Total Particle Number ($particles/cm^3$) | 148998 | 91127 [0.61] ** | 88315 [0.59] *** |
| Particle Number >23 nm ($particles/cm^3$) | 50411 (33.83%) | 41882 (45.96%) | 42873 (48.55%) |
| Particle Number <23 nm ($particles/cm^3$) | 98587 (66.17%) | 49425 (54.04%) ** | 45442 (51.45%)*** |

* = Different to ULSD ($p < 0.05$).
# = Different to other biodiesel ($p < 0.05$).

reading at 10 min 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 (Fig. S1). All fuels displayed similar trends over time with $O_2$ decreasing rapidly in the first 10 min as it becomes used up in combustion reactions, CO peaking in the first 10 min before decreasing rapidly to near undetectable concentrations as the catalytic converter warms and $NO_x$ (NO and $NO_2$), $CO_2$ and $SO_2$ increasing rapidly in the first 10 min of the exposure before trending to general increase as the cold start effect wears off. 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 and $SO_2$, when compared to Tallow biodiesel exhaust ($p < 0.001$) and except for CO when compared to ULSD ($p < 0.001$). In contrast, Tallow and ULSD exhaust were only different for $SO_2$ ($p < 0.05$).

### 2.2. Exhaust particle characteristics

Particle size spectra were obtained for all exhausts between the sizes of 10–340 nm (Fig. S2). We observed significantly decreased particle numbers in both biodiesel fuels compared to ULSD (Table 1), which is mostly caused by an decreased number of nucleation mode particles ($p < 0.01$). Particle mass concentrations were also highest in ULSD, although little difference was observed between median particle sizes.

### 2.3. Mouse weights

Mice were weighed before exposure and the day after exposure (Fig. S3). All groups of mice gained weight, Canola and Tallow exhaust exposure groups significantly so. After exposure, Tallow biodiesel exhaust exposed mice were significantly heavier than Air control mice ($p < 0.05$).

### 2.4. Lung function at functional residual capacity

Thoracic gas volume (TGV) and lung mechanics (airway resistance ($R_{aw}$), tissue damping (G), tissue elastance (H) and η/hysteresivity) at functional residual capacity (FRC) were measured (Table S1) 24 h post exposure. Differences were observed between Air and Canola for $R_{aw}$ (mean ± SD 235.6 ± 51.4 and 382.1 ± 36.5 hPa·s−1 respectively, $p < 0.05$)and between Canola and Tallow for H (mean ± SD 37135 ± 4477 and 33930 ± 4464 hPa respectively, $p < 0.05$).

### 2.5. Volume dependence of lung function

Respiratory pressure-volume curves, specific lung compliance and the volume dependence of $R_{aw}$, G and H (Fig. 1) 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 Canola exhaust exposed mice were significantly lower than Tallow exhaust exposed mice ($p < 0.01$), but there were no other significant differences ($p > 0.05$ in all cases; Fig. 1a). There was also no effect of treatment on specific compliance ($p > 0.05$ in all cases). At a volume of 0.7 mL (largest lung volume with data for all mice) there was no difference between treatments for $R_{aw}$ ($p > 0.05$ in all cases), however Canola had significantly higher G and H than Tallow ($p < 0.05$; Fig. 1c and d).

### 2.6. Responsiveness to methacholine

$R_{aw}$, G and H were measured after exposure to increasing doses of methacholine (Fig. 2). There were significant effects of exposure on responsiveness to MCh with respect to airway resistance in that Tallow were significantly more responsive than Air and Canola ($p < 0.01$ in both cases), but not ULSD (Fig. 2A). ULSD were also more responsive than Air ($p < 0.05$) but not Canola. This pattern was replicated in terms of sensitivity to MCh with both Tallow and ULSD requiring a significantly lower dose of MCh to reach a 50% increase in $R_{aw}$ (17.37 ± 7.43 mg/mL and 17.48 ± 5.06 mg/mL respectively compared to Air at 23.34 ± 7.14 mg/mL, $p < 0.05$).

### 2.7. Bronchoalveolar lavage cells, mediators, protein and phospholipid concentrations

Total and differential cell counts were performed on bronchoalveolar lavage (Fig. 3). Significantly more cells were found in the BAL of Tallow exhaust mice compared to Air ($p < 0.05$). This was mainly due to an increase in macrophages, however significant increases in both neutrophils and lymphocytes were also observed ($p < 0.05$). Canola exhaust exposed mice also had significantly more neutrophils compared with Air ($p < 0.05$). Tallow exhaust exposed mice had significantly more neutrophils and lymphocytes than both ULSD and Canola ($p < 0.05$). No other cell types were detected.

In terms of BAL mediator concentrations (Table 2), the majority of significant differences were found in Tallow compared to Air with 9 out of 21 mediators being significantly different ($p < 0.05$). All 9 mediators for which there were significant differences (IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-13, IFN-γ, GM-CSF and MIP-1β) were lower after Tallow biodiesel exhaust exposure compared with Air. Exposure to ULSD exhaust led to significantly lower levels of IL-10 and IL-13 ($p < 0.05$) compared with Air, while mice exposed to Canola biodiesel exhaust had significantly lower levels of IL-13 and GM-CSF ($p < 0.05$) compared with Air.

Total protein and phospholipid concentrations within the BAL were also measured. There was no effect of treatment on total protein in BAL ($p > 0.05$ in all cases), however Tallow exhaust exposed mice had significantly higher phospholipid concentrations in the BAL compared with Air (41.04 ± 6.85 and 35.73 ± 4.10 respectively, $p < 0.05$, Table 2).

### 2.8. Systemic mediators

Similar to what was observed in the BAL, changes in systemic

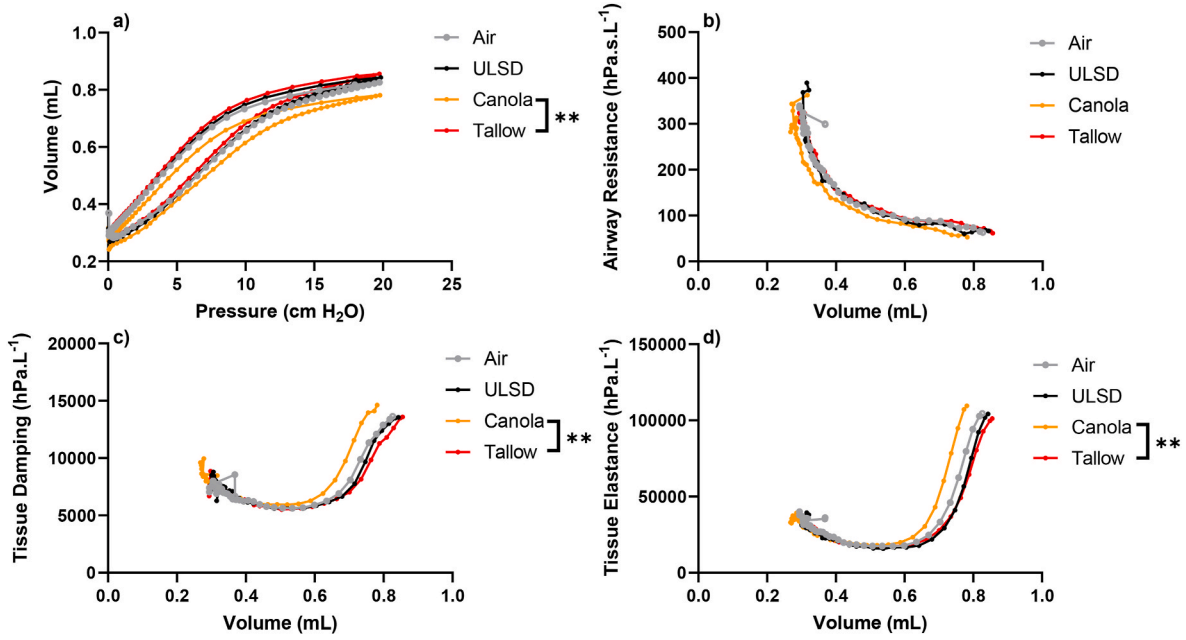


**Fig. 1. Pressure volume loops and volume dependence of lung function.** a) Pressure-volume loops and volume dependence of lung function for b) airway resistance, c) tissue damping and d) tissue elastance for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust. Data are group means; n = 23 for all groups except ULSD (n = 22) and Tallow (n = 21). Changes in thoracic gas volume were analysed at Prs = 20 cm $H_2O$. (** = $p < 0.01$). Differences between groups for volume dependence measurements were analysed at a lung volume of 0.7 mL, representing the highest volume for which data was available for each individual (** = $p < 0.01$).

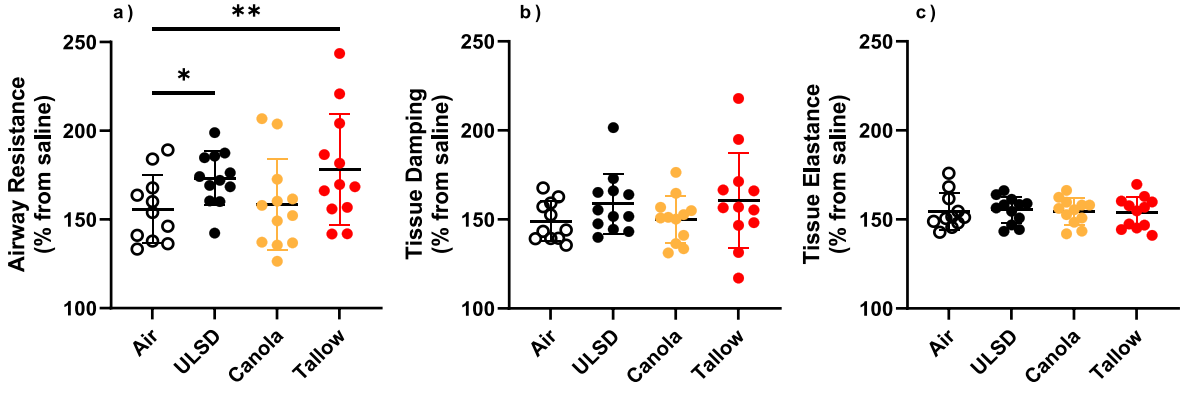


**Fig. 2. Responsiveness to methacholine.** Responsiveness to 30 mg/mL methacholine for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust a) airway resistance, b) tissue damping and c) tissue elastance for all exposure groups (n = 12, except Air where n = 11). All data are shown as mean ± SD increase/decrease from saline (* = $p < 0.05$, ** = $p < 0.01$).

mediator levels (Table S2) after exhaust exposures were mostly decreases compared with Air controls. The biggest changes were observed in Tallow compared to Air with 4 of 20 mediators being significantly lower in Tallow (IL-9, G-CSF, eotaxin and MIP-1β ($p < 0.05$)). Exposure to ULSD led to significantly lower levels of two immune mediators, IL-12p70 and G-CSF ($p < 0.05$) compared with Air controls. Exposure to Canola biodiesel resulted in significantly lower levels of IL-9, IFN-γ and eotaxin ($p < 0.05$) compared with Air controls. There was no significant effect of treatment on the concentrations of the three tested immunoglobulins ($p > 0.05$ in all cases).

## 3. Discussion

The results of this study show that a single, 2-h exposure to diluted exhaust from an engine running on different types of biodiesel or diesel fuel is enough to induce negative health effects in mice and that these effects are detectable 24 h after exposure. This has concerning implications for anyone (or anything) exposed daily to diesel or biodiesel exhaust as it highlights that 24 h is not enough to fully recover from the negative impacts of a single exposure. This implies that each new exposure could build upon the previous day's effects to eventually culminate in much larger health impacts. This study also found exhaust specific differences, further highlighting that biodiesel feedstock type impacts the resulting toxicity of the generated exhaust (Landwehr et al., 2021b, 2022b, 2023b). Tallow biodiesel exhaust exposure was the most toxic and resulted in the greatest number of health impacts with increased airway hyperreactivity, increased sensitivity to MCh, increased inflammatory response and decreased mediator concentration in the BAL (9 cytokines) and serum (4 cytokines) alongside increased phospholipid concentration in the BAL. In comparison, ULSD exhaust resulted in increased airway hyperreactivity, increased sensitivity to MCh, and decreased cytokine concentration in the BAL (2 cytokines) and serum (2 cytokines). Exposure to Canola biodiesel exhaust was the least toxic overall due to only causing relatively small and inconsistent

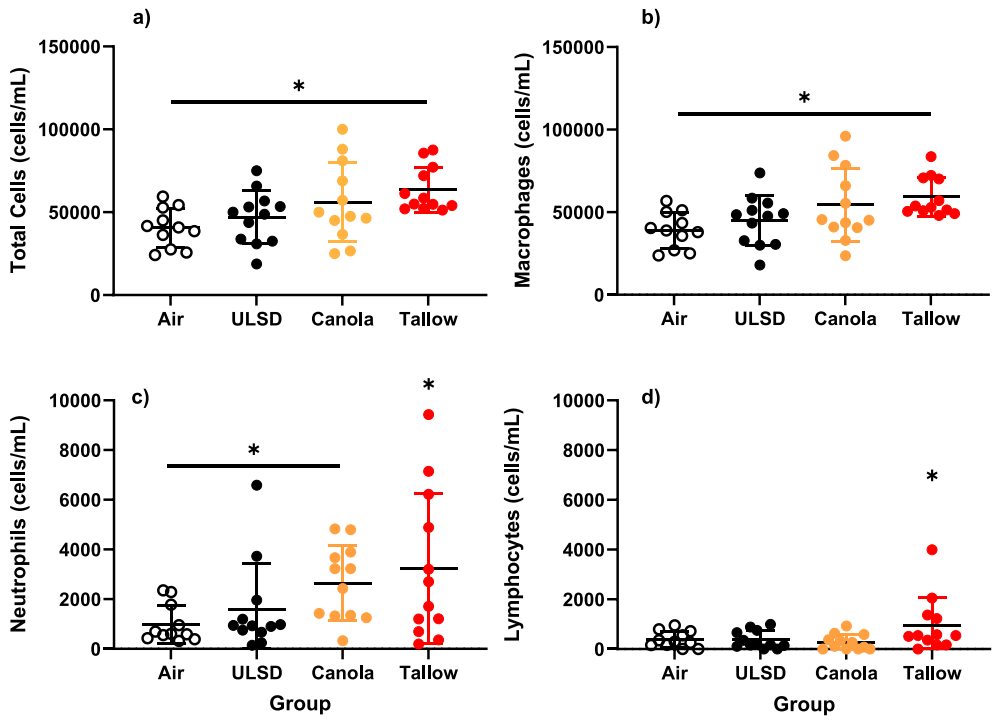


**Fig. 3. Cellular inflammation in bronchoalveolar lavage.** Results are shown as mean ± SD for a) total cells, b) macrophages, c) neutrophils and d) lymphocytes (n = 12, except Air where n = 11) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). Note different scales.

differences in measured health effects, with exposure resulting in a slight increase in $R_{aw}$ at FRC, a small inflammatory response in the BAL (proportion of neutrophils only) and decreased cytokine concentration in the BAL (2 cytokines) and serum (3 cytokines).

The different types of exhaust used in this study varied in terms of physico-chemical properties. While Tallow biodiesel exhaust had few differences compared to ULSD, with a lower concentration of $SO_2$ and smaller particle number concentration, it was Canola that was the most different with almost all exhaust gases and particle number being significantly lower compared to ULSD exhaust. This is similar to what has been found in previous studies within and outside of our lab (Hemmingsen et al., 2011; Landwehr et al., 2022a, 2023b), where biodiesel made from animal fat was found to have a more similar exhaust profile to diesel compared to biodiesel made from rapeseed oil. This pattern persisted in both pure B100 and B20 blended biodiesel.

A concerning implication from this study is that we measured significant biological effects 24 h after exposure, and yet the exhausts used were diluted enough to be below occupational exposure limits in many countries (US OSHA; European Agency for Safety and Health at Work, 2013; Safe Work Australia, 2019). The Safe Work Australia limits, which are similar or more stringent than the limits used by the USA and the European Union (US OSHA; European Agency for Safety and Health at Work, 2013; Safe Work Australia, 2019), recommend exposures of no more than 30 ppm CO, 5000 ppm $CO_2$, 25 ppm NO, 3 ppm $NO_2$ (peak concentration not exceeding 5 ppm) and 2 ppm $SO_2$ (peak concentration not exceeding 5 ppm) averaged over 8 h. Oxygen concentration should be above 19.5% (Safe Work Australia, 2018). In the European Union, particle mass is recommended to be below 50 μg/$m^3$ elemental carbon (EU, 2004; European Union, 2019) averaged over 8 h. The exhaust concentrations used in this study meet occupational guidelines in all cases except for slightly too low oxygen levels, which due to mice being burrowing animals and thus adapted to breathing lower oxygen and higher $CO_2$ than humans, is unlikely to have an effect (Arias-Reyes et al., 2021).

Changes in weight before and after exposure were also observed in both biodiesel exhaust exposed groups, with the Tallow exhaust exposed mice being significantly heavier than the Air controls (Fig. S3). This may indicate that exposure to biodiesel exhaust increases mouse appetite, although changes in food consumption were not measured directly. As whole-body exposures were used for this study, mice could also be grooming unburnt fuel from their fur. Biodiesel has a high calorific value, and generally more unburnt biodiesel fuel makes it into the exhaust than unburnt diesel fuel (McCormick et al., 2001; Fontaras et al., 2009; Ozcanli et al., 2013). A third explanation could be that mice were more lethargic after exposure to biodiesel exhaust and did not expend as much energy as the control mice. A study assessing behavioural changes after different types of biodiesel exhaust exposure would be an interesting future direction to explore.

One of the most significant findings of this study is that a single, highly diluted 2-h exposure to ULSD or Tallow biodiesel exhaust induces hyperreactivity of the airway with higher peak responses to MCh in airway resistance compared to Air controls (Fig. 2). This suggests that very little exhaust is needed to induce a hyperresponsive phenotype, especially considering the observed response is similar to what was seen in our sub-chronic exposure experiment (Landwehr et al., 2023b). Compared to previous studies measuring airway hyperreactivity after diesel exhaust exposure, the peak response observed in this study is much lower (Lambert et al., 2003; Nemmar et al., 2011; Brandt et al., 2013, 2015). This is unsurprising as we used inhalation instead of instillation as the exposure method at a much lower dose of exhaust. That said, we still measured a significant response after exhaust exposure alone, when some previous studies require co-exposure of diesel exhaust particles and house dust mite extract to elicit an exhaust specific response. This suggests that inhalation of whole exhaust may be a much more potent sensitiser than instillation of exhaust particles alone. As exposure to diesel exhaust has been linked with the development of asthma (Muñoz et al., 2019), a similar hyperreactive response seen after exposure to Tallow biodiesel exhaust suggests that asthma may also be a concern for some biodiesel exhaust exposures. This is especially true when compared to Canola, which did not elicit increased airway hyperreactivity, highlighting that only certain types of biodiesel may be a concern for asthma incidence. Although small but significant changes

**Table 2**
**Mean (standard deviation) mediator levels in bronchoalveolar lavage fluid**. Mediator levels in bronchoalveolar lavage fluid for mice exposed to Air, ULSD, Canola or Tallow biodiesel exhaust (n = 12).

| BAL Measurement | Treatment | | | |
|---|---|---|---|---|
| | Air | ULSD | Canola | Tallow |
| Total Protein Concentration (mg/mL) | 357.9 (50.24) | 388.9 (101.0) | 378.4 (78.70) | 368.8 (99.18) |
| Phospholipid Concentration (nMol/mL) | 35.73 (4.095) | 39.25 (5.990) | 39.27 (6.212) | 41.04 (6.852)* |
| IL-1α (pg/mL) | 3.173 (1.863) | 2.583 (0.651) | 2.758 (0.710) | 2.326 (0.682) |
| IL-2 (pg/mL) | 3.166 (1.235) | 2.813 (1.001) | 2.916 (0.870) | 2.586 (0.822) |
| IL-4 (pg/mL) | 0.390 (0.138) | 0.294 (0.113) | 0.298 (0.101) | 0.255 (0.111)* |
| IL-5 (pg/mL) | 0.948 (0.427) | 0.692 (0.338) | 0.675 (0.315) | 0.695 (0.332) |
| IL-6 (pg/mL) | 1.922 (1.285)b | 1.180 (1.085) | 1.694 (0.814) | 0.991 (0.930)* |
| IL-9 (pg/mL) | 4.319 (1.765) | 3.272 (1.490) | 3.546 (1.347) | 2.780 (1.430)* |
| IL-10 (pg/mL) | 5.386 (1.502) | 4.121 (1.114)* | 4.412 (1.394)a | 3.149 (1.350) ***a |
| IL-12(p40) (pg/mL) | 43.234 (6.989) | 51.991 (28.060) | 47.163 (28.882) | 38.00 (10.190) |
| IL-12(p70) (pg/mL) | 12.074 (5.163) | 9.529 (5.099) | 10.708 (3.223) | 7.160 (4.536)* |
| IL-13 (pg/mL) | 27.139 (14.320) | 18.399 (6.412)* | 19.843 (7.798)* | 18.593 (2.876)* |
| IL-17 (pg/mL) | 1.785 (0.590) | 1.568 (0.614) | 1.523 (0.513) | 1.369 (0.578) |
| Eotaxin (pg/mL) | 5.606 (1.809) | 4.902 (1.007) | 4.901 (1.120) | 4.781 (1.405) |
| G-CSF(pg/mL) | 3.846 (1.488) | 3.288 (0.907) | 3.325 (1.043) | 3.623 (1.399) |
| GM-CSF(pg/mL) | 5.228 (2.221) | 3.811 (1.565) | 3.570 (1.455)* | 3.412 (1.161)* |
| IFN-γ (pg/mL) | 3.569 (1.164) | 2.925 (1.018) | 3.233 (0.949) | 2.615 (0.924)* |
| KC (pg/mL) | 37.466 (8.131) | 47.665 (11.315) | 41.541 (14.035) | 46.682 (15.752) |
| MCP-1 (pg/mL) | 18.00 (12.725) | 17.119 (6.479) | 13.502 (6.549) | 14.587 (8.137) |
| MIP-1α (pg/mL) | 2.205 (0.555) | 2.596 (0.804) | 2.574 (0.699) | 2.325 (0.974) |
| MIP-1β (pg/mL) | 13.754 (5.341) | 9.912 (4.322) | 11.734 (6.019) | 7.531 (5.267)* |
| RANTES (pg/mL) | 9.315 (3.046) | 8.578 (1.607) | 8.180 (2.142) | 7.259 (2.077) |
| TNF-α (pg/mL) | 9.713 (3.011) | 8.093 (2.330) | 8.030 (2.144) | 7.652 (2.523) |

* = Different to Air (* = $p < 0.05$, *** = $p < 0.001$).
# = Different to ULSD (# = $p < 0.05$).
a = Different to other biodiesel ($p < 0.05$).

were observed in $R_{aw}$ at FRC between Air and Canola, in the volume dependence of lung function no differences between Canola and Air were statistically significant which complicates interpretation of results. Lung volume at $P_{rs}$ = 20 cm $H_2O$ was statistically significantly lower in Canola compared with Tallow, however the mean difference was only 0.074 mL, which is potentially biologically insignificant, and may be partially reflective of differences in size between animals. Surprisingly, G and H at a lung volume of 0.7 mL were significantly *higher* in Canola compared with Tallow (Fig. 1B and C). This would normally be interpreted as a greater impairment in lung function – i.e. higher resistance and stiffness of the lung parenchyma at a lower lung volume, which does not easily reconcile with our responsiveness to MCh data. Reasons for these seemingly contradictory findings are difficult to elucidate, and are indicative of a complex interplay between exhaust type and lung function responses in mice.

Similar to what has been observed previously in both a diesel exhaust exposure study (Boylen et al., 2011) and in our sub-chronic exposure study (Landwehr et al., 2023b), a single exposure to diesel and/or biodiesel exhaust resulted in direct impacts to proportions of cell types in the BAL and induced indications of immune dysregulation in the local and systemic immune responses. This was especially true after Tallow biodiesel exhaust exposure, with increased numbers of macrophages, neutrophils and lymphocytes compared to both Air and ULSD. Canola induced increased numbers of neutrophils whereas ULSD did not have a significant impact compared to Air, indicating that both biodiesel exhaust exposures caused greater pulmonary inflammation than ULSD at the single timepoint investigated. In contrast to the cell numbers in the BAL, the majority of significantly different mediator responses in both the BAL and serum showed decreases in concentration, which may be explained as the mediators being "used up" in response to exposure (Boylen et al., 2011). Again, Tallow was the most concerning exposure, inducing the greatest number of significantly decreased mediators both locally and systemically compared to Air. Similarly, the increased phospholipid concentration in the BAL after a single exposure to Tallow biodiesel exhaust indicates that 24 h is not enough time for mice to fully recover from the lung chemistry altering effects of exposure. A study assessing blended biodiesel exhaust exposure in human lung epithelial cells also found that exposure to biodiesel exhaust particles can alter lipid chemistry by disrupting lipid metabolism (Wang et al., 2023), suggesting that the toxic effects of some biodiesel exhaust exposures may be more variable than current literature suggests. Although the changes measured in local and systemic mediator response and lung lipid chemistry were small, and a greater response may have been measured closer to the time of exposure, especially for cytokine levels (Boylen et al., 2011), when considering that these effects were found 24 h after a single exposure this implies a longer time period is needed to fully recover from exposure effects. This has concerning implications for daily exposure effects, implying that ongoing exposure may result in the inability to mount an appropriate protective response in case of other insults such as infection or even cancer (Gowdy et al., 2010; Zarcone et al., 2017; Dai et al., 2018; Shears et al., 2020).

This study has several limitations. Firstly, we used highly diluted exhaust concentrations in order to simulate real-world relevant exposures, which has resulted in relatively subtle downstream effects. A more concentrated exhaust exposure may allow for more differences between exposure groups to become apparent at the risk of negating real-world applicability. Secondly, this study lacks a comprehensive chemical analysis of the particulate matter. This is due to the high exhaust dilutions used, which meant it was not possible to collect enough particles to perform these analyses. The data that have been collected, where no one exhaust component stands out as toxic, suggests that linking exhaust exposure health effects to exhaust components is complex whereby both particle and gaseous components are contributing (Table 1). We have previously linked exhaust toxicity to the number of double bonds in the biodiesel fuel (Landwehr et al., 2021b) which is an avenue for future research. Finally, the choice of feedstocks was based on both current global use and our previous work (Landwehr et al., 2021b), selecting the most and least toxic of current commonly used biodiesel types from a suite of six.

In conclusion, a single 2-h exposure to highly diluted Tallow biodiesel exhaust was enough to induce hyperreactive airway physiology and the beginnings of both systemic and local immune dysregulation. In contrast, exposure to Canola biodiesel did not induce as many health impacts even when compared to ULSD exhaust exposure, highlighting that biodiesel exhaust toxicity changes depending on the feedstock used to create the biodiesel fuel. Tallow biodiesel exhaust exposure resulted in more toxic outcomes than exposure to ULSD exhaust, which raises concerns for the future use of biodiesel. Controls around what biodiesel can be made from need to be tightened to prevent investment into the more toxic feedstock types. More research is needed in what the most

toxic biodiesel types are, and future studies should focus on biodiesel types that are likely to be implemented in future, including those that come from food waste such as used cooking oil.

## 4. Materials and methods

### 4.1. Animals

This animal study was approved by the Curtin University Animal Ethics Committee (approval number ARE2020-16, approved July 23, 2020). Briefly, 96 seven-week-old male BALB/cARC mice were purchased from the Animal Resources Centre (Murdoch, WA, Australia). They were housed in individually vented cages (set at 22–23 °C with 30–31% humidity, 50 air changes per hour, 12:12 h cycling (IVC Allentown XJ model, ECO FLO air handling)) and left to acclimatise for one week before being weighed and randomly assigned into one of 4 different treatments (n = 24 per group). These groups were exposed for 2 h to the diluted exhaust of an engine running on ultra-low sulfur diesel (ULSD), Canola or Tallow biodiesel as previously described (Landwehr et al., 2021a, 2023b) or exposure to Air as a control. Twenty-four hours after exposure, mice were weighed and prepared for lung function measurements (Larcombe et al., 2008).

### 4.2. Engine configuration

The diesel fuel was obtained from a local supplier (SHELL, Australia, <10 ppm sulfur). Both biodiesel types were created by following a previously published sodium methoxide transesterification process (Knothe et al., 2015) using feedstocks obtained from local suppliers (Campbells Wholesale Reseller, WA, Aus and Range Products, WA, Aus). Detailed FAME profiles have been previously published (Landwehr et al., 2021a, 2021b). All exhaust exposures were run from cold start using a single cylinder, 435 cc 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) with a constant load of 40% and a speed of 2000 rpm. Air exposures were done simultaneously alongside exhaust exposures.

### 4.3. Exposure protocol

As previously described (Landwehr et al., 2023b), collected exhaust from the engine was pumped into a mixing chamber attached to the exhaust piping and diluted with air from an air pump. This mixture was then vacuumed through an isokinetic sampling point at a rate of 5 L/min into a 27 L exposure chamber with the mice inside divided into individual cubicles to even out each individual's exposures and prevent fighting. This exposure chamber was contained inside a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) maintained at 28 °C to optimise mouse comfort and to help dampen the noise of the engine. Exhaust exiting the exposure chamber was analysed for gas and particle physico-chemical properties. 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. 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.

### 4.4. Gas and particle analysis

Exhaust exiting the exposure chamber was analysed every 10 min for particle concentrations between the sizes of 10–340 nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Count-median particle size was calculated using the number of particles mean. Particle mass was calculated from particle spectra (number and size), assuming sphericity and using previously analysed 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). Simultaneously to particle measurements, exhaust gas concentration was analysed every 10 min for concentrations of combustion gas products including $O_2$, $NO_x$ (NO and $NO_2$), CO, $CO_2$ and $SO_2$ using a combustion gas analyser (TESTO 350, Testo, Lenzkirch, Germany).

### 4.5. Lung function measurements

Measurement of thoracic gas volume (TGV) and lung mechanics were conducted as previously described (Larcombe et al., 2011a, 2017; Landwehr et al., 2023b). In brief, mice were anesthetized via intraperitoneal injection of a solution containing ketamine (40 mg/mL; Troy Laboratories, New South Wales, Australia) and xylazine (2 mg/mL; Troy Laboratories, New South Wales, Australia) at a dose 0.1 mL/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 (Jánosi et al., 2006). 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 et al., 1997; Sly et al., 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 ($P_{rs}$), 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 $P_{rs}$ = 8 $cm/H_2O$ minus lung volume at $P_{rs}$ = 3 $cm/H_2O$ on the deflationary arm (Limjunyawong et al., 2015).

### 4.6. Methacholine challenge

After measurement of TGV and lung mechanics at FRC, 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 et al., 2011b). 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 (0.1, 0.3, 1, 3, 10 and 30 mg/mL). Peak responses to MCh at each dose were used to construct dose response curves.

### 4.7. 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 centrifuged at 400 g for 4 min to pellet the cells and the supernatant removed and stored at −80 °C for mediator, protein and phospholipid analysis. 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, stained with DiffQuik as per kit protocol (Thermofisher Scientific) and scanned using a Panoramic MIDI® scanner and paired software (3DHISTECH Ltd.) A randomised portion of 300 cells were counted to determine proportion of cell types within the lavage.

### 4.8. Serum collection

Following 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 min. This tube was then centrifuged at 2000 g for 10 min to separate serum which was collected and immediately stored at −80 °C for future mediator analysis.

### 4.9. Phospholipid, protein and mediator analysis

Phospholipid (choline containing) concentration within the BAL was analysed as per kit protocol using a Colorimetric Phospholipid Assay Kit (Abcam). Protein concentration of the BALs was assessed as per kit protocol using a Pierce™ BCA protein assay kit (Thermofisher Scientific). Serum immunoglobulin concentration was analysed as per kit protocol using Mouse Immunoglobulin Isotyping Magnetic Bead Panel (Milliplex, MERCK). 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).

### 4.10. Statistical analysis

All statistical analyses were performed using R statistical software (v3.4.3) (R Core Team, 2018) loaded with the packages "lme4" and "mgcv". Excluding gas concentration data, analyses 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. Data are presented as mean ± standard deviation. All statistical analyses P-values less than 0.05 were considered significant.

## Declarations

### Ethics approval

The animal study protocol was approved by the Curtin University Animal Ethics Committee (approval number ARE2020-16, approved July 23, 2020).

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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## CRediT authorship contribution statement

**Katherine R. Landwehr:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ryan Mead-Hunter:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Rebecca A. O'Leary:** Writing – review & editing, Methodology. **Anthony Kicic:** Writing – original draft, Methodology, Funding acquisition, Data curation, Conceptualization. **Benjamin J. Mullins:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Alexander N. Larcombe:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142621.

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