Particle characterization and toxicity in C57BL/6 mice following instillation of five different diesel exhaust particles designed to differ in physicochemical properties

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Abstract
Background Diesel exhaust is carcinogenic and exposure to diesel particles cause health effects. We investigated the toxicity of diesel exhaust particles designed to have varying physicochemical properties in order to attribute health effects to specific particle characteristics. Particles from three fuel types were compared at 13% engine intake O 2 concentration: MK1 ultra low sulfur diesel (DEP13) and the two renewable diesel fuels hydrotreated vegetable oil (HVO13) and rapeseed methyl ester (RME13). Additionally, diesel particles from MK1 ultra low sulfur diesel were generated at 9.7% (DEP9.7) and 17% (DEP17) intake O 2 concentration. We evaluated physicochemical properties and histopathological, inflammatory and genotoxic responses on day 1, 28 and 90 after single intratracheal instillation in mice compared to reference diesel particles and carbon black.

Results Moderate variations were seen in physical properties for the five particles: primary particle diameter: 15-22 nm, specific surface area: 152-222 m 2/g, and count median mobility diameter: 55-103 nm. Larger differences were found in chemical composition: organic carbon/total carbon ratio (0.12-0.60), polycyclic aromatic hydrocarbon content (1-27 mg/mg) and acid-extractable metal content (0.9-16 mg/mg). Intratracheal exposure to all five particles induced similar toxicological responses, with different potency. Lung particle retention was observed in DEP13 and HVO13 exposed mice on day 28, with less retention for the other fuel types. RME exposure induced limited response whereas the remaining particles induced dose-dependent inflammation and acute phase response on day 1. DEP13 induced acute phase response on day 28 and inflammation on day 90. DNA strand break levels were not increased as compared to vehicle, but were increased in lung and liver compared to blank filter extraction control. Neutrophil influx on day 1 correlated best with estimated deposited surface area, but also with elemental carbon, organic carbon and PAHs. DNA strand break levels in liver on day 90 correlated with acellular particle-induced ROS.

Conclusions We studied diesel exhaust particles designed to differ in physicochemical properties. Our study highlights particle size, elemental carbon content, PAHs and ROS-generating potential as physicochemical predictors of diesel particle toxicity.

Introduction
The classification of diesel exhaust as carcinogenic [1] and the reported diesel particle airway toxicity and systemic effects in both humans [2, 3, 4] and in mice [5, 6, 7, 8, 9, 10, 11, 12] necessitates further studies. The ultrafine particle fraction has been suggested to be a main mediator of the carcinogenic effects and to contribute to other adverse health effects [13], but it is less known if it can be related to specific physicochemical particle properties.

Diesel engine exhaust consists of a particulate phase containing insoluble high surface area carbonaceous particles (elemental carbon; EC, also known as black carbon) with absorbed metal oxides and an adsorbed liquid fraction containing low volatility organic matter, including polycyclic aromatic hydrocarbons (PAH) formed in the combustion process and branched alkanes originating from the lubrication oil. In addition, diesel exhaust consists of gases including carbon monoxide, nitrogen oxides (NOx), and volatile organic compounds (VOCs) [14, 15]. Particulate matter and gas emissions can be reduced either by altering the combustion process or by installing after-treatment systems. Exhaust gas recirculation (EGR) is commonly used as a NOx reduction technique, but as the reduction of oxygen concentration and temperature reduce NOx emissions, soot emissions increase [13, 21]. Hence, the physicochemical characteristics of the emitted particles, such as content of PAH, metals and ratio of elemental and organic carbon [14, 16, 17], depend on engine combustion conditions. Particle size and thereby SSA is a driver of pulmonary inflammation [18] and acute phase response [19, 20] whereas certain metals and PAH, as well as ROS formation are linked to genotoxicity [21, 22]. Several PAH compounds including benzo[a]pyrene are classified as carcinogenic or possibly carcinogenic by IARC [23].

Emissions also depend on fuel type [17, 24, 25, 26], and recently, renewable diesel fuels have been introduced on large scales to replace fossil diesel [27, 28, 29]. There is incomplete knowledge on the potential adverse health effects of emissions from these new types of renewable diesel fuels. Renewable diesel fuels are based on vegetable oils, animal fats or waste products and are used in conventional engines as full substitution or in blends. Rapeseed methyl ester is a fatty acid methyl ester and differs from fossil diesel, as it has a high O₂ content in the fuel (~ 10%). More recently,
Second generation renewable diesel fuels have become available where the oxygen content is removed by hydrogen treatment. One such example is hydrogen treated vegetable oil (HVO) which is a synthetic/paraffinic diesel produced from plant and animal sources and chemically similar to fossil diesel, except it has no aromatic content and shorter carbon chains [25].

In this study, we investigated the toxicological effects following pulmonary exposure to diesel exhaust particles collected without after-treatment from a controlled modern heavy-duty diesel engine in a laboratory environment. A detailed description of the generation procedure and physicochemical analysis is described by Gren LM, Malmborg VB, Jakobsen NR, Shukla PC, Bendtsen KM, Shamun S, Eriksson AC, Essig Y, Krais AMK, Loeschner K, Strandberg B, Vogel U, Tunér M and Pagels J in “Effect of renewable fuels and inlet O$_2$ concentration on diesel engine emission characteristics and reactive oxygen species (ROS) formation” (submitted manuscript, forwardly referred to as “Gren et al, submitted manuscript”), where focus is on relationships between combustion conditions and fuel types with different emission levels and physicochemical properties, including ROS formation in vitro. Briefly, the engine was operated in different modes to vary the physical and chemical properties of five different diesel exhaust particles (DEP). Three different levels of EGR were chosen in order to generate diesel exhaust particles with a) high fraction of PAH and refractory organic carbon (OC) relative to EC, b) high fraction of EC and c) high fractions of lubrication oil related OC and metals relative to EC. As such, three particle types were generated by changing the combustion conditions measured as intake O$_2$ concentration in the combustion cylinder of 9.7% (DEP9.7), 13% (DEP13) and 17% (DEP17) with MK1 low-sulfur fossil diesel as fuel. In order to investigate the effect of renewable fuels, two additional particle types were generated at 13% intake O$_2$ concentration in the combustion cylinder, using renewable rapeseed methyl ester (RME13) and hydrotreated vegetable/animal oil (HVO13) as fuel. Thus, the diesel exhaust particles were designed to differ in primary particle size and in content of PAHs, OC, EC and metals.

The aims of the study were: 1) to study the toxicity of five diesel exhaust particle samples designed to differ in physicochemical properties and 2) to identify physicochemical properties driving the toxicity
of the particles. Toxicity was evaluated in terms of reactive oxygen species generation and inflammatory and genotoxic responses in mice on day 1, 28 and 90 after exposure to 6, 18 and 54 µg collected particles by single intratracheal instillation.

Results

Physicochemical properties

The characterization of the five different combustion particles in terms of particle mobility size in air, morphology by transmission electron microscopy (TEM), elemental carbon (EC)/organic carbon (OC) to total carbon (TC) ratio, PAH and metal contents are described in detail in Gren et al. (submitted manuscript) and shown in Table 1. Overall, combustion conditions were the most important factors for all particle characteristics on a relative basis. The combustion conditions heavily affected the mobility size, OC, EC, metal and PAH content. The engine emissions measured as total PM1 were reduced by 65% for the renewable diesel fuels compared to the fossil diesel fuel (Table 1).

Analysis of OC, EC, metal- and PAH content was carried out on extracted particles, whereas TEM analyses was done on diluted exhaust particles.

Electron microscopy

The five different particle samples from experimental combustion emissions and the CB reference sample were visualized by transmission electron microscopy (Figure 1). The morphology of the particles generated with 13% intake O₂ concentration, namely DEP13, HVO13 and RME13 (Figure 1 b, d, e) and DEP17 with 17% intake O₂ concentration (Figure 1 c) were all similar in appearance and showed typical soot agglomerates (diameter ~50-300 nm) of smaller primary particles (diameter ~10-30 nm). In contrast, the soot agglomerates from low temperature combustion (DEP9.7) had less defined primary particles and appeared more fused (bridging between primary particles) compared to the other samples (Figure 1 a).

Organic and elemental carbon, primary particle size and specific surface area

Primary particle size and specific surface area (SSA) were estimated and calculated by measuring the diameters of well-defined spherical primary particles from the TEM images (assuming no voids inside the primary particles). The primary particle size decreased with the intake O₂ concentration for the
MK1 diesel samples. The largest primary particle size was found for HVO13, 23 nm, followed by 17 nm for DEP13 and 16 nm for RME13 (Table 1). Estimated SSA was overall rather similar, with the largest SSA for RME13 with 222 m²/g and the lowest for DEP9.7 with 152 m²/g. The EC fraction was highest for the particles generated at 13% intake O₂, and the lowest for 9.7% intake O₂. The EC fraction was higher for DEP13 compared to HVO13 and RME13.

Table 1. Mass, size, carbon composition and surface area of particles

| Particles | Fuel | Intake O₂ (%) |
|-----------|------|---------------|
|           |      |               |

| Average emitted exhaust PM1 mass concentration (mg/m³)ᵃ |
|---------------------------------------------------------|
| Particle mobility diameter (GMD) (nm)ᵇ |
| Primary particle diameter (GMDₚ) (nm) * |
| Estimated specific surface area (SSA) (m²/g)* |
| Elemental to total carbon (EC/TC) |
| Organic to total carbon (OC/TC) |

ᵃThe average exhaust PM mass concentration (mg/m³), before dilution and particle mobility diameter (nm) with ± 1 std. b
ᵇMobility particle (agglomerate) size based on the number concentration. Measured with the DMS in the dilution tunnel. c
The specific surface area (SSA) was estimated by using the primary particle size (dₚp) distribution and diesel soot densit

**Metal contents**

Semi-quantitative analysis of elemental contents by inductive coupled plasma mass spectrometry (ICP-MS) showed the highest concentrations for Cu and Fe (Table 2). For Cu, Fe and several other trace elements, DEP17 showed 5-17 fold higher metal mass fractions compared to the other four samples. DEP13 had the lowest amount of Cu. RME13 and DEP13 had the lowest amount of Fe.
Table 2. Extracted elemental concentrations (µg/g)

| Particles | DEP | HVO | RME | NIST2975<sup>a</sup> |
|-----------|-----|-----|-----|-----------------------|
| Fuel      |     |     |     |                       |
| Intake O₂ % | 9.7 | 13  | 17  | 13                    | 13                    |
| V         | 14  | 6   | ND  | 3                     | 2                     | ND                    |
| Cr        | 8   | 7   | 52  | 11                    | 7                     | 7/4                   |
| Mn        | 92  | 53  | ND  | 39                    | 43                    | 6/3                   |
| Fe        | 220 | 137 | 2.115 | 247                  | 116                   | 663/516               |
| Co        | 2   | 1   | 88  | 1                     | 1                     | 0/0                   |
| Ni        | 15  | 6   | 118 | 9                     | 25                    | 4/4                   |
| Cu        | 2.349 | 629 | 13.160 | 1.632               | 2.291                 | 23/13                 |
| Ga        | 1   | 1   | 1   | 1                     | 1                     | ND                    |
| As        | ND  | ND  | ND  | ND                    | ND                    | ND                    |
| Se        | 2   | 0   | ND  | ND                    | 0                     | ND                    |
| Rb        | 2   | 1   | 1   | 1                     | 1                     | 13.926/17.003         |
| Sr        | 99  | 54  | ND  | 41                    | 37                    | 2/1                   |
| Ag        | 0   | 0   | 1   | 0                     | 0                     | 0/0                   |
| Cd        | ND  | ND  | ND  | ND                    | ND                    | ND                    |
| In        | 0   | 0   | 0   | 0                     | 0                     | 0/0                   |
| Cs        | 0   | 0   | ND  | 0                     | 0                     | ND                    |
| Ba        | 15  | 10  | ND  | 9                     | 6                     | 26/ND                 |
| Hg        | 0   | 0   | ND  | 0                     | ND                    | 0/0                   |
| Tl        | 0   | 0   | 0   | 0                     | 0                     | ND                    |
| Pb        | ND  | ND  | ND  | ND                    | ND                    | 21/4                  |
| Bi        | 0   | 0   | 0   | 0                     | 0                     | 0/0                   |
| U         | ND  | 0   | ND  | ND                    | ND                    | ND                    |

Elemental concentrations determined by semi-quantitative analysis by ICP-MS (µg/g particle) (ND = not detectable). Blanl and CB were analyzed in duplicates (separated by slash).<sup>a</sup>Results previously published in Bendtsen et al. (2019) [12].<sup>b</sup>Reference values from Ball et al. (2000)<sup>c</sup> (the study only analyzed Co, Cu, Fe, Ni, V, and Zn). Note that we extracted for significantly longer time (several days vs. overnight nitric acid).<sup>d</sup>Reference values from the MAK-Collection for Occupational Health and Safety (written communication of unpublished data of Degussa).<sup>e</sup>

**Content of polycyclic aromatic hydrocarbons (PAHs) in the collected particles**

The samples were analyzed for native PAHs and PAH derivatives by gas chromatography–mass spectrometry (GC-MS). In total, particle extracts were analyzed for 20 native PAHs, 13 alkylated PAHs
(alkyl-PAHs), 14 nitrated PAHs (nitro-PAHs), 10 oxygenated (oxy-PAHs) and 6 dibenzothiophenes (DBTs). Table 3 shows the total amount of different groups of PAH derivatives in µg per g collected particle mass. Values of the individual compounds are given in Additional File A. DEP9.7 showed the highest mass fractions of native PAHs, which was expected due to the low temperature combustion mode caused by the lower intake O₂ concentration. DEP9.7 also contained the highest levels of nitro-PAHs, while DEP17 contained the lowest mass fraction of all measured PAHs.

The highest level of the sum of all PAHs were found in DEP9.7, followed by HVO13, DEP13, RME13 and DEP17. PAHs were inversely proportional to the intake O₂ for DEP9.7, DEP13, and DEP17, which agrees well with a more complete combustion at higher intake O₂ concentration.

Compared to DEP13, HVO13 particles contained higher amounts of all PAHs, especially native PAHs and oxy-PAHs, while RME particles contained lower concentration of total PAHs.

The levels of DBTs followed a different trend than the other PAH derivatives, by increasing with increasing intake O₂ concentrations. DBT levels were highest for DEP17, followed by DEP13 and DEP9.7, while DBT levels were similar for DEP13, HVO13 and RME13.
Table 3. Summary of PAH content (µg/g) in the PM samples. A full list of all PAH derivatives can be found in Additional File A.

| Particles | DEP | HVO       | RME       | NIST2975 |
|-----------|-----|-----------|-----------|----------|
| Fuel      |     |           |           |          |
| Intake O₂ % | 9.7 | 13        | 17        | 13       |
| Native PAHs | 23700 | 2470      | 858       | 9960     | 1180     |
| Alkyl-PAHs | 400  | 483       | 77        | 644      | 150      |
| DBTs      | 47   | 78        | 128       | 86       | 94       |
| Nitro-PAHs | 131  | 21        | 8         | 65       | 40       |
| Oxy-PAHs  | 2490 | 1450      | 314       | 2630     | 596      |
| Total PAHs | 26800 | 4500      | 1390      | 13400    | 2060     |
| BaPeq* (µg/g) | 4685  | 165       | 59        | 1067     | 60       |

*Sum of BaPeq for 12 PAH (see Additional File L) out of in total 63 different measured PAHs and PAH derivatives. Levels of native PAHs and PAH derivatives analyzed by GC-MS analysis (µg/g particles). Particle samples, blank control filters and standard reference material NIST2975 were analyzed for 20 native PAHs, 13 alkylated PAHs (alkyl-PAHs), 14 nitrated PAHs (nitro-PAHs), 10 oxygenated PAHs (oxy-PAHs) and 6 dibenzothiophenes (DBTs). PAH concentrations of blank control filters were substracted. A full list of all PAH derivatives can be found in Additional File A.

Reactive oxygen species (ROS) generation

Reactive oxygen species generation by the five different particle samples and by CB was measured acellularly, where generated ROS causes formation of 2’,7’ dichlorofluorescein (DCF) from DCFH₂ which can be spectrofluorimetrically measured. The initial slope of the curve (alfa values) of measured fluorescence of the five particles are given in Table 4. In comparison, the alfa value of CB was 41554. Detailed ROS results are discussed in detail in Gren et al. (submitted manuscript).

Table 4. ROS generation (in arbitrary units)

| Particles | DEP | HVO       |
|-----------|-----|-----------|
| Fuel      |     |           |
| Intake O₂ % | 9.7 | 13        | 17        |
| ROS (alfa) | 2685 | 14682     | 24039     |

The initial slope of the curve (alfa values) of measured fluorescence of the five particles are given in arbitrary units.

Particle size distribution in dispersion
For the in vivo study, the diesel exhaust particles were collected on Teflon filters with a PM1 preseparator, extracted using methanol, and dispersed in vehicle and diluted. The particles were dispersed in 0.1% Tween in Nanopure water and sonicated to achieve stable dispersions [33, 34]. The hydrodynamic number and intensity size distributions were measured by Dynamic Light Scattering (DLS) for particle concentrations of 0.12, 0.36 and 1.08 mg/ml corresponding to the doses of 6, 18 and 54 μg particulate matter in 50 μL instillation volume per mouse. Similar distributions of particles sizes corresponding to agglomerates/aggregates were observed for the five different particles, in the same range as CB and NIST2975, where CB was the smallest (Additional File, B).

**Pulmonary exposure of C57BL/6 mice**

Mice were exposed by intratracheal instillation to 6, 18, and 54 μg of dispersed particles and euthanized on day 1, 28 and 90. Exposure to the vehicle itself (Nanopure water with 0.1% Tween) was included as exposure control (vehicle). In addition, exposure to blank filter extraction dispersed in 0.1% Tween was included as blank filter extraction control as well (extract). Carbon black Printex90 particles dispersed in the same vehicle (0.1% Tween) were included at a single dose level as reference particle (CB).

**Pulmonary histopathology**

Pulmonary histopathology was evaluated on day 28 (Figure 2) and day 90 (not shown). Generally, only minor histopathological changes were observed. Most particle retention on day 28 was observed in DEP13 and HVO13 exposed mice (Figure 2b, d). Histopathological changes observed for these particles were related to macrophage and lymphocyte infiltration. For DEP9.7, DEP17 and RME13, particles were scarce and no apparent histological changes were observed (Figure 2a, c, e). All five particle types appeared as black micron-sized agglomerates mainly phagocytized in macrophages (Figure 2a₁-e₁). In addition, some larger dense aggregates were observed for RME13 (Figure 2e₂).

**Cell composition in bronchoalveolar lavage (BAL) fluid**

Pulmonary inflammation was evaluated 1, 28 and 90 days post-exposure by differential cell count of BAL fluid cell composition (Figures 3 and 4, and Additional File C and D).

*Day 1 post-exposure*
Neutrophil influx was significantly increased in mice exposed to 54 µg of DEP9.7, DEP13, DEP 17, and HVO13 compared to vehicle control with similar, significant dose-response relationships. The 54 µg doses exceeded the level of response to the CB reference at the same dose level. DEP9.7 exposure resulted in most mice with a high response (Figure 3 a). In contrast, RME13 did not cause significantly increased neutrophil influx as compared to vehicle. No consistent differences were found for influx of lymphocytes and macrophages compared to vehicle (Figure 3 b and c). For eosinophils, only DEP13 at 54 µg significantly increased the influx compared to vehicle, but large variations were seen for all particles with factor 10 difference between low- and high responding mice (Figure 3 d).

Day 28 post-exposure

On day 28, some particle exposures seemingly resulted in reverse dose response relationships for neutrophil influx, with significant increase for 6 µg of RME13 and DEP13 compared to vehicle (Additional File, D 1). For 18 µg DEP13, there was a very low response, even significantly decreased compared to vehicle mice. CB exposure was not statistically different from vehicle for neutrophils. However, lymphocytes were significantly increased for CB exposed mice compared to vehicle (Additional File, D 2). No statistical differences were found for macrophages and eosinophils (Additional File, D 3 and 4).

Day 90 post-exposure

On day 90 following exposure, DEP13 had a significantly increased numbers of neutrophils and lymphocytes compared to vehicle (Figure 4 a and b). Presence of macrophages was also observed for all exposed mice at levels around 40’000 cells, including vehicle mice, where HVO13 had a noteworthy lower cell number (Figure 4 c). Except for some individually high responsive animals, no statistically significant differences were seen for eosinophils (Figure 4 d).

**Serum Amyloid A in lung**

Day 1 post-exposure

Saa3 mRNA levels were used as biomarkers of acute phase response [19, 35] in lung tissue. On day 1, significant, dose-dependent increase in Saa3 mRNA levels compared to vehicle was observed in lung tissue for all exposures, except for RME13 (Figure 5 a). DEP9.7 (p<0.0003), DEP13 (p<0.0003), DEP17
(p<0.0003), and HVO13 (p<0.0001) of 54 µg all exceeded the level of CB. DEP13 (p=0.0110) and HVO13 (p=0.0074) of 18 µg were also significantly increased compared to vehicle. Saa3 mRNA levels in lung correlated well with neutrophil influx (R²=0.5902, p=0.0002) (Additional File E).

Test for linear dose-response was significant for all exposures, except for RME13 (Figure 5 a).

**Day 28 and 90 post-exposure**

On day 28, DEP13 of 54 µg (p=0.0034) and CB (p=0.0007) were still increased compared to vehicle (Figure 5 b). No significant differences were seen on day 90 (Figure 5 c).

**DNA damage**

Genotoxicity was evaluated as DNA strand breaks in the comet assay, using comet tail length and % tail DNA in BAL derived cells, lung cells and liver cells on day 1, day 28 and day 90 post-exposure (Figure 6 and 7, and Additional File F). Generally, variations were observed between exposures and within the vehicle control groups over doses and time points. At no time points were any exposures or CB significantly increased compared to vehicle, and some were decreased. There were no differences on day 1 (data not shown). There was a significant difference between vehicle and blank filter extraction control, especially for liver on day 28 (p=0.0023), and for all tissues on day 90 (p-values: 0.0005-0.0305), with blank filter extraction control samples having significantly lower DNA strand break levels compared to vehicle (Figure 6 and 7).

When compared to the blank filter extraction control DEP13 at 6 µg was increased on day 28 for tail length in BAL cells (p=0.078). For tail length in liver cells, RME13 (p=0.0017), HVO (p=0.004), DEP13 (6 and 18 µg: p=0.0480), DEP17 (18 µg: p=0.0072; 54 µg: p<0.0001), and CB were increased (p=0.0405) (Figure 6). DEP17 and HVO13 were increased in both lung (DEP17: p=0.0008; HVO13: p=0.0187) and liver cells (DEP17: p=0.0026; HVO13: p=0.0062) compared to blank filter extraction control (Figure 7).

**Correlations**

Linear regression analyses were carried out in order to assess which physicochemical properties best predicted inflammation and acute phase response. The estimated deposited specific surface area (SSA), elemental carbon (EC), organic carbon (OC) and PAHs were calculated by multiplying the
physicochemical values in Table 1 by the dose (EC and OC: dose in µg * EC fraction = deposited EC in µg; for SSA: dose in g * SSA m²/g = deposited SSA in g; for PAH: dose in g * PAH µg/g = deposited PAH in µg). In this study, the SSA of CB was estimated to 230 m²/g, although other values have been reported [5, 11, 36].

Neutrophil influx correlated well with estimated deposited SSA on day 1 (Figure 8 a), where 50-60% of the variation in neutrophil influx could be explained by estimated deposited SSA. To compare with known reference particles, the plot was made with either inclusion ($R^2 = 0.6388, p<0.0001$) or exclusion ($R^2=0.5523, p=0.0010$) of previously published data on surface area and neutrophil influx on standard reference material (SRM) NIST2975 and NIST1650, which are diesel exhaust particles derived from a diesel-powered industrial forklift and a heavy duty truck, respectively. The vehicle used for this historical data was Nanopure water, without the addition of Tween80 [12]. In a previous study comparing the effect of vehicle on carbon black-induced neutrophil influx, there was no differences between 0.1%Tween80 and Nanopure water [33]. Similar significant correlations with neutrophil influx, with 40-50 % of the variation explained, were seen when deposited elemental carbon (EC), organic carbon (OC) and deposited PAHs were used as dose metrics (EC: $R^2=0.522, p=0.0016$; OC: $R^2 =0.4688, p=0.0049$), Total PAHs: $R^2 = 0.4944, p=0.0035$) (Figures 8 b-d).

Deposited metals (sum of most abundant metals measured) were poorly correlated with neutrophil influx on day 1 (Additional File G a).

Saa3 mRNA levels on day 1 correlated well with deposited EC ($R^2=0.5041, p=0.0021$), but less with deposited surface area, OC, and PAHs (surface area: $R^2=0.3847, p=0.0104$; OC: $R^2=0.1771, p=0.1182$, PAHs: $R^2=0.3429, p=0.0218$) (Figure 9 a-d).

As no significant differences were seen on day 28 for neutrophil influx and for genotoxicity on day 1, these data were not subjected to correlational analysis.

On day 28, ROS formation (Additional File H), deposited Total PAHs (Additional File I) or BaPeq (not shown) did not correlate well with genotoxicity, measured by Tail length in BAL, lung and liver in the
Comet assay. On day 90, Tail length did not correlate with Total PAHs (Additional File J), BaPeq (not shown) or with ROS (Figure 10 a-b). Data from day 90 included very few data points. However, ROS correlated well with Tail length in the liver (Figure 10 c) (R²=0.8348, p=0.0301). On day 90, neutrophil influx did not correlate with surface area or deposited elemental carbon (Additional File G b-c).

**Discussion**

Five particle samples were designed to differ in regards to their chemical and physical properties in order to assess the toxicity of the different particle properties. The diesel exhaust particles were generated from experimental diesel engine combustion with different levels of intake O₂ concentrations, by using different amounts of EGR, and different fuel types. The toxicity was evaluated in terms of inflammation, acute phase response, DNA strand breaks and histopathological changes. In order to investigate the effect of renewable fuels, HVO and RME exhaust particles were generated with the same engine settings used for generating diesel exhaust particles with 13% intake O₂, resulting in EC dominated particles.

**Physicochemical properties**

*Organic and elemental carbon, primary particle size and surface area*

A qualitative overview of the results of the physicochemical analyses and the toxicity parameters are given in Table 5, in relative comparison between the five different particle samples. The obtained variations in the physical properties (primary particle size: 15-22 nm, SSA 152-222 m²/g and count median mobility diameter: 55-103 nm) were moderate, but are within the variations observed for the accumulation mode of real-world engine out emissions. Much larger variations were obtained for the organic carbon fraction (0.12-0.60), PAH fraction (1-27 mg/mg) and metal contents (0.9-16 mg/mg). Organic carbon emissions in diesel exhaust are mainly lubrication oil-derived under lower/medium EGR (higher intake O₂ concentrations) and can therefore be expected to show less dependence on fuel type. The combustion conditions strongly affected the exhaust particle composition.

The particles generated at 13% intake O₂ concentration had the highest EC fraction, and DEP9.7 had
the highest PAH fractions as a consequence of a higher degree of incomplete combustion process.

The OC/EC fraction (Table 1) was higher for the renewable fuels compared to DEP13. These observations are consistent with previously reported reduced EC/BC emissions from renewable diesel fuels [37].

The ROS potential was highest for DEP17 and lowest for DEP 9.7 thus increasing with increasing combustion temperature.

Table 5. Qualitative relative comparison of data on particles from combustion of three fuel types.

| Physical-chemical properties   | DEP           |   |   |   |
|-------------------------------|---------------|---|---|---|
|                               | MK1 low sulfur diesel | Hydrotre: |
| Intake O₂ %                   | 9.7           | 13 | 17 | 13 |
| Oxygen content in fuel        | 0             | 0  | 0  | 0  |
| PAHs                          | +++           | +  | -/+| ++ |
| OC                            | +++           | +  | ++ | ++ |
| EC                            | +             | +++| +  | ++ |
| ROS                           | +             | ++ | +++| +++|
| Metals                        | ++            | +  | +++| ++ |
| Surface Area                  | ++            | ++ | ++ | ++ |

In vivo response

| Histopathology (lung)         | -/+           | ++ | -/+| ++ |
| Material presence             | +             | ++ | +  | ++ |
| Large agglomerates            | -             | -  | -  | -  |
| Inflammation as neutrophils and lymphocytes in BAL fluid | + | ++ | +  | +  |
| Eosinophil response           | -             | +  | -  | +  |
| Inflammation as acute phase response | ++ | +++| ++ | +++|
| DNA damage                    | -             | +  | ++ | ++ |

PAHs=polycyclic aromatic hydrocarbons, OC=organic compounds, BC=black carbon, ROS=reactive oxygen species, BAL= categories: “-“: not present, “-/+” = very low content/response, “+” = low content/response, “++” = medium content/response, “+++” = high content/response.

Metals

The most abundant metals were Cu and Fe, with these and several other trace elements being increased 5-17 fold in DEP17 compared to the other four samples. Similarly, the metal fraction was 2.5-3.5 times higher for both renewable diesel fuels compared to DEP13. Increased concentrations of Cu and Fe in the used lubricating oil was found in a previous study using the same engine setup [38].

The emissions of metals (µg/m² in undiluted exhaust) are within a factor 2 for all samples, but as the PM emission was much lower for DEP17, and lower for HVO13 and RME 13, compared to DEP13, the
metal fraction of the total collected PM was increased. The highest summed mass fraction of the elements analyzed with ICP-MS is only ~1.5% (DEP17). Thus, in all cases the particle composition was strongly dominated by carbonaceous constituents. The Zn and Mg measurements were excluded from the data set, because very high concentrations were detected in the blank filter control samples. As we only included one blank sample in the analysis, it was not possible to conclude whether the Zn and Mg came from the particles or were the result of background contamination. In turn, several of the constituents of lubrication oil and diesel particles were not measured, including P and Ca, although the mass percentages of these would be expected to be low.

**PAHs**

The sum of benzo(a)pyrene equivalents (BaPeq) largely follow the sum PAHs (see Table 3 and Additional File L) which is due to the fact that benzo(a)pyrene itself constitute the major part of the sum BaPeq (37-80%). However, the ratio of BaPeq compared to the total PAHs are higher for DEP9.7 compared to the other samples, which indicates that the low temperature combustions favor the formation of larger more genotoxic PAHs.

The BaP toxicity equivalent weighted emissions shows that there was a higher fraction of larger and more toxic PAHs compounds for DEP9.7, compared to the other particles. The PAHs fraction was substantially higher for HVO13 than for DEP13. This can partly be explained by higher PM1 emission levels from DEP13 compared to HVO13. RME13 contained lower amounts of PAHs compared to both DEP13 and HVO13. It is possible that the oxygen content in the RME fuel has additional effects on the soot formation process such that the PAHs formation is reduced more strongly than PM and EC. The results clearly demonstrate that PAHs emissions in this study were primarily combustion-derived, since HVO has essentially no PAHs and no aromatic content in the fuel (precursors of PAHs). As combustion temperature were reduced (intake O₂ decreased) with the addition of EGR, the PAHs/PM and PAHs/EC ratios increased strongly. This suggests that DEP9.7 may be a proxy for exposure situations with particles from other low temperature combustion processes, where the PAHs mass fractions are high. It also suggests that PAHs emissions may not be well predicted from measured EC and PM concentrations in epidemiological and exposure measurements.
In this study, only one sample was analyzed so inter-sample variations were not accounted for, however, at least two filter collections from the engine were pooled to account for engine variability.

**Toxicity**

The toxicity was evaluated in terms of inflammation, acute phase response, DNA strand breaks and histopathological changes at different dose levels and time points following pulmonary deposition in mice. Additionally, generation of ROS was measured in an acellular assay. Inhalation is the golden standard for testing pulmonary toxicity, whereas pulmonary intratracheal instillation allows control of the deposited dose, and is thus suitable for comparison of hazard potential between different particles. We used 0.1% Tween as vehicle [33, 39], since all five diesel exhaust particle samples could be dispersed using this vehicle. Furthermore, two different control groups were included, vehicle controls and blank filter extraction controls, which contained extracts from blank filters, which were blank filters placed in the high volume cascade impactor (HVCI) and extracted using the same extraction protocol. No air was sampled on them.

Pulmonary exposure to the five different DEP overall induced similar responses with different potency.

**Histopathology and particle retention**

Black particles compatible with DEP were readily observed in lungs 28 days post-exposure to DEP13 and HVO13. In RME13-exposed lungs, some large aggregates of particles were observed, but overall there seemed to be less particles present at day 28 in RME13, DEP9.7 and DEP17 exposed lungs than in DEP13 and HVO13. As the amount of particles appears to be greatest for the two DEP types with the most pronounced inflammatory response, differences in biopersistence of the particles could potentially explain the observed differences in pulmonary responses at day 28. Unfortunately, we do not have data to address this further in the current study, but DEP13 and HVO13 were the two diesel exhaust particles with highest content of EC, reflecting the insoluble carbon core.

It could be suggested, that EC is the most biopersistent part of the diesel exhaust particle as such, reflected in the continued presence of neutrophils in BAL on day 90. However, RME13 was similar in OC/EC ratio to DEP13 and HVO13, and if the response was dependent on the EC content, we should expect a similar response. There could be a difference in the EC nanostructure for RME particles,
considering the content of 10% O₂ in the fuel itself. This might change the combustion characteristics and the soot oxidation, and previous studies have reported different nanostructures depending of renewable diesel fuels compared to diesel [40, 41].

**Inflammation and acute phase response**

The deposited surface area is an important predictor of neutrophil influx into the lung for insoluble particles [18]. Interestingly, RME13 did not induce inflammation in terms of neutrophil or lymphocyte influx at the assessed dose levels despite having similar SSA as the other particles. In addition, DEP13 appeared to be more inflammogenic than the other diesel exhaust particles, as increased influx of neutrophils and lymphocytes was still observed 90 days post-exposure.

Acute phase response is the systemic response to various types of insults including bacterial infections, virus, infarction and all types of chronic inflammatory conditions [42], and acute phase response is a risk factor for cardiovascular disease in prospective epidemiological studies [43]. The acute phase protein serum amyloid A is causally related to atherosclerosis by promoting formation of atherosclerotic plaques [44]. We have previously shown that inhalation and pulmonary exposure to particles induce a dose dependent acute phase response [10, 19, 45], and we use Saa3 mRNA levels as a biomarker of pulmonary acute phase response [19]. All the diesel exhaust particles except RME13 induced increased Saa3 mRNA levels in the lung at day 1, and DEP13 even induced increased Saa3 mRNA levels at day 28 alongside CB. It was previously shown that Saa3 mRNA levels correlated closely with neutrophil influx and deposited surface area for insoluble particles such as CB and TiO₂ nanoparticles and carbon nanotubes [10, 19, 35, 46]. However, in the current study, although Saa3 mRNA levels correlated closely with neutrophil influx, they correlated poorly with estimated deposited surface area on day 1 post-exposure, but well with deposited EC. RME had similar specific surface area as the other diesel exhaust particles but did not induce increased Saa3 mRNA levels, and DEP13 induced more long lasting acute phase response than the others despite similar SSA. This could suggest that other particle components affecting the nanostructure in relation to the organic carbon content, in addition to SSA, are important contributors to the acute phase response of diesel exhaust.
particles. In turn, inflammation and acute phase response are general biomarkers of unspecific toxicity, as means of clarifying cause and effect relationships from different particles. None-the-less, acute phase response is an important cardiovascular disease risk factor, and inflammation may be linked to other pathologies including fibrosis [47] and secondary genotoxicity [48].

**DNA damage and ROS generation**

The comet assay detects single strand breaks in DNA, and these can be caused by bulky PAH-DNA adducts and by particle-induced oxidative DNA damage. DNA damage in BAL and lung tissue may be caused by particles, metal ions, or PAHs released from the particles. DNA damage in the liver may be caused by primary genotoxicity caused by translocated particles [22] and by PAHs released into systemic circulation [49, 50]. In the present study, DEP9.7 had the highest content of PAHs, but PAH levels did not correlate with DNA strand break levels. When blank filter extraction control was used as reference, DEP13, DEP17 and HVO13 induced DNA strand breaks in lung and liver tissues. RME13 also induced DNA strand breaks to a lesser extent. Particles with a diameter <100 nm are able to translocate from the lung to the systemic circulation [51]. Subsequent accumulation in liver has previously been shown to occur for particles of similar size as the particles in the current study (approx. 20 nm), and particle-dependent ROS generation was suggested as the likely cause of the observed DNA strand breaks in liver following pulmonary exposure to carbon nanoparticles [22]. This suggests that carbon nanoparticles induce primary genotoxicity likely caused by ROS. In the current study, ROS generation potential was highest for DEP17 and lowest for DEP9.7, and particle-induced DNA strand break levels in the liver, but not in the lung, correlated well with particle-induced ROS on day 90. Thus, the observed DNA strand break levels in liver tissue correlated better with ROS potential than with PAH levels. Carbon black nanoparticles did not induce DNA strand breaks in the current study, but we have previously found CB-induced DNA strand breaks in BAL, lung and liver following inhalation and instillation [5, 34, 52, 53].

Carbon black nanoparticles are very efficient ROS generators [54] and induce oxidative DNA damage [55, 56] in vivo and in vitro. Carbon black nanoparticles and diesel exhaust particles have similar mutagenic potential in cell studies [21, 55] and diesel exhaust and carbon nanoparticles had similar
carcinogenic potency in chronic inhalation studies in rats [57, 58]. This suggests that insoluble carbon nanoparticles such as the carbon core of diesel exhaust particles has the same mutagenic and carcinogenic potency as intact diesel exhaust. The present study supports the notion that particle-generated ROS contributes to the particle-induced genotoxicity.

**Particle mass concentration and importance of fuel**

The main effect of fuel type was that the PM concentration in the undiluted exhaust was reduced by 65% for the two renewable diesels (HVO13 and RME13), compared to DEP13. RME13 clearly had less toxicological potential per mass for the endpoints included in this study, observed by less inflammation and acute phase response, which are risk factors for cardiovascular disease. However, when taking into account the reduced PM emission factor, HVO also had a reduced toxicological response for most endpoints compared to MK1 diesel. Most other chemical components showed a much lower reduction than PM1 and EC with renewable fuels and were therefore enriched in the two renewable diesel samples.

The mass emission should be considered when assessing the toxicity potential of the engine “output”, where reduced mass emission from the engine may lead to reduced toxicity potential of the engine output, even if the absolute toxicity per ng of particles is higher. In this study, the mice exposure dosed varied by a factor of three. The PM emission factor for DEP13 was the same three-fold higher compared to RME13 and HVO13. Taking this into account, a comparison of engine emissions corresponds to the comparison of neutrophil influx following exposure to DEP13 at 54 µg and RME13 and HVO13 at 18 µg (Figure 3 a), showing that DEP13 results in significant inflammation compared to RME13 and HVO13. However, a similar comparison for Saa3 mRNA levels does not show as clear a picture.

In summary, exposure to particles from RME fuel with 13% intake O₂ concentration resulted in the least inflammation and acute phase response, whereas the physicochemically similar HVO13 and DEP13 induced more inflammation and acute phase response. The small differences in the assessed physicochemical properties of RME13 compared to HVO13 and DEP13 cannot explain the lower inflammatory response. However, the RME fuel has oxygen content in the fuel itself, which might
affect the soot oxidation and resulting surface properties, which can possibly affect the particle toxicity. This can be either by changing the surface structure of the carbon core, for example in terms of the frequency of edge sites and the composition of strongly bound surface oxides or by changes in the chemical composition of the liquid organic coating. The major part of the organic coating is lubrication oil derived (Gren et al., submitted manuscript), but the presence of minor fractions of fuel derived oxygenated organic compounds cannot be excluded. Future studies should include direct measurements of the surface properties of the collected samples for example by X-Ray Photo Electron Spectroscopy.

DEP17 and HVO13 had the highest ROS production and 90 days post-exposure DNA strand breaks were increased in both lung and liver compared to blank filter extraction controls. The particle-induced ROS production correlated with DNA strand break levels in liver tissue on day 90.

Conclusions
We conclude that fuel type and combustion conditions are important factors for the physicochemical particle properties measured in this study, and that combustion conditions were more important than the fuel type. RME fuel induced the least toxic response. We aimed at clarifying the relationship between physicochemical properties and toxicity. Estimated deposited surface area, elemental carbon, organic carbon, and PAHs correlated well with neutrophil influx, and reactive oxygen species were found to correlate with genotoxicity in liver on day 90. PAH content did not correlate with genotoxicity. Our study highlights particle size, elemental carbon content and ROS-generating potential as physicochemical predictors of diesel particle toxicity. These results may guide safe-by-design decisions for combustion engines.

Material And Methods

**Particle generation, collection and extraction**

The particles were generated, collected and extracted as described in detail in Gren et al. (submitted manuscript), where the same particles are studied with other endpoints (in Gren et al. the particles are named differently). Briefly, particles were generated with a modern heavy-duty diesel engine, operating on petroleum diesel (Swedish ultra low sulfur MK1) and two types of renewable diesels,
hydrotreated vegetable oil (HVO) and rapeseed methyl ester (RME) with no external exhaust aftertreatment system. The engine was operated at a constant low load, and particle properties adjusted by varying the amount of exhaust gas recirculation (EGR). The amount of EGR changes the intake $O_2$ concentration to the combustion cylinder, which in turn changes combustion temperature and thus the combustion conditions. An increase in EGR results in a decrease in intake $O_2$ concentration and combustion temperature.

Reference particles

Carbon black Printex90 was provided by Evonik Degussa GmbH (Frankfurt, Germany) [5, 55, 59]. Diesel particle SRM 2975 (referred to as NIST2975) was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The certificate of analysis is available at http://www.nist.gov.

Transmission electron microscopy (TEM) and analysis of organic (OC) and elemental (EC) carbon

The organic carbon (OC) and elemental carbon (EC) of the extracted particles were measured with a thermal-optical carbon analyzer (Sunset Laboratory Inc.), using the EUSAAR_2 protocol. To analyze the soot particles microstructure, samples were collected with electrostatic precipitation (Nanometer Aerosol Sampler, model 3089, TSI Inc.) on Cu-grids with lacey carbon coating (Whatman PTFE, 150 mm, pore size 5 µm) as well as the extracted particles were deposited in instillation vehicle on Cu-grids with lacey carbon coating (data not shown) and imaged by transmission electron microscopy (JEOL 3000F) operating at 300kV. The primary particle determination and estimation of specific surface area (SSA) is described in Gren et al. (submitted manuscript). In brief, the primary particle diameter was manually measured with ImageJ software [60]. The specific surface area (SSA) of each primary particle was estimated by using the primary particle size ($d_{pp}$) and diesel soot density ($\rho_{pp}$) of 1.8 µg/m3 [30] with the formula $SSA = \frac{6}{(\rho_{pp} \cdot d_{pp})}$ [61]. By assuming point contact between the primary particles in the agglomerates, the geometric mean of the lognormal distribution of SSA of all measured primary particles within a sample was used as the mean SSA. The particle
mobility size distribution in the diluted exhaust was measured from the dilution tunnel with a fast particulate analyzer (model DMS500, Cambustion Ltd.). The fast particulate analyzer measures the number size distribution of 5-1000 nm by classification of particles by their electrical mobility.

**PAHs and metal analysis**

**PAHs**

Particle samples, blank control filters and standard reference material NIST2975 were solvent-extracted using dichloromethane, as described in detail in Gren et al. (submitted manuscript). Extracts were analyzed for 20 native PAHs, 13 alkylated PAHs, 14 nitrated PAHs, 10 oxygenated PAHs and 6 dibenzothiophenes (DBT), using an Agilent 5975C mass spectrometer (MS) coupled to a 7890A gas chromatograph (GC, Agilent Technologies). Names, nominal masses, retention times and the associated deuterium labeled internal standards (IS) and recovery standards (RS) for all investigated compounds are shown in Additional File K.

**Metals**

The metal analysis was carried out as previously described in Bendtsen et al (2019) [12], but with slightly modified extraction times. Briefly, as it was not possible to transfer the amount of ≤ 1 mg particle matter from the received vials to containers suitable for microwave-assisted acid digestion, a volume of 1 mL of 25 % (v/v) nitric acid was directly added to the vials. For the preparation of reference material NIST2975 and CB (n=2), 1 mg of material were weighed into 13 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) and 1 mL of 25 % (v/v) nitric acid added. Samples were first agitated at 600 oscillations per min overnight (Stuart Scientific SF1 shaker), then incubated for approximately 7 hours at room temperature without agitation and then shaken for another 72 hours and transferred with 6 mL of ultrapure water into polypropylene tubes. Before analysis, the samples were centrifuged for 5 min at 4500 x g (Heraeus Multifuge X3 FR, Thermo Scientific), because no complete digestion of the particles was achieved. A volume of 5 mL of the supernatant was transferred to a new polypropylene tube and diluted 5-fold with 5 % nitric acid. A triple quadrupole inductive coupled plasma mass spectrometer (ICP-MS) (Agilent 8900 ICP-QQQ, Santa Clara, USA) equipped with a MicroMist borosilicate glass concentric nebulizer and a Scott type double-pass water-
cooled spray chamber was run in no gas (Cd, Hg, Pb, Bi, U) or helium (remaining elements) mode with 0.1 - 3 s integration time per mass. Quantification was performed based on external calibration.

**Reactive oxygen species (ROS) assay**

The level of ROS generated by the DEPs and CB were determined using the acellular 2′,7′ dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) assay. We used the protocol previously described in detail [54, 62] with the addition, that all materials were tested for auto-fluorescence as this may interfere with the assay. CB Printex 90 was tested alongside the DEPs as benchmark particle. Briefly, the DCFH$_2$-DA (#D399, Invitrogen) was chemically hydrolyzed in the dark with NaOH to generate 2′,7′ dichlorodihydrofluorescein (DCFH$_2$), which was further diluted with phosphate buffer (pH 7.4) to 0.04 mM. The PM suspensions were prepared using 16 min sonication (Branson S-450D) in Hank’s balanced saline solution (HBSS, without phenol, #H6648, Sigma Aldrich). NPs were further diluted in HBSS and tested at 0 µg/ml and eight doubling PM concentrations from 1.05 up to 101.25 µg/ml. The final concentration of DCFH$_2$ at assay start was 0.01 mM. Generated ROS caused formation of 2′,7′ dichlorofluorescein (DCF) from DCFH$_2$ that was spectrofluorimetrically measured following 3h of incubation in the dark (37 °C and 5% CO$_2$). Excitation and emission wavelengths were $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm, respectively (Victor Wallac-2 1420; PerkinElmer, Skovlunde, Denmark). Auto-fluorescence was measured by replacing the ROS probe by Hank’s balanced saline solution (HBSS) at the highest tested PM concentrations.

**Dynamic Light Scattering**

Dynamic Light Scattering (DLS) was used to analyze hydrodynamic size distributions of particles in suspension (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., UK). Determinations were carried out directly in the solutions used for instillation in 1 ml polystyrene cuvettes at 25° C. Six repeated same sample measurements were analyzed and average was calculated. For the calculation of hydrodynamic size, values from reference particle carbon black Printex90 was used (refractive (Ri) = 2.020, absorption indices (Rs) = 2000) for all particles, with standard optical and viscosity properties for H$_2$O.
Mice

The study was in agreement with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of mice used for scientific purposes, and the Danish Animal Experimentation Act (LBK 474 15/05/2014). The study was approved by The Animal Experiments Inspectorate under The Ministry of Environment and Food of Denmark (License: 2015-15-0201-00465) and the local Animal Welfare Committee responsible for ensuring implementation of 3R policy at the National Research Center for the Working Environment.

For this study, 492 female C57BL/6Tac mice were used. They were 7 weeks old at arrival and group-housed in standard cages with 6-8 mice with ad libitum access to tap water and Altromin 1324 rodent diet. All mice were housed in 1290D euro standard Type 3 cages on saw dust bedding with mouse house, wooden chew blocks and Enviro Dri nesting material as enrichment. The mice were kept at 21±1°C and 50±10% humidity in a 12 hour light-dark circle.

Study design

After one week of acclimatization, mice were exposed to a single dose of collected particles of either 6 µg, 18 µg or 54 µg per mouse by intratracheal instillation (6-8 mice per dose per exposure).

As it is only possible to expose a certain number of mice per day, the study was spread out on several days. First cohort was exposed to RME13 and CB, second cohort was exposed to DEP13, third cohort was exposed to DEP9.7, fourth cohort was exposed to DEP17, and fifth cohort was exposed to HVO13. For each exposure cohort there were four vehicle control mice, which were pooled together as one final control group for each post-exposure day for data evaluation. All cohorts were euthanized on day 1, day 28 and day 90 post-exposure. On day 28 and day 90, five mice per exposure were used separately for histology (except for CB). In groups subjected to 1-day exposure to DEP13, DEP9.7, DEP17 and HVO13, some animals were initially not correctly dosed or cell recovery procedures failed upon euthanization. Therefore, a new cohort of mice was exposed, comprising two animals per dose for 1-day exposures, supplementing the groups from the original study.

Instillation procedure

Particles were suspended in Nanopure Diamond Water with 0.1% Tween80 and sonicated for 16
minutes using a Branson Sonifier S-450D (Branson Ultrasonics Corp, Danbury, CT, USA) (see description in [53]). The suspensions were diluted and re-sonicated for 2 minutes. Nanopure Diamond Water with 0.1% Tween80 was prepared similarly as vehicle. All solutions were prepared fresh for each instillation day and instilled within 1 hour.

Instillation procedure was carried out as previously described [12, 63]. In brief, mice were exposed during isoflurane anesthesia to the suspended particles in vertical position with back support. A diode light was placed at the larynx visualizing the breathing pattern, to ensure correct delivery. 200 µl air was placed in the syringe after the instillation volume and administered post-exposure, to ensure maximum delivery into the lung. The mice were then returned to the home cage, placed on a heating plate, to ensure optimal recovery from anesthesia. Following the procedure and until euthanization, the mice were under observation for signs of discomfort. In case of weight loss (maximum 20%) and/or clear signs of discomfort (ruffled fur, isolation, facial pain expression, changed respiration, reduced activity), the mouse was taken out of the study and euthanized.

Organ harvest and preparation

Sedation, bronchoalveolar lavage (BAL), euthanization, organ harvest and procedures for analysis of mRNA, protein, and DNA strand breaks in the Comet assay were carried out as previously described [12].

**Statistical analysis of in vivo data**

Data was analyzed with GraphPad Prism (GraphPad Prism, version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). BAL fluid data was log10 transformed to achieve normal distribution. If no cells were counted across all cell types for one mouse, for instance due to high number of erythrocytes, it was considered as a cell processing error and the mouse was removed from the particular dataset. Values of zero within one cell type were replaced by the value of 0.25, generating an arbitrary low value of <500 cells, considered as the detection limit of the differential count. Parametric data were analyzed by one-way ANOVA followed by Dunnett’s (comparison to control group). Nonparametric data was analyzed by Kruskal-Wallis followed by Dunn’s multiple comparisons test. Linear regressions were performed on log-transformed data, after testing
the normality of the residuals.

Declarations

**Ethics approval and consent to participate**

The study was in agreement with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of mice used for scientific purposes, and the Danish Animal Experimentation Act (LBK 474 15/05/2014). The study was approved by The Animal Experiments Inspectorate under The Ministry of Environment and Food of Denmark (License: 2015-15-0201-00465) and the local Animal Welfare Committee responsible for ensuring implementation of 3R policy at the National Research Center for the Working Environment.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analyzed 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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**Authors’ contributions**

Idea and study design: UBV, JP. Engine operating points: MT & PCS, Engine operation: PCS, Electron Microscopy: LG. Light Microscopy: TB and HW. Metal contents: KL. PAH analyses: PAC, JE and AK. Particle collection and emission characterization: LG, JP, VBM, PCS, MT. ROS assay: NRJ. In vivo data: KMB and UBV. Interpretation of data: KMB, UBV, LG, VBM, JP. KMB drafted the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

TEM images of DEP9.7 (a), DEP13 (b), and DEP17 (c), HVO13 (d), RME13 (e) and CB (f). The morphology of the particles generated with 13% and 17% O2 conditions (b, c, d, and e) are similar, while the soot agglomerates generated with 9.7% O2 (a) have less defined primary particles and appear more aggregated compared to the other samples.
Figure 2

Mouse lung histology 28 days post-exposure to 54 µg DEP9.7 (a), DEP13 (b), DEP17 (c), and HVO13 (d) and RME13 (e). (a1)-(e2) are high magnification images of black particles in exposed lungs. Haematoxylin and eosin stain.
Cell counts of broncho-alveolar lavage of mice day 1 post-exposure to 6, 18, and 54 µg DEP9.7, DEP13, DEP17, HVO13 and RME13. a) Neutrophils. Four values of zero were excluded due to the log10 axis chosen to depict the large response range. b) Lymphocytes. c) Macrophages d) Eosinophils. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.
Figure 4

Cell counts of broncho-alveolar lavage of mice day 90 post-exposure to 54 µg DEP9.7, DEP13, DEP17, HVO13 and RME13. a) Neutrophils. b) Lymphocytes. c) Macrophages d) Eosinophils. * = p<0.05. ** = p<0.01, *** = p<0.001, **** = p<0.0001.
Saa3 mRNA levels in lung tissue of mice day 1 (a), 28 (b) and 90 (c) post-exposure to 6, 18
and 54 µg DEP9.7, DEP13, DEP17, HVO13 and RME13. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Figure 6

DNA strand breaks assessed in the Comet assay by %Tail DNA and Tail length in broncho-alveolar lavage cells, and lung and liver tissue of mice day 28 post-exposure to 6, 18 and 54 µg DEP9.7, DEP13, DEP17, HVO13 and RME13. ¶ = compared to extract, * = compared to VEH, ## = compared to CB.
Figure 7

DNA strand breaks assessed in the Comet assay by % Tail DNA and Tail length in bronco-alveolar lavage cells, and lung and liver tissue of mice day 90 post-exposure to 6, 18 and 54 µg DEP9.7, DEP13, DEP17, HVO13 and RME13. * = compared to extract, * = compared to VEH, ## = compared to CB.
Figure 8

Deposited surface area, EC, OC, and PAH correlations with neutrophil influx on day 1. a.

Estimated deposited surface area, original data (left panel) and linear regression plots with
and without NIST references (right panels). b. Estimated deposited elemental carbon, original data (left panel) and linear regression plot (right panel). c. Estimated deposited organic carbon, original data (left panel) and linear regression plot (right panel). d. Estimated deposited Total (native) PAH, original data (left panel) and linear regression plot (right panel). The estimated deposited specific surface area (SSA), elemental carbon (EC), organic carbon (OC) and PAHs were calculated by multiplying the physicochemical values by the dose (EC and OC: dose in µg * EC fraction = deposited EC in µg; for SSA: dose in g * SSA m²/g = deposited SSA in g; for PAH: dose in g * PAH µg/g = deposited PAH in µg). The data was log-transformed for the linear regression analysis.
Deposited surface area, EC, OC, and PAH correlations with Saa3 mRNA on day 1. a. Estimated deposited surface area, original data (left panel) and linear regression plot (right panel). b. Estimated deposited elemental carbon, original data (left panel) and linear regression plot (right panel). c. Estimated deposited organic carbon, original data (left panel) and linear regression plot (right panel). d. Estimated deposited Total (native) PAH, original data (left panel) and linear regression plot (right panel). The estimated deposited specific surface area (SSA), elemental carbon (EC), organic carbon (OC) and PAHs were calculated by multiplying the physicochemical values by the dose (EC and OC: dose in µg * EC fraction = deposited EC in µg; for SSA: dose in g * SSA m²/g = deposited SSA in g; for PAH: dose in g * PAH µg/g = deposited PAH in µg). The data was log-transformed for the linear regression analysis.
Figure 10

ROS formation correlations with Tail Length in Comet assay on day 90. a. BAL, original data (left panel), linear regression plot (right panel). b. Lung, original data (left panel), linear regression plot (right panel). c. Liver, original data (left panel), linear regression plot (right panel). The ROS is given in arbitrary alfa-values. The data was log-transformed for the linear
regression analysis.

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