Re-assessing the toxicity of particles from biodiesel combustion: a quantitative analysis of *in vitro* studies

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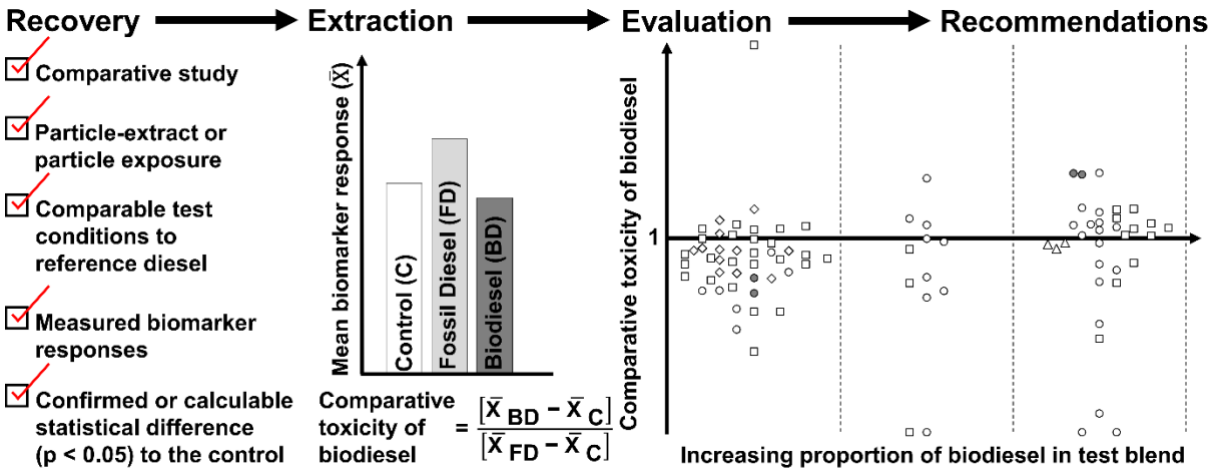
Graphical abstract



Highlights

- Previous reviews highlight uncertainty over biodiesel comparative toxicity.
- We systematically compare biological responses from particle or extract exposure.
- Biodiesel and fossil diesel responses are paired for comparable methodologies.
- Overall, biodiesel may reduce PM toxicity towards *in vitro* and acellular models.
- Toxicologists and combustion researchers must coordinate to resolve the uncertainty.

Keywords

HVO, FAME, Particulate, PAH, *in vitro*, Systematic review.

Abstract

Biofuels may reduce road transport carbon intensity; however, it is uncertain whether displacing fossil diesel would alter the engine-derived particulate toxicity. The primary objective of this work was to determine whether there is a fuel effect on the comparative *in vitro* toxicity of biodiesel exhaust particulates relative to those from fossil diesel. A secondary aim was to determine qualitatively whether the observed outcome is related to the organic phase, namely Polycyclic Aromatic Hydrocarbons (PAHs). In vitro and acellular exposure studies were recovered from a literature survey following the PRISMA framework. Biological responses attributable to biodiesel and paired fossil diesel particles, including particle-

extracts were selected. To qualify for inclusion, either of the paired responses must differ statistically significantly ($p < 0.05$) from the control or each other. Paired responses were assigned to one-of-five categories which best represents the pathophysiological role of the biomarker: inflammation, oxidative stress, cytotoxicity, genotoxicity and mutagenicity. Biodiesel reduced particle toxicity in two-thirds of paired responses, however, there were large differences between biodiesels for category-specific biomarkers. Particles derived from Rapeseed oil Methyl Ester (RME) were less inflammatory, whereas Soybean oil Methyl Ester (SME) particles were more inflammatory than fossil diesel on average. Conversely, SME reduced oxidative stress while few trends emerged for mutagenicity and genotoxicity. The largest fuel effect was observed for cytotoxicity: Waste Cooking Oil Methyl Ester (WCOME) increased and Palm oil Methyl Ester (PME) decreased particle cytotoxicity. Particle-phase PAH emissions compiled on a mass-of-soot basis also followed this trend, however, literature focusing on both these aspects is limited; careful consideration of fuel composition and use of normal primary human cell types and omics technologies, could resolve this open question. This assessment systematically compares biological responses from particulate only exposure, with co-exposure necessarily excluded due to an absence of understanding of how gaseous components modify particulate toxicity.

1.0 Introduction

Signatories at the Paris Climate Conference (COP21) of 2015 agreed to mitigate global warming to substantially below 2 °C above pre-industrial levels. However, current commitments are inadequate resulting in a predicted increase of 2.0–4.9 °C by 2100 (Raftery et al. 2017). The transport sector represents a significant contribution towards global greenhouse gas emissions, accounting for 24 % of direct CO_2 emissions, of which up to 75 % (6.0 Gt_{CO2}) was attributed to road transport in 2018 (IEA 2019). In addition to zero-emission vehicles and improvements in fuel economy, biofuels are promoted by legislators to reduce the carbon intensity of current vehicles. As an example, EU Directive 2018/2001

established a mandatory target for all member states to achieve at least 14 % contribution of final energy consumed by the transport sector from renewable sources (European Parliament and Council 2018). Furthermore, under the sustainable development scenario, biofuels are targeted to contribute 10 % towards transport fuels by 2030 (IEA 2019). By the end of 2019, global biofuel production reached 91.8 Mtoe with bioethanol and Fatty Acid Methyl Ester (FAME) biodiesel representing a share of 62 % and 38 % respectively (BP 2020).

Unlike fossil diesel, which is comprised mostly of paraffins and aromatics, FAME biodiesel contains esters with varying chain length up to 24 carbon atoms and up to 4 carbon-carbon double bonds (Dubois et al. 2007). On the other hand, subjecting fats and oils to hydrogenation rather than transesterification, leads to the production of Hydrotreated Vegetable Oil (HVO), a fuel containing straight and branched paraffins (Aatola et al. 2008). A wide variety of feedstocks with unique fatty acid profiles may contribute towards biodiesel production including vegetable oils, non-edible oils, waste oils, animal fats and algal oils (Elgharbawy et al. 2021). Variability in feedstock fatty acid profile influences the physiochemical properties of the corresponding biofuel (i.e. FAME, HVO), including the flash point, oxidative stability, cloud point, cetane number and heating value (Hellier and Ladommatos 2015; Karmakar et al. 2010).

Air pollution is a leading risk factor for mortality associated with cardiovascular and respiratory diseases, most prominent with Particulate Matter (PM) smaller than 2.5 μm ($PM_{2.5}$) (Cohen et al. 2017; Landrigan et al. 2018; Mannucci et al. 2015). Exposure to ambient $PM_{2.5}$ was attributed to a global burden of 4.2 million deaths (7.6 % of total global deaths) and 103.1 million disability-adjusted life-years in 2015 (Cohen et al. 2017). Furthermore, emerging evidence suggests a possible link between air pollution and non-cardiopulmonary adverse outcomes through systemic effects such as inflammation, oxidative stress, immune modulation and epigenetic alterations (Thurston et al. 2017).

Beyond mortality and physical morbidity, poor air quality is detrimental to cognition, mental well-being, productivity and social conduct in exposed communities (Lu 2020).

Legislators are motivated to improve ambient air quality by establishing progressively more stringent emissions standards such as EURO I to VI (Council of the European Communities 1993; European Parliament and Council 1996, 1998, 2008a, 2008b). Regulators have defined emission limits for Carbon Monoxide (CO), Total Hydrocarbons (THC), non-methane Hydrocarbons (NMHC), Oxides of Nitrogen (NO_X), PM and lately, Particle Number (PN). Relative to fossil diesel, biodiesel combustion tends to reduce regulated emissions of PM, CO and THC, and increase NO_x; these trends are often attributed to the higher oxygen content of FAME biodiesel (Xue et al. 2011), although reductions in fuel sulphur and aromatic content may also contribute to some of these observations (Lapuerta et al. 2008; Li and Gulder 1998; Tan et al. 2009). Furthermore, investigations with single component esters have shown key structural features (e.g. number of double bonds, alkyl chain length, alcohol chain length) to influence regulated emissions (Hellier and Ladommatos 2015; Schönborn et al. 2009). For single component esters, NOx emissions increase with increasing number of double bonds and decreasing alkyl chain length, whereas PM emissions increase with increasing alkyl- and alcohol-chain length.

Displacement of fossil diesel by biodiesel may increase the proportion of organic carbon (OC) on emitted particles. Measurements of elementary carbon (EC) and OC in particulates have indicated a reduction of EC/OC ratio with biodiesel use for a wide variety of fuel blends and engine operating conditions (Kooter et al. 2011; Traviss et al. 2014; Vojtisek-Lom et al. 2015; Zhang and Balasubramanian 2014a, 2014b, 2014c, 2018). These measurements typically show both EC and OC are reduced, but with a greater reduction of EC compared to OC with biodiesel use; however, the results are not clear-cut and mixed results have been reported (Gali et al. 2017; Martin et al. 2017; Tsai et al. 2012), with biodiesel increasing the EC/OC ratio in two studies (Bhavaraju et al. 2014; Yang et al. 2017). Further support for

higher organic component of biodiesel PM compared to fossil diesel is provided by measurements of the soluble organic fraction (SOF), also known as extractable organic matter (EOM), which is higher in both polar and nonpolar solvents (Adenuga et al. 2016; Bünger et al. 2000a, 2000b, 2006; Jalava et al. 2012; Krahl et al. 2002; Kuang et al. 2017; Liu et al. 2008; Turrio-Baldassarri et al. 2004; Westphal et al. 2012, 2013). Overall, these studies indicate that biodiesel may increase the organic component of engine-out PM.

Polycyclic Aromatic Hydrocarbons (PAHs) are an important constituent of the organic component of combustion particulates. PAHs are ubiquitous environmental pollutants formed in the fuel-rich regions of combustion engines and are known, or reasonably anticipated, to be carcinogenic towards humans (Heywood 2018). Within the cell, PAHs activate the Aryl hydrocarbon Receptor (AhR) and upregulate phase I and II metabolic enzymes which transform the PAHs into bioactive metabolites including diol-epoxides and o-quinones. Diol-epoxides may form bulky DNA lesions, whereas o-quinones generate Reactive Oxygen Species (ROS) through redox cycling which deplete cellular antioxidants, once overwhelmed, ROS may damage DNA and proteins. An array of DNA repair mechanisms function to maintain genetic integrity (Fleck and Nielsen 2004); however, unrepaired DNA lesions may lead to mutations in daughter cells and may promote neoplasia if the mutation upregulates proto-oncogenes or inhibits tumour suppressor genes (Klaassen 2018). Cells exposed to stress beyond the capacity to function may undergo programmed (i.e. apoptosis) or accidental (i.e. necrosis) cell death (Galluzzi et al. 2018). Moreover, AhR activation may alter an inflammatory response through genomic and non-genomic signaling (Puga, Ma and Marlowe 2009).

There is sufficient evidence in the literature for the particulate component of diesel exhaust and outdoor air pollution to be classified as carcinogenic towards humans by the International Agency for Research on Cancer (IARC 2014, 2016). A reduction of PM emissions with biodiesel blends would therefore suggest an improvement in public health;

however, recent reviews have highlighted conflicting evidence in toxicological reports. Madden (2016), reviewing literature published between 2007 and 2016, found a comparable number of reports showing higher or lower biodiesel particulate toxicity compared to those produced by fossil diesel combustion. Additionally, the authors concluded that it was unclear whether biodiesel blends were more potent than neat fuels. An updated review by Godri Pollitt et al. (2019) concluded that biodiesel induced a comparable or higher level of cytotoxicity, inflammation and oxidative stress compared to fossil diesel in exposed lung cells. However, the authors summarised that "there is no scientific consensus on the health effects associated with biodiesel exposure" (Godri Pollitt et al. 2019).

Differences in experimental variables may contribute towards the apparent disagreement in the literature regarding the comparative toxicity of biodiesel. Researchers have a choice of fuels, engine or vehicle models, engine operating conditions, exhaust aftertreatment devices, particle collection apparatus or dilution factor for direct exposure, cell model for *in vitro* assessment, exposure duration and load, and selection of adverse outcome biomarkers representative of cellular responses. Reviewers have called for standardised testing to partially eliminate the observed uncertainty due to experimental design (Bünger et al. 2012; Godri Pollitt et al. 2019; Larcombe et al. 2015); however, comparing investigations undertaken in a variety of engines and operating conditions may be more representative of real-world exposure and could allow greater correspondence with epidemiological studies.

The primary aim of this study is to assess whether there is a fuel effect on the comparative toxicity of biodiesel particulates relative to those for fossil diesel, to support legislator aims to reduce air pollution and improve public health. Gaseous exposure (i.e. direct exposure of cell cultures, animals or human subjects to filtered/unfiltered exhaust gases) is outside the scope of this work as it is uncertain how the particle and gaseous phases interact. Components of the gaseous phase may modify particulate responses and because of this uncertainty, *in vivo* models, human exposure studies and epidemiological evidence were excluded from this

assessment. Therefore, this assessment focuses on biological responses from acellular and *in vitro* models exposed to biodiesel and fossil diesel particulates, or the corresponding particle extracts.

A literature survey was performed and a ratio of the biodiesel response to the fossil diesel response was compared between studies for findings which were confirmed to be statistically different ($p < 0.05$) from control cells. Biomarkers were categorised into one of five categories: inflammation, oxidative stress, cytotoxicity, genotoxicity and mutagenicity. Lastly, particle-phase PAH profiles were compiled from literature data, to determine whether there was a tentative qualitative link between the comparative toxicity of biodiesel and the organic components of the particles. The uncertainty within literature regarding toxicity of biodiesel and lack of a systematic assessment were the two primary motivating factors for this work.

2.0 Methods

2.1 Search strategy

The search strategy was focused through Scopus and supplemented by searches on the PubMed and Web of Science databases. To identify appropriate keywords, a preliminary search on Scopus was performed and the frequency of keywords were recorded. Variants of these keywords were identified and categorised into fuel, health, chemical components and PM; common factors were generated and provided inputs to the search queries described below.

Searches on Scopus were first performed on the 19th November 2018 and search strings were constructed by selecting only one entry (separated by commas) of each input per search query from Table 1, into the following formats:

- (TITLE(biodiesel) OR TITLE-ABS-KEY(biodiesel AND *input 2*)) AND TITLE-ABS-KEY(*input 3*) AND NOT TITLE-ABS-KEY(microalgae OR alga*)
- (TITLE(*input 1*) OR TITLE-ABS-KEY(*input 1* AND *input 2*)) AND TITLE-ABS-KEY(toxic*) AND NOT TITLE-ABS-KEY(microalgae or alga*)
- (TITLE(biodiesel) OR TITLE-ABS-KEY(biodiesel AND *input 2*)) AND TITLE-ABS-KEY(toxic*) AND NOT TITLE-ABS-KEY(microalgae OR alga*)

TITLE-ABS-KEY is the default field for Scopus search queries which return documents that contain the enclosed text either in the title, abstract or listed in the keywords, whereas the TITLE field only returns documents with the specified text in the TITLE. The asterisk (*) wildcard modifies the specified text to return documents that contain the text within a string of characters: for example, TITLE(Toxic*) would return any indexed document containing the words toxic, toxicity, toxicological, toxicology and toxicants among others, within the title.

The effectiveness of this strategy was assessed by comparing the recovery of relevant research cited by the latest review available at the time of the literature search (Madden 2016). Of the 32 relevant articles cited, 30 were retrieved from the current search strategy; the two remaining articles reported exhaust emissions and the search strategy was expanded by inclusion of the following search strings:

- (TITLE(biodiesel) OR TITLE-ABS-KEY(biodiesel AND exhaust)) AND TITLE-ABS-KEY(toxic*) AND NOT TITLE-ABS-KEY(microalgae OR alga*)
- (Title(*input 1*) OR TITLE-ABS-KEY(*input 1* AND exhaust)) AND TITLE-ABS-KEY(toxic*) AND NOT TITLE-ABS-KEY(microalgae or alga*)

Search queries on the Web of Science and PubMed databases were performed on the 10th July 2019 and constructed by combining biodiesel with *input 2*; however, the “PM or partic*” query generated in excess of 5000 records from the two databases and therefore an additional term “toxic*” was included to refine the results. Scopus alerts were setup for the

following queries to find additional records and last checked on the 2nd October 2020: TITLE-ABS-KEY(toxic AND *input 2* And biodiesel), TITLE-ABS-KEY(toxic* AND exhaust AND biodiesel). Following a PRISMA style framework (Moher et al. 2009), the search results were combined, filtered and assessed for eligibility against the selection criteria.

2.2 Study selection criteria

The primary inclusion criteria was a study which compares biological responses *in vitro* from exposure to particulates generated from biodiesel and fossil diesel powered direct injection compression ignition engines under comparable conditions. Articles were assessed for suitability in three rounds of screening: assessing the contents of the title, abstract and full-text consecutively.

In the first round of screening, the titles of the articles were reviewed for eligibility and any articles clearly unrelated to the current study were eliminated from the compiled list; examples include articles related to biodiesel production, computational studies, fuel storage stability and life cycle assessments. The second stage of screening reviewed the abstracts of the remaining articles to assess suitability against the primary inclusion criteria.

Full-text articles were then reviewed to determine the suitability for inclusion in the present analysis. Articles were suitable if the reference fuel contained no more than 10% FAME content and within each study all of the following conditions were comparable between experiments: engine model and running conditions, presence of exhaust gas after treatment, protocol of particle collection and extraction (if applicable), cell line utilised, cell treatment, culture time and lag period between last dose and measurement. Studies which sampled atmospherically, rather than collecting from the exhaust, were excluded due to potential sample contamination with particulates generated from other sources. Additional constraints

included explicitly reporting the form of biodiesel, reporting responses from the control (i.e. unexposed cells) and the uncertainty of each measured response.

In vivo models were excluded primarily due to the exposure method deployed in these studies: animals were exposed in exposure chambers (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; Lecureur et al. 2020; Magnusson et al. 2019; Shvedova et al. 2013) and thus would be co-exposed to the gaseous phase of the exhaust which was outside the scope of this work. Two studies administered particles via pharyngeal aspiration (Kisin et al. 2015; Yanamala et al. 2013) while only one study focused on pulmonary responses (Yanamala et al. 2013). Yanamala et al. (2013) measured pulmonary responses from inhaled PM derived from a neat corn-based biodiesel and fossil diesel fueled Isuzu C240 engine equipped with a Diesel Oxidation Catalyst (DOC), following an 8-mode test cycle (ISO 8178 C1). Enhanced inflammation, oxidative stress and cytotoxicity was observed with biodiesel PM exposure compared to fossil diesel exposure.

2.3 Data confirmation and extraction

Biodiesels were defined by the feedstock oil, rather than the composition of individual FAMEs as few studies speciated the test fuels. Similarly, all HVO fuels were grouped together due to the absence of detailed compositional analysis. The fuels were subdivided into blend intervals of 10 % biodiesel content (e.g. 01–10 %, 11–20 %).

Mean responses, standard deviation and number of repeats of biodiesel and reference fuel treatments, along with the control group were extracted from qualifying articles identified through the literature survey. Responses from the two treatment groups were paired according to the same experimental conditions (e.g. engine setpoint, treatment dose, time of measurement). The required data were extracted from both graphical and tabulated formats

depending on the chosen presentational style of the authors; if both formats were available, the latter option was preferred. In the circumstances of the former, the figure scale was determined by dividing the highest reported y-value (Y_{MAX}) by the distance between Y_{MAX} and the x-axis (L_{MAX}). Where displayed graphically, the mean and standard deviation for each group was estimated from the measured scale as demonstrated by Figure 1. Figures with a logarithmic y-axis were converted into linear scales before calculating the scaling factor; under these circumstances the mean and standard deviation were calculated from the logarithmic base raised to the product of the scale factor and measured length L_1 or L_2 respectively. The number of independent repeats were found within the main body of text.

To provide greater certainty in this analysis all paired responses were required to meet a minimum standard of statistical significance ($p < 0.05$). It was necessary for either one of the paired responses to differ significantly from the control, or for there to be a significant difference between the paired responses. Statistical tests performed by the original authors took precedence over our calculations, as raw data were seldom available. An exception to the previous statement applied for paired responses without prior statistical analysis; therefore, either a two-tailed student's t-test or an analysis of variance (ANOVA) followed by post-hoc Dunnett's t-test was performed. Insufficient reporting of the number of repeats/replicates or measure of uncertainty prohibited inclusion because the requirement above could not be confirmed. However, there was a special case for the number of reverts in TA98 or TA100 tester strains exposed to particle extracts following the standardised Ames assay (Mortelmans and Zeiger 2000). The mean and variance of spontaneous reverts (i.e. reverts due to DMSO + S9 fraction exposure) were estimated for articles without control data due to the consistency from articles reporting control responses. Measurements of the mean spontaneous reverts (±SD) include 27 ± 5 (Westphal et al. 2013), 26 ± 15 (Krahl et al. 2009), 26 ± 9 (Westphal et al. 2012), 22 ± 7 (Bünger et al. 2007), 21 ± 5 (Bünger et al. 2006) and 27 ± 3 (DeMarini et al. 2019) for TA98, while 148 ± 19 (Westphal et al. 2013), 129 ± 28 (Krahl et al. 2009), 141 ± 35 (Westphal et al. 2012), 119 ± 26 (Bünger et al. 2007) and 127 ±

19 (Bünger et al. 2006) were observed for TA100. Therefore, the average number of spontaneous reverts for these studies were 25 ± 8 and 133 ± 26 for TA98 and TA100 respectively. Each estimated standard deviation was calculated from the average variance between these studies (Bland and Altman 1996).

The absolute difference between treatment groups may vary by several orders of magnitude depending on the chosen cell line, biomarker and treatment dose, aptly demonstrated by Hemmingsen et al. (2011) from ROS measurements with DCFH-DA in acellular models and transformed and primary cells. The variation of scale may mask fuel effects when comparing between studies; therefore, we chose a ratio of the mean responses rather than the absolute difference. Furthermore, the direction of response was assigned by considering the absolute difference between the treatment and control cells to account for circumstances where an enhancement or reduction of a measured biomarker are both considered adverse (e.g. proliferation and cell death are opposing effects on cell viability). Considering both scaling and directionality, comparisons between biodiesel and reference fuel were calculated from the equation presented within Figure 1. Lastly, biomarkers were assigned into one of five categories depending on the physiological role including inflammation, oxidative stress, cytotoxicity, genotoxicity and mutagenicity.

2.4 Acquisition of particle-phase PAH profiles

To assess whether there is a tentative link between particulate organic phase and the comparative toxicity of biodiesel, the profiles of comparative particle-phase PAHs identified in the literature search were compiled. Additional PAH profiles were recovered from studies without measurements of biological responses, through supplementary searches in the Web of Science database (data not shown). PAH emission profiles were collated on the mass-of-soot basis as we deemed it to be the most suitable basis on which to quantify the toxicity of biodiesel particulates. PAH profiles reported on alternative bases were converted onto the

mass-of-soot basis if the original authors provided the corresponding mass emission rates. To minimise the influence of the engine model and operating conditions, the PAH emission profiles were reported as a ratio relative to fossil diesel, combusted under comparable conditions.

3.0 Results and discussion

3.1 Literature search and data extraction from in-vitro studies

The combined literature searches through the three databases (Scopus, PubMED and Web of science) recovered 1469 unique results of which 34 were included in this analysis, as shown by the PRISMA statement in Figure 2. A comparison between the frequency of papers reporting each test fuel before and after the confidence screen showed only modest changes (supplementary information). In total, 578 comparisons were generated from the qualifying articles and split between the five biomarker categories and respective fuels as shown by Table 2. HVO and Rapeseed oil Methyl Ester (RME) were the predominant biodiesels assessed in this evaluation, whereas, Linseed oil Methyl Esters (LiME), Coconut oil Methyl Ester (CoME) and Jatropha oil Methyl Ester (JME) were seldom investigated. An overview of the biomarkers assigned to each category is provided in Table 3.

A summary of the data set is presented in supplementary file (Excel Table S1) along with the fuel specifications (biodiesel test blend, fossil diesel sulphur and FAME content), engine characteristics (engine type and emissions standard, presence of aftertreatment and operating conditions), cell line and exposure conditions (exposure method and solvent used if applicable, exposure load and time), source of data (i.e. tabulated or graphical), a summary of the comparison (the biomarker, biomarker category, relative ratio and confirmation of significance). It is important to note that some articles contributed more towards the quantitative analysis than others depending on the availability of data; for

example, Jalava et al. (2010, 2012) and Hemmingsen et al. (2011), contributed 70, 100 and 68 data points for this analysis respectively. In comparison, the median number of data points per article was 6 for this assessment.

3.2 Analysis of literature to quantify biodiesel comparative toxicity

Supplementary Table S1 and S2 present descriptive statistics for each fuel and biomarker category. Biodiesels and test blends absent from Table S1 and Table S2 either did not meet the inclusion criteria or were not recovered in the literature survey. The listed biodiesels include CoME, Palm oil Methyl Ester (PME), Animal Fat Methyl Ester (AFME), JME, Waste cooking oil Methyl Ester (WCOME), Soybean oil Methyl Ester (SME), LiME, RME and HVO.

A ratio mean value below 1.0 would indicate on average that the biodiesel (or biodiesel blend) induced a smaller deviation from the control cells in a measured biomarker compared to the reference fuel; whereas above 1.0, that biodiesel induced a greater deviation from the control cells compared to the reference fuel. As the strength of this approach arises from averaging a wide range of responses, mean values acquired from only a limited number of comparisons should be interpreted with caution. Additional insight is provided by comparing the contribution from the modal article to the number of articles contributing towards each test blend. Greater confidence can be assigned to mean responses with a lower contribution from the modal article as this suggests various studies are showing a similar trend; conversely, caution is advised against mean responses from a single study and highlights the need for future research. Unless an arbitrary threshold is established, interpretation of the analysis will be subjective to whether there is sufficient data to validate the reported mean.

Figure 3 to 7 show plots of the mean biodiesel potency compared to reference diesel for biomarkers of inflammation, oxidative stress, cytotoxicity, genotoxicity and mutagenicity

407 respectively. For each biodiesel-diesel dataset these plots contain a marker which
408 represents the geometric mean and error bars which represent the multiplicative standard
409 deviation. Data points on each figure are firstly ordered by increasing blend interval and
410 secondly by increasing average molecular weight although the spacing intervals do not
411 represent the magnitude of change in mean molecular weight of FAME. The molecular
412 weight of FAME was based on an average of literature profiles presented in Table S3. Each
413 plot has a logarithmic scale (log_2) to provide equal spacing (symmetric y-axis) above and
414 below the x-axis.
415
416 *3.2.1 Overview of inflammatory biomarkers*
417
418 Particulates derived from neat or blended RME appear to be less inflammatory than those
419 derived from diesel combustion (30 out of 33 data points with a ratio below 1.0). Three
420 articles (Hemmingsen et al. 2011; Jalava et al. 2010, 2012) contributed a similar number of
421 comparisons to this observation (8, 12, 13 data points) suggesting good overall agreement.
422 On the other hand, only 3 out of 18 SME data points have a ratio below 1.0 suggesting that
423 neat and blended SME particulates may provoke a greater inflammatory response than fossil
424 diesel. A majority of these comparisons were derived from Vogel et al. (2019) whom
425 contributed 12 out of 18 data points and showed consistently higher responses from SME
426 particulates; an observation further supported by Fukagawa et al. (2013) but contrasted by
427 Bhavaraju et al. (2014).
428
429 HVO particulates appear to be comparable to fossil diesel regarding inflammatory potency,
430 with good agreement between the five contributing articles (Jalava et al. 2010, 2012; Lankoff
431 et al. 2017; Skuland et al. 2017; Vogel et al. 2019). On the other hand, particulates derived
432 from AFME may be less inflammatory than those derived from fossil diesel; however, only
433 two articles contribute to this observation (Hemmingsen et al. 2011; Vogel et al. 2019) and 8
434 out of 14 comparisons were provided by one study (Hemmingsen et al. 2011). Furthermore,

all of the comparisons reporting higher inflammatory potency of AFME particulates were derived from Vogel et al. (2019), therefore further research is required to resolve this conflict, although different biomarkers were assessed.

Researchers have focused on the potential of biodiesel and reference diesel particulates to upregulate the production and secretion of pro-inflammatory cytokines (Table 3), however, the effect on immuno-suppressant cytokines (e.g. TNF-β, IL-10) were seldom investigated. Ambient $PM_{2.5}$ may increase IL-10 secretion (Xian et al. 2019), therefore, an overreliance on pro-inflammatory cytokines in toxicity assessments may overlook a wider immune response from PM exposure.

3.2.2 Overview of oxidative stress biomarkers

Reduced oxidative stress from particulates derived from neat or blended SME was found in 13 out of 14 comparisons obtained from five articles (Agarwal et al. 2018; Fukagawa et al. 2013; Holmén et al. 2017; Karavalakis et al. 2017; Vogel et al. 2019). Pooled together, SME particulates had a two-fold reduction of oxidative potency compared to reference diesel. All other biodiesels with more than one comparison (including AFME, RME and HVO) induced on average, responses comparable to fossil diesel particulates.

3.2.3 Overview of cytotoxic biomarkers

Biomarkers of cytotoxicity show the largest variation between fuels. Particulates derived from neat or blended PME had reduced cytotoxicity, particularly at high blend ratios, compared to fossil diesel in 54 out of 62 comparisons. Five articles (Liu et al. 2008, 2009, 2011; Zhang and Balasubramanian 2014a, 2018) reporting biological responses from PME particle exposure showed overall good agreement; however, Liu et al. (2009) consistently reported higher cytotoxicity with PME10 at four engine loads (0, 10, 33 and 55 %). Other work from

463 the authors (Liu et al. 2008, 2011) with similar research methods tend to show lower or
464 comparable cytotoxicity from PME use, therefore the cause of the discrepancy is unknown.
465
466 In contrast to PME, WCOME particulates consistently increased cytotoxicity compared to
467 fossil diesel with low variance (as shown by Figure 5); however, caution is required as these
468 comparisons were derived from one study (Betha et al. 2012) investigating various
469 biomarkers of cytotoxicity. Biodiesels derived from animal fat also enhanced particle
470 cytotoxicity from one study (Hemmingsen et al. 2011), although less convincingly due to
471 higher variability and fewer comparisons than WCOME. RME and HVO on the other hand,
472 were consistently comparable to fossil diesel with good agreement between studies. Further
473 research is required to confirm whether WCOME and AFME enhances particle toxicity
474 compared to fossil diesel.
475
476 *3.2.4 Overview of genotoxic and mutagenic biomarkers*
477
478 Overall, particulates collected from biodiesel combustion induced either comparable or lower
479 genotoxicity compared to fossil diesel, although there were few consistent trends for specific
480 biodiesels. RME particulates were less genotoxic than fossil diesel in 18 out of 32
481 comparisons with a pooled average ratio of 0.64. Comparisons for SME and HVO are
482 inconsistent showing higher and lower potency depending on the blend level, however, only
483 one article was suitable for SME (Ross et al. 2015) therefore there is scope for further work
484 with this biodiesel.
485
486 SME may be less mutagenic than fossil diesel as mutagenicity decreased with increasing
487 biodiesel content, although this is a tentative observation which is undermined by the
488 variance observed for SME20 (Agarwal et al. 2018). In addition to SME, RME may reduce
489 the mutagenic potency of particle extracts with increasing biodiesel content. Overall, there is
490 insufficient data to make meaningful comparisons except for SME and RME.

The high relative mutagenicity of PME may be misleading and presents an example where this assessment may produce sporadic results. PME induced a lower number of reverts (54 ± 9 graphical estimate (Schröder et al. 2012)) than the expected number of spontaneous reverts based on published work with TA100 strain (133 ± 26), whereas fossil diesel produced a higher number of reverts (137 ± 15). Therefore, PME was calculated to be more mutagenic than fossil diesel although in this case it is reasonable to argue for the opposite conclusion.

3.2.5 Overall effect of biodiesel use

Overall, there is a downward trend of lower toxicity from biodiesel use. Two-thirds of paired responses have a ratio below 1.0 (Excel Table S1); whilst, one-third of paired responses are above 1.0, thereby showing an overall reduction in toxicity compared to fossil diesel particulates. Subdividing the pooled paired responses into ratios below 0.25, 0.25 to 0.50, 0.50 to 1.0, 1.0 to 2.0, 2.0 to 4.0 and above 4.0, sees individual contributions of 9, 13, 42, 28, 5 and 3 % (of total paired responses) respectively. Therefore, the pooled paired responses appear to have a log-normal distribution skewed towards lower biodiesel toxicity. 70 % of paired responses had lower than two-fold change in either direction (i.e. a ratio between 0.5 and 2.0), whereas 50 % of ratios are within a range of 0.7 – 1.4 (Figure S1).

The data suggests non-linear cell responses with respect to blend ratio. Non-linear responses have been observed previously (Liu et al. 2008) and present a challenge to extrapolating findings to other blend ratios thereby requiring further testing; however, the direction of change of blends tends to follow the same direction as B100 (Table S4). Blends between 11 – 20 % and 91 – 100 % FAME are the most common test blends explored by researchers included in this analysis, as 41 % and 56 % of research articles report biological

responses from these test blends respectively. Whereas, no authors tested blends between 51 – 60 %, and blends between 01 – 10 %, 31 – 40 % and 71 – 80 % were seldom studied.

Pooled paired responses show reduced toxicity (ratios below 1.0) from particles derived from neat or blended PME (67 % of 63 data points), PME/SME (100 % of 7 data points), AFME (68 % of 47 data points), SME (56 % of 62 data points), RME (73 % of 158 data points), HVO (58 % of 201 data points); in contrast, only 14 % of 37 data points for WCOME had a ratio below 1.0 suggesting higher biodiesel toxicity. All of the comparisons for WCOME above 1.0 were derived from measures of cytotoxicity and oxidative stress reported in a Singapore study (Betha et al. 2012); whereas, all those below 1.0 were taken from two articles published by researchers based in the United States (Holmén et al. 2017; Karavalakis et al. 2017) measuring oxidative stress, inflammation or mutagenicity. Unfortunately, it is not possible to determine whether the discrepancy is due to different assessments (e.g. GSH/GSSG ratio, DTT assay) or geographical variation in the feedstock oils; however, this observation does highlight a need for further research with WCOME, an increasingly important biodiesel.

3.3 Comparative emissions of particle-phase PAHs

In total, 23 studies were identified from the literature search and supplementary searches, to have extractable particle-phase PAH profiles, reported on the mass-of-soot or convertible to the mass-of-soot basis (see Excel Table S2). Additionally, 13 studies reported overall particle-phase PAH emissions on the mass-of-soot or convertible basis (see Excel Table S3). In total there were 216 comparisons between biodiesel and fossil diesel and 89 unique PAHs were quantified. For each study, the engine model, operating conditions, particle extraction and the test fuels were recorded to aid interpretation of the results. Figure 8 presents the change in total particle-bound PAHs in biodiesel extracts compared to fossil diesel under comparable conditions.

Ratios of total particle-phase PAHs have lower variability compared to ratios of biological responses, which may be due to higher consistency or accuracy of measured endpoints. The difference in total particle-phase PAH emissions between biodiesel and fossil diesel increased with increasing blend ratio. At low blend levels only small deviations are observed, whereas at higher blends the difference between the paired profiles increases on average. The tentative trend for total particle-phase PAH emissions with respect to blend ratio is in contrast to some biological reports, which show potency to initially increase then decrease with biodiesel use (Schröder et al. 2013). There are differences between biodiesels: SME and PME are consistently lower or comparable whereas WCOME may lead to comparable or higher particle-phase PAH emissions relative to fossil diesel. Furthermore, these trends are also observed on a scatter graph showing the ratio of genotoxic PAHs between biodiesel and reference diesel on the mass-of-soot basis (Figure S2).

Only a few comparative studies analysed PAH profiles and associated biological responses. PAH metabolism was identified as the top-ranked pathway in BEAS-2B cells after 4- and 24-h exposure to particle extracts derived from fossil diesel, neat and blended HVO (Libalova et al. 2016). Tsai et al. (2011) exposed U937 cells to size-segregated particle extracts derived from neat fossil diesel and SME20 fueled Yanmar TF110E engine operating at two steady state setpoints (0 kW and 3 kW). The authors reported positive correlations between the cell death rate measured by the MTT assay and total PAHs (r^2 = 0.659 – 0.741) and B(a)P-equivalent (r^2 = 0.672 – 0.749) in the extract. Additionally, Jalava et al. (2012) exposed murine macrophages (RAW264.7 cell line) to particle extracts and reported several statistically significant correlations (MIP-2 secretion and genotoxic PAHs, cell apoptosis and genotoxic PAHs, intracellular ROS and genotoxic PAHs, DNA damage and Total PAHs, DNA damage and genotoxic PAHs); however, only weak correlations were found for cytotoxicity measured by the MTT assay. Genotoxic PAHs were defined by WHO-IPCS criteria (World Health Organisation and International Programme on Chemical Safety 1998).

574 Overall, these studies indicate that PAH levels may correlate with adverse outcome
575 biomarker responses. However, due to variations in methodology and analysis there is a
576 high level of uncertainty to make any conclusive extrapolations, suggesting any trend with
577 the compiled PAH profiles would be difficult to observe. In fact, a preliminary regression
578 analysis between the compiled PAH profiles and biomarker categories only achieved weak
579 correlations (data not shown). We therefore suggest that a more detailed analysis of PAH
580 profiles in studies of this kind are required to determine whether differences in particulate
581 associated chemical composition can be corelated to measurable adverse outcomes.
582
583 The influence of engine model and operating conditions cannot be understated and remains
584 a prominent hurdle for comparing trends between studies. Other sources of variation include
585 the ambient conditions (e.g. temperature, pressure, humidity), method of sampling,
586 extraction from soot (including equipment, solvent, time), selection of PAHs chosen to study,
587 sample clean-up procedure, method of quantification. Variations in experimental design and
588 choice of comparison basis may contribute towards the apparent disagreement within the
589 literature (Singh et al. 2016). For example, contrasting trends are observed if one compares
590 SME20 PAH profiles reported in supplementary file Excel Table S2.
591
592 Similarly, to the comparison of biological responses, it is advantageous to compare PAH-
593 profiles generated under a wide variety of combustion conditions and engine models, to
594 better understand the overall burden on human health. One approach to aid comparisons
595 between studies would be to measure PAH emissions at a well-defined set point to
596 supplement the measured PAH-profiles of the chosen conditions. Measurements over a
597 range of well-defined engine conditions would indicate the effect of the engine on PAH
598 profiles. These could include urban driving conditions (e.g. 1600 rpm and 4 bar imep) as well
599 as motorway driving at higher load and speed conditions. Total PAH emissions would be
600 unsuitable for this purpose, due to the variable number of PAHs characterized in the
601 literature; instead, a single or range of PAHs could be selected as representative, for

example pyrene and fluoranthene may be chosen as both species were consistently reported and no study failed to detect either of these species. Alternatively, benzo(a)anthracene and chrysene or benzo(a)pyrene could be used as markers of the engine effect on larger predominantly particle-phase PAH emissions. Quantification of a six-membered ring and a five-membered ring may also identify whether there is any bias towards five- or six-membered rings. Regardless of which PAH(s) is selected as the standard, it would allow authors to compare to a control.

3.4 Critique of the present assessment

To the best of our knowledge this assessment is a novel attempt to systematically compare the toxicity of biodiesel and fossil diesel particles from a variety of experimental designs reported in literature.

There are inherent limitations of this assessment which warrant discussion. Firstly, comparing biodiesel responses between two or more studies inherently assumes a consistent fuel composition and properties. It is conventional to define biodiesel by the feedstock, which may be acceptable for clearly defined esters such as those derived from coconut oil but problematic for waste cooking oils due to geographic variance. Furthermore, the difficulty comparing between biodiesels defined by feedstock is exacerbated by the uptake of novel fuels: whether that be from feedstocks not represented by the toxicology literature (e.g. edible oils, non-edible oils, microalgae (Selvaraj et al. 2019)) or through biodiesel blends (e.g. PME/CoME (Karavalakis et al. 2009)), SME/PME (Soriano et al. 2020)). A definition of biodiesel based on fuel characteristics accounting for the average chain length (or average molecular weight), average number of double bonds (i.e. iodine value) and level of oxidation, would improve this assessment as these properties are feedstock agnostic and broaden the applicability of any findings. Moreover, establishing a minimum fuel standard for the reference fuel would be an additional improvement.

The choice to define valid comparisons as those which meet a minimum standard of statistically significant difference ($p < 0.05$) between either of the test fuels and the control is subjective. Without a minimum threshold this analysis would include 877 paired responses from 40 articles. A significance threshold was defined to exclude responses where the uncertainty within the experiment undermines the confidence in the comparison. Alternative approaches include a minimum fold change, a minimum absolute difference to the control or no threshold for a change which would include unchanged endpoints; however, these alternative approaches are subject to the same criticism as the present assessment and the latter would need to distinguish between a true no effect or an apparent no effect due to exposure below a threshold dose. Not including investigated endpoints which remained unchanged from the control, may have inadvertently introduced bias by reducing the influence of these endpoints on the assessment.

Assignment of biomarkers to explicit categories may be contentious. Adverse effects within tissues in response to toxicological insult involve multiple signalling events and cell types. Cell type specific biomarkers exist for resident and inflammatory cell types (Travaglini et al. 2020), that can be highly relevant in directing outcome but are unlikely to be captured by examining single cell types and a restricted set of biomarkers as occurs in many in vitro studies. In addition, there is some overlap between certain biomarkers across signalling pathways, for example genes such as NQO1, a prototypical Nrf2 dependent genes has also been observed as regulated by the AHR (Jennings et al. 2013). This may be particularly important for assignment of either oxidative stress or direct AHR activation as responsible for NQO1 levels in response to stimulation from chemicals such as PAHs. Ultimately, the use of more complete cellular models (multiple cell types) combined with comprehensive biomarker assessment, such as provided by omics technologies is likely to give a more complete picture of how toxicological effects can be modelled. Lastly, the present methodology does

not distinguish between biomarkers within the same health category, for example, secretion of TNF and IL-8 are considered comparable.

3.5 Recommendations for future research

Future work comparing more than one biodiesel at multiple blend levels would be desirable to build a knowledge base to help identify fuel structural features that may be responsible for biodiesel apparent toxicity. Improved reporting of fuel composition (e.g. ester profile) would permit correlations between the pooled biological responses and fuel characteristics (e.g. average chain length, degree of unsaturation). As an example, genotoxicity may decrease with increasing average molecular weight of FAME biodiesel (Figure 6). Additionally, the establishment of an engine test set point to measure PAH-emission profiles would provide further insight into the effect of the engine model on measured profiles, assisting future comparisons. Further development of this assessment by accounting for experimental variables and inclusion of co-exposure and *in vivo*, would close the gap between the laboratory and the field of epidemiology; to provide the necessary information to support legislators to reduce net CO_2 emissions and improve public health.

The *in vitro* models captured within this assessment comprise common mammalian cell lines and bacteria, typically used for cursory toxicological assessment. Limitations exist however in how adverse cellular outcomes derived from endpoints in these models can be interpreted to inform human hazard potential. For example, the airway epithelial BEAS-2B and A549 models are SV-40 transformed and tumour derived cells respectively, and do not represent normal cells within the human lung. Together with a lack of appropriate culture differentiation and characterisation, needed to establish normal physiological, metabolic and detoxification functions, such cultures typically lack the ability to respond as corresponding airway cells would *in vivo*. Future *in vitro* assessments relevant for organ specific toxicity, should therefore encourage the use of normal primary human cell types and a minimum standard of

cellular differentiation. An additional limitation regards a lack of understanding how gaseous components may modify particulate toxicity and thus exclude studies that report whole exhaust exposure from this assessment. Furthermore, the use of a narrow selection of toxicological endpoints within these studies is limiting. This is particularly important, as recent advances in biochemical analyses including omics technologies, has allowed for a more comprehensive assessment of cellular adverse effects, allowing for more accurate and informative hazard assessments. Adverse outcome pathway approaches incorporating this mechanistic information (Leist et al. 2017), as well as dosimetric estimates should also be encouraged in the future, as a framework to translate often abstracted biochemical information, towards a more regulatory decision-making understanding.

4.0 Conclusions

This work presents an assessment of the comparative toxicity of biodiesel particulate relative to fossil diesel under comparable conditions. Mean biodiesel responses were compared to the reference fuel within each study. This assessment includes all paired responses that are confirmed to statistically differ ($p < 0.05$) from the control. Each paired response was assigned to an appropriate biomarker category: inflammation, oxidative stress, cytotoxicity, genotoxicity or mutagenicity. Ratios above 1.0 suggest biodiesel enhances particle toxicity, whereas, ratios below 1.0 suggest the reverse conclusion.

Overall, two-thirds of paired responses had lower particle toxicity with neat or blended biodiesel. A log-normal distribution skewed towards lower biodiesel toxicity with 50 % of paired responses possessing a ratio between 0.7 and 1.4. Ratios were above or equal to the pooled average reduction for RME (73 % of 158 comparisons), AFME (68 % of 47 comparisons) and PME (67 % of 63 comparisons) whereas WCOME was appreciably below the pooled average (14 % of 37 comparisons below unity), suggesting differences between biodiesels may alter the physical or chemical properties of particles. Improved reporting of

fuel composition may identify which features are responsible for the apparent change in toxicity.

Large differences between fuels were observed for category-specific biomarkers, as shown in the summary of the literature analysis (Figure 9). Particles derived from neat or blended RME induced a lower inflammatory response compared to reference diesel (30 out of 33 ratios were below 1.0), in contrast SME particulates were mostly more inflammatory (3 out of 18 ratios were below 1.0). SME may reduce particle-induced oxidative stress compared to reference diesel. Biomarkers of cytotoxicity showed the largest fuel effect including PME reducing particle cytotoxicity while WCOME and AFME were notably more cytotoxic than reference diesel. HVO was mostly comparable to fossil diesel, which may be explained by the absence of oxygenates compared to FAME biodiesels.

Additionally, this analysis suggests a tentative link between the trends observed in the biological responses and particle-phase PAH profiles. WCOME may increase particle-phase PAH emissions which may increase the overall toxicity towards human lung cells, whereas the opposite may be true for PME. Mixed results were observed for the other biodiesels. However, a comparison between particle composition and adverse effects is limited as only a few studies report PAH profiles and biological responses.

Overall, this work highlights the fragmented nature of the biodiesel literature and identifies understudied test blends and fuels. We recommend the community coordinate its efforts to focus on both the environmental and social impacts of transport fuels. At present, we cannot say with certainty which fuels are more or less toxic than fossil diesel, however, the present analysis does demonstrate quantitatively that we cannot assume biodiesels are interchangeable from a health perspective. Standardised reporting of fuel composition and careful consideration of the biological assay would support an overall consensus on how the fuel structure may alter particle toxicity, aiding the development of novel fuels beyond

741 traditional FAME biodiesel. A fundamental understanding of this manner would support
742 legislators' aims by reducing greenhouse gas emissions without further detriment to public
743 health.
744
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749
750 **Nomenclature**
751
752 Animal fat methyl ester, AFME; Coconut oil methyl ester, CoME; Diesel oxidation catalyst,
753 DOC; Elementary carbon, EC; Extractable organic matter, EOM; Fatty acid methyl ester,
754 FAME; Hydrotreated vegetable oil, HVO; Jatropha oil methyl ester, JME; Linseed oil methyl
755 ester, LiME; Non-methane hydrocarbons, NMHC; Sum of nitrogen oxides (NO and NO_2),
756 NO_X; Organic carbon, OC; Polycyclic aromatic hydrocarbons, PAH; Palm oil methyl ester,
757 PME; Particulate matter, PM; Particulate number, PN; Preferred reporting items for
758 systematic reviews and meta-analyses, PRISMA; Rapeseed oil methyl ester, RME; Reactive
759 oxygen species, ROS; Soluble organic fraction, SOF; Soybean oil methyl ester, SME; Total
760 hydrocarbon, THC; Waste cooking oil methyl ester, WCOME.
761
762 **Supplementary material**
763
764 Supplementary material is available from the published version of this article:
765 https://doi.org/10.1016/j.atmosenv.2021.118570.

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1219 Table 1: Database inputs to complete search strings described in-text for article recovery.

Input 1	Input 2	Input 3
HVO or “hydrotreated vegetable oil”, RME or “rapeseed methyl ester”, SME or “soy methyl ester”, PME OR “palm oil methyl ester” OR “palm methyl ester”, FAME or “fatty acid methyl ester”, “waste cooking oil” OR “used cooking oil”	PM or Partic*, PAH or “polycyclic aromatic hydrocarbons”	Airway, Allergen, Asthma, Carcinogen*, Cyto*, DNA, Gene, Genotoxic*, Inflammation, Lung, Mutagen*, Oxidative stress, Respiratory

1220

1221 Table 2: The number of comparisons for each biodiesel and biomarker category included in
1222 this evaluation.

Fuel	Inflammation	Ox. stress	Cytotoxicity	Genotoxicity	Mutagenicity	Total
HVO	46 (5)	32 (7)	82 (4)	40 (4)	1 (1)	201 (9)
RME	33 (3)	36 (5)	37 (4)	32 (5)	20 (6)	158 (13)
PME	0 (0)	0 (0)	62 (6)	0 (0)	1 (1)	63 (7)
SME	18 (3)	14 (5)	1 (1)	4 (1)	25 (3)	62 (10)
AFME	14 (2)	17 (3)	4 (1)	12 (1)	0 (0)	47 (3)
WCOME	0 (0)	13 (3)	24 (1)	0 (0)	0 (0)	37 (3)
SME/PME	0 (0)	0 (0)	0 (0)	3 (1)	4 (1)	7 (1)
LiME	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
CoME	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
JME	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
Total	111 (8)	112 (14)	210 (13)	91 (9)	54 (9)	578 (34)

1223 Note: Parentheses denote the number of articles which have contributed to the number of
1224 comparisons stated for that biodiesel and biomarker category.

1225 Table 3: Summary of the assays assigned to the five discrete biological categories.

Biological category	Summary
Inflammation	Primarily focused on pro-inflammatory cytokine (e.g. IL-6, IL-8, TNF-α) gene expression or secreted protein content.
Oxidative stress	Oxidative potential was assessed with cellular and acellular assays. Acellular assays measured the rate of Dithiothreitol (DTT) or ascorbic acid consumption, whereas cellular assays primarily assessed mRNA levels for *Nrf2* responsive genes, GSH/GSSG ratio or utilised fluorescent probes.
Cytotoxicity	Membrane permeable or impermeable dyes distinguished between viable and membrane compromised cells. Alternatively, the microtox test, caspase activity, pro- and anti-apoptotic gene expression and markers of cellular metabolism were investigated.
Genotoxicity	Measurements of direct and in-direct chromosomal damage with the comet assay, H2AX phosphorylation, micronucleus assay, mRNA expression of genes belonging to the DNA damage response pathway and formamidopyrimidine DNA glycosylase (FPG) activity.
Mutagenicity	The standardised Ames salmonella assay (Mortelmans and Zeiger 2000) was widely adopted. Tester strains TA98 and TA100 were included with metabolic activation (+ S9 fraction).

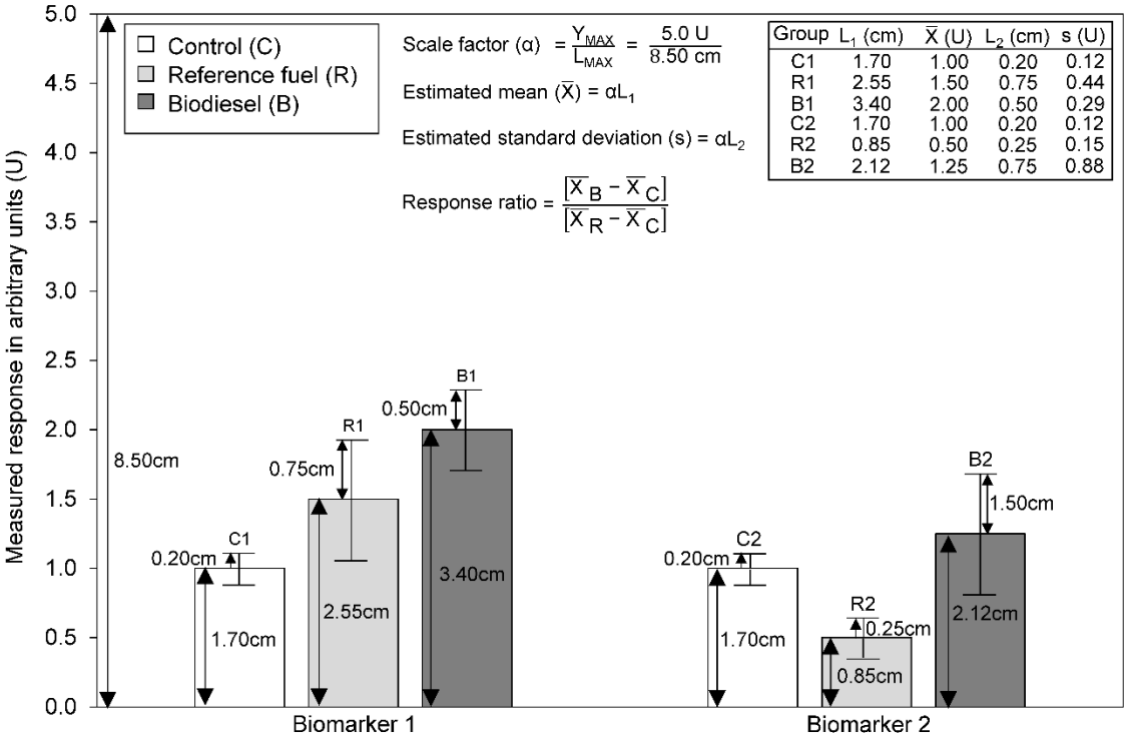


Figure 1: An example of the procedure to extract the mean response and standard deviation from graphical data reported in literature, and estimation of the comparative potency of biodiesel. L_2 may represent other measures of variability which are converted to standard deviation accordingly. Y_{MAX} is the maximum value shown on the figure Y-axis, whereas L_{MAX} is the measured distance from the X-axis to Y_{MAX}.

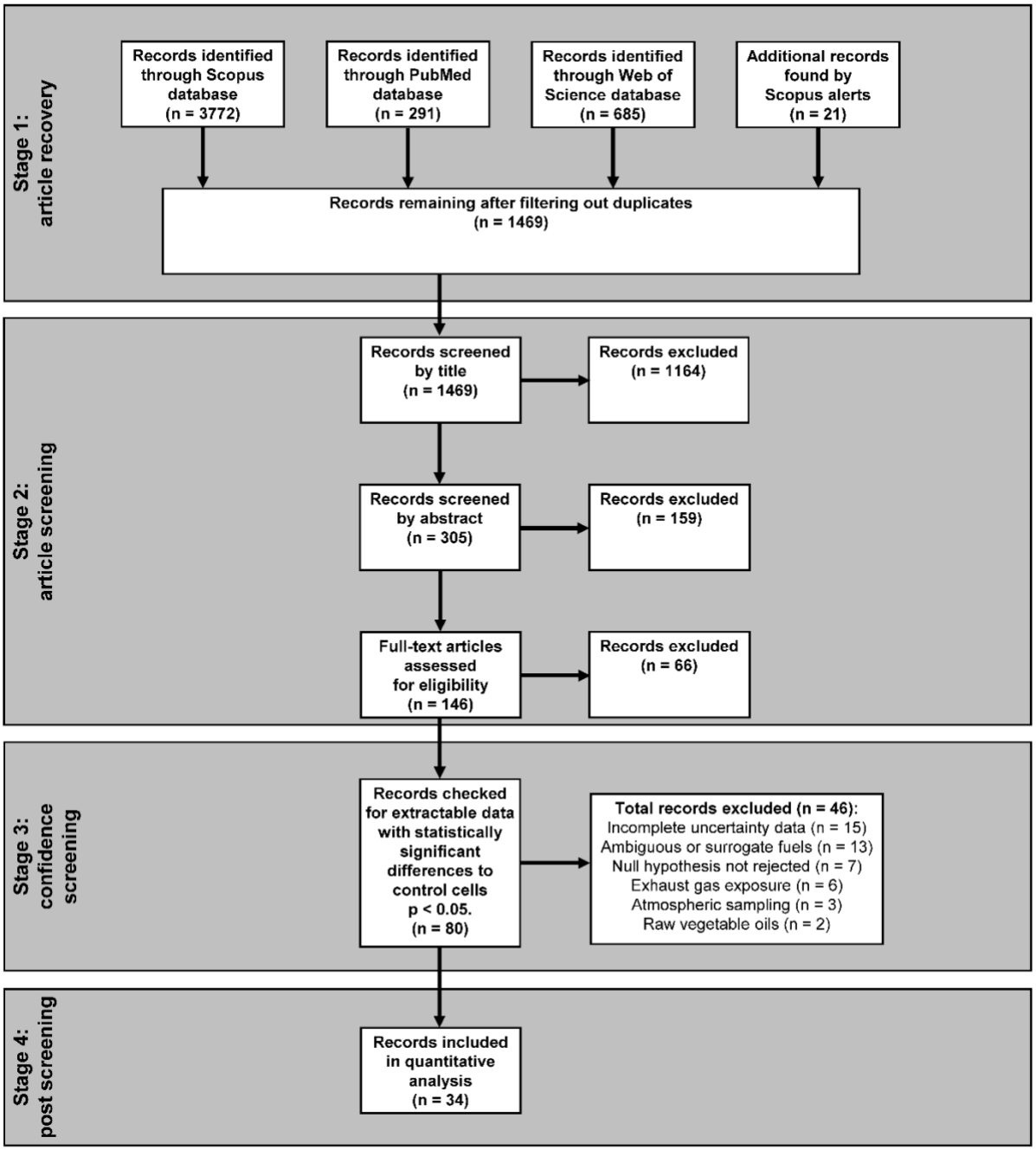
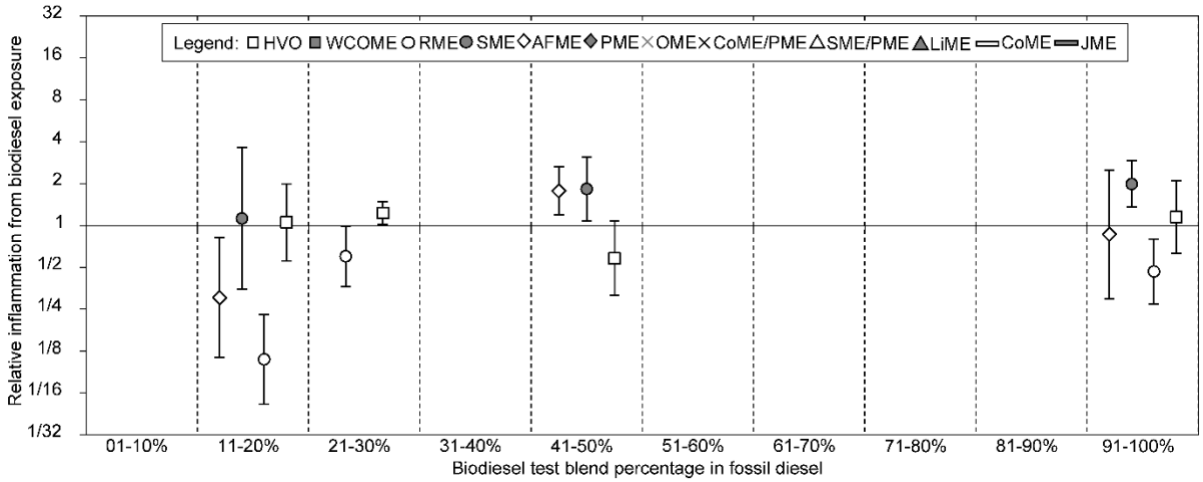


Figure 2: Flowchart of the literature search and article selection process, following the guidelines outlined in the PRISMA framework (Moher et al. 2009).



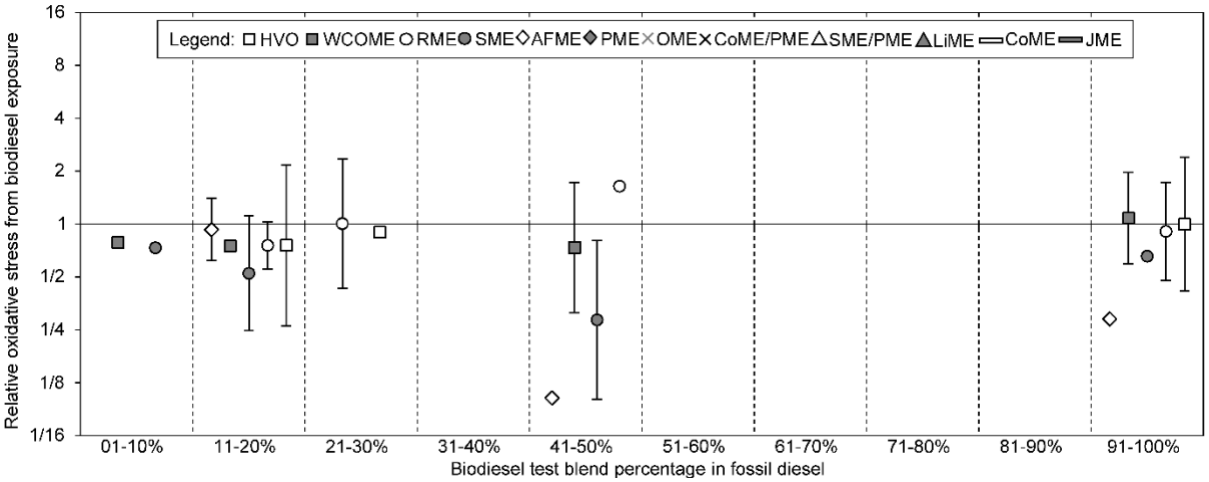
1235

1236 Figure 3: Geometric mean of ratios of the mean biodiesel response with respect to the

1237 reference fuel for biomarkers of inflammation (see Table 3) induced by exposure to particles

1238 or particle-extracts. Error bars represent ± 1 multiplicative standard deviation (s*).

1239



1240

1241 Figure 4: Geometric mean of ratios of the mean biodiesel response with respect to the

1242 reference fuel for biomarkers of oxidative stress (see Table 3) induced by exposure to

1243 particles or particle-extracts. Error bars represent ± 1 multiplicative standard deviation (s*).

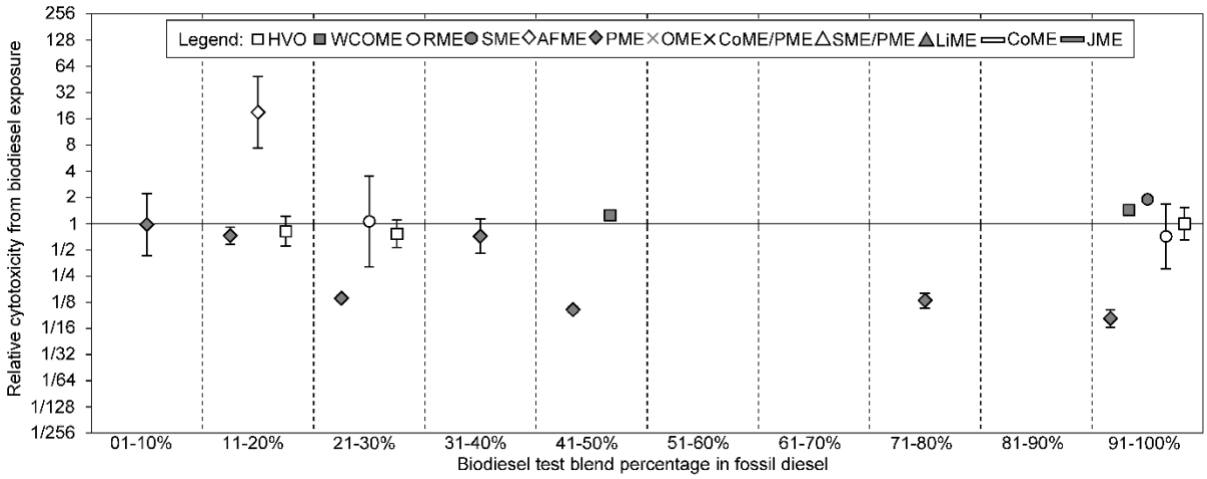


Figure 5: Geometric mean of ratios of the mean biodiesel response with respect to the reference fuel for biomarkers of cytotoxicity (see Table 3) induced by exposure to particles or particle-extracts. Error bars represent ± 1 multiplicative standard deviation (s*).

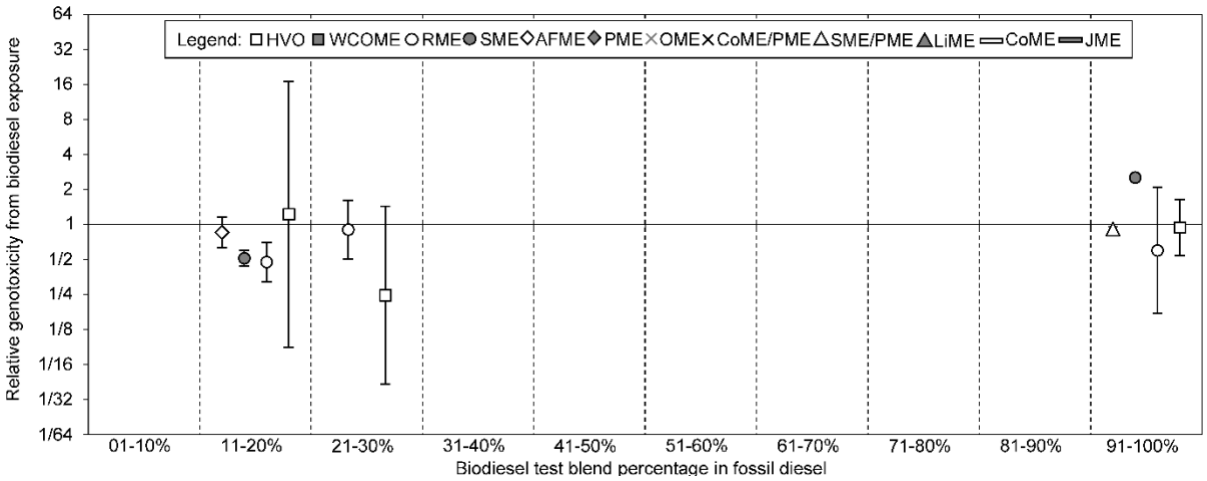


Figure 6: Geometric mean of ratios of the mean biodiesel response with respect to the reference fuel for biomarkers of genotoxicity (see Table 3) induced by exposure to particles or particle-extracts. Error bars represent ± 1 multiplicative standard deviation (s*).

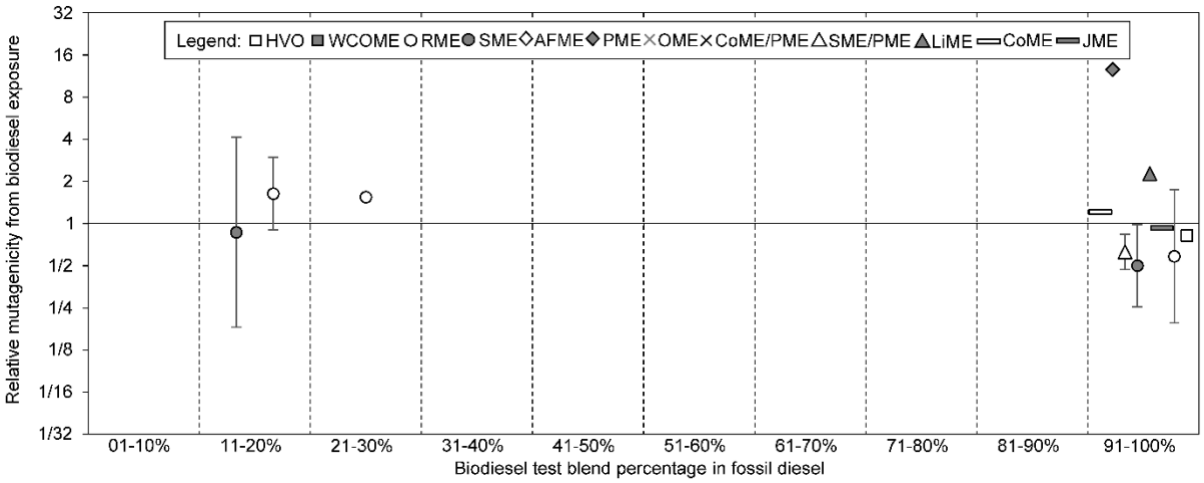


Figure 7: Geometric mean of ratios of the mean number of reverts (i.e. mutagenicity) induced by exposure to biodiesel and reference fuel particle-extracts. Error bars represent ± 1 multiplicative standard deviation (s*).

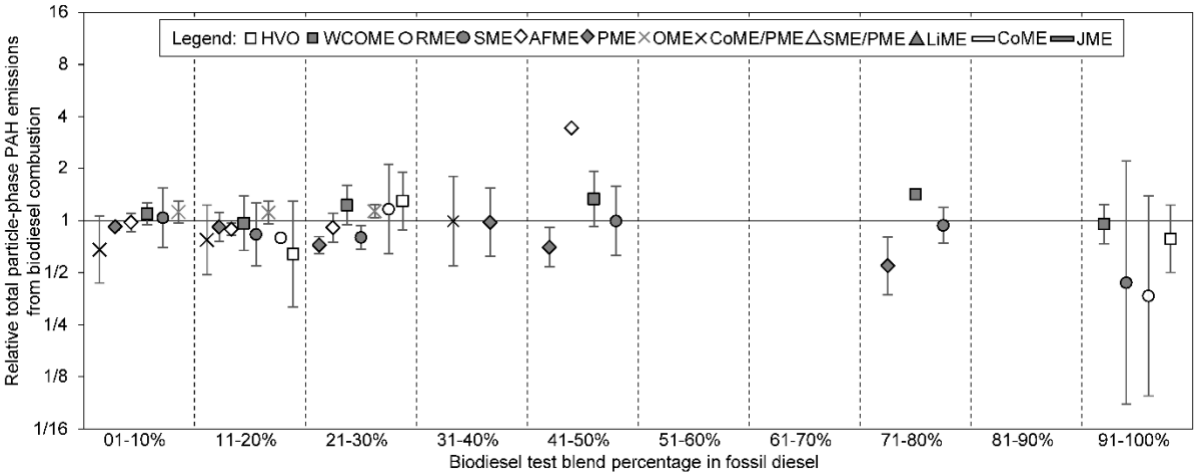


Figure 8: Total PAH emissions on biodiesel particles compared to fossil diesel under comparative conditions. Notes for symbols: Data was extracted directly from graphical/tabulated data reported in literature and converted into PAH ng/mg-soot for comparison purposes. Markers indicate geometric mean and error bars represent ± 1 multiplicative standard deviation (s*).

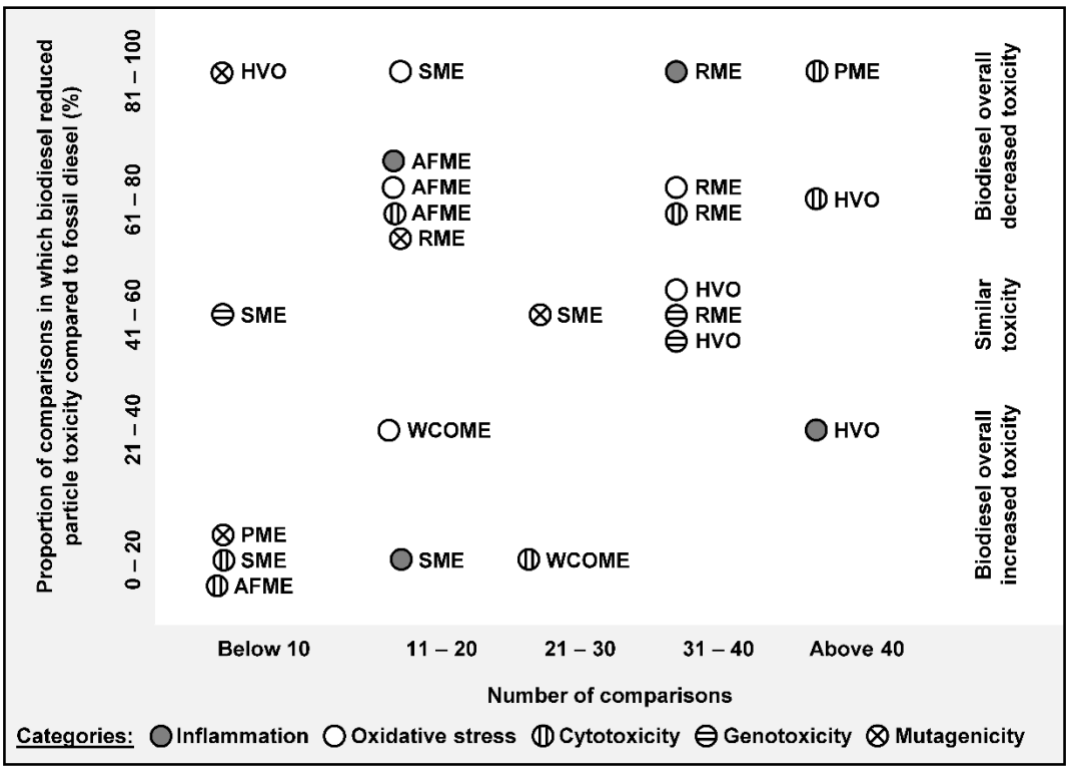


Figure 9: Comparisons in which biodiesel induced a lower response in a measured biomarker compared to fossil diesel tested under the same experimental conditions, plotted against the total number of comparisons for that biodiesel and biomarker category.