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Comparison of the toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells *in vitro*

Sandro Steiner [a, *], Jan Czerwinski [b], Pierre Comte [b], Olga Popovicheva [c], Elena Kireeva [c], Loretta Müller [d], Norbert Heeb [e], Andreas Mayer [f], Alke Fink [a], Barbara Rothen-Rutishauser [a, d]

[a] *Adolphe Merkle Institute, University of Fribourg, Route de l' ancienne Papeterie, 1723 Marly, Switzerland*
[b] *Bern University for Applied Sciences, Switzerland*
[c] *Institute of Nuclear Physics, Moscow State University, Russia*
[d] *University of Bern, Department of Clinical Research, Switzerland*
[e] *EMPA, Swiss Federal Laboratories for Materials Testing and Research, Switzerland*
[f] *TTM, Technik Thermischer Maschinen, Switzerland*

HIGHLIGHTS

- Biodiesel (rapeseed methyl-ester) affects particle emissions by diesel engines.
- The blending ratio (bio-/fossil diesel) influences this effect.
- Quantitative effects on the gas-phase exhaust composition are weak.
- The pro-inflammatory potential of the exhaust is influenced by the changes.
- Strong changes in particle emissions translate into weak changes in exhaust toxicity.

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ABSTRACT

Alternative fuels are increasingly combusted in diesel- and gasoline engines and the contribution of such exhausts to the overall air pollution is on the rise. Recent findings on the possible adverse effects of biodiesel exhaust are contradictive, at least partly resulting from the various fuel qualities, engine types and different operation conditions that were tested. However, most of the studies are biased by undesired interactions between the exhaust samples and biological culture media. We here report how complete, freshly produced exhausts from fossil diesel (B0), from a blend of 20% rapeseed-methyl ester (RME) and 80% fossil diesel (B20) and from pure rapeseed methyl ester (B100) affect a complex 3D cellular model of the human airway epithelium *in vitro* by exposing the cells at the air–liquid interface. The induction of pro-apoptotic and necrotic cell death, cellular morphology, oxidative stress, and pro-inflammatory responses were assessed. Compared to B0 exhaust, B20 exhaust decreased oxidative stress and pro-inflammatory responses, whereas B100 exhaust, depending on exposure duration, decreased oxidative stress but increased pro-inflammatory responses. The effects are only very weak and given the compared to fossil diesel higher ecological sustainability of biodiesel, it appears that – at least RME – can be considered a valuable alternative to pure fossil diesel.

1. Introduction

The International Energy Agency reports that in the period from 2005 to 2011, the global consumption of alternative fuels in road transport has doubled from approximately 70–140 million tons of oil equivalents, which corresponds to approximately 8.8% of the total road transport consumption. The dominant alternative fuels are ethanol and natural gas, but with an estimated contribution of 3.5%, biodiesel is important as well (IEA advanced motor fuels, annual report 2011). Biodiesel is defined as the monoalkyl esters of vegetable oils or animal fat (American Society for Testing and Materials (ASTM) Standard D6751) and can be produced from various sources such as algae, used frying oil, soy beans, palm kernels, or rapeseed. The choice of feedstock thereby strongly

* Corresponding author. Tel.: +41 26 300 95 15; fax: +41 26 300 96 24.
E-mail address: sandro.steiner@bfh.ch (S. Steiner).

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influences the composition of a biodiesel, which in turn influences its quality as a fuel. Recently, the technologies for biodiesel production have advanced to a state where engine compatibility does not pose considerable problems any more or where biodiesel is even superior to fossil diesel in certain aspects (Basha et al. 2009; Dwivedi et al. 2011; Lin et al. 2011; Valentino et al. 2011).

Biodiesel has a higher oxygen content than fossil diesel, which results in a more complete fuel combustion. As a consequence, biodiesel and biodiesel blends tend to reduce the emissions of soot and polyaromatic hydrocarbons. Simultaneously, other hydrocarbon emissions like formaldehyde, acetaldehyde and acrolein have been reported to increase together with nitrogen oxides (NO_x) (Bakeas et al. 2011; Dwivedi et al. 2006; Karavalakis et al. 2011; Macor et al. 2011; Zhang et al. 2011). Given such profound changes in exhaust composition, a detailed risk assessment, particularly considering human health aspects of biodiesel-derived emissions is important. The question on how the use of biodiesel affects exhaust toxicity to lung cells was not conclusively answered so far. The use of biodiesel has been reported to result in decreased (Bunger et al. 1998; Krahl et al. 2002), comparable (Jalava et al. 2010; Topinka et al. 2012) and increased (Kooter et al. 2011) exhaust mutagenicity compared to fossil diesel. Similarly, (pro-) inflammatory responses to exhaust exposure have been reported to be weaker (Jalava et al. 2010), unchanged (Brito et al. 2010) or stronger (Swanson et al. 2009). Further, Kooter et al. (2011) reported a lower oxidative potential for biodiesel exhaust, whereas Jalava et al. (2010) observed the opposite. This inconsistency is certainly due to the different fuel types, engines and engine operation conditions used in the different studies, but also to the various experimental approaches that have been used. Many studies conducted so far were carried out using organic extracts from exhausts, which bears the risk of biased sample generation. Furthermore, in most studies using eukaryotic cells, exposures were conducted with submersed cell cultures, which does not represent the *in vivo* situation and allows for interactions between culture medium and exhaust sample, thereby introducing an additional level of bias (Holder et al. 2008; Muller et al. 2011).

The aim of the present study therefore was to minimize experimental biases as much as possible, in order not only to obtain a clearer picture on the effects of RME, but also to provide a suitable method for the testing of further biodiesel types. We used freshly produced, complete exhaust, including gaseous, liquid and particulate components. Exposures were conducted at the air–liquid interface using a 3D *in vitro* model of the human epithelial airway barrier (Blank et al. 2007; Lehmann et al. 2010). In this first series of experiments, we investigated the toxicity of diesel exhaust generated from low-sulfur fossil diesel (B0), a blend of 80% fossil diesel and 20% rapeseed methyl ester (RME, B20) or pure RME (B100) under low load conditions.

2. Materials and methods

Exposure experiments using B20 and B100 were repeated five times, experiments using pure fossil diesel ten times.

2.1. Cell cultures

A 3D *in vitro* model of the human epithelial airway barrier was used as previously described (Blank et al. 2007; Lehmann et al. 2010). Briefly, a confluent layer of 16HBE14o$^-$ epithelial cells was cultured on an insert membrane. Human whole blood monocyte derived macrophages (MDMs) were cultured on the apical side and human whole blood monocyte-derived dendritic cells (MDDCs) on the basal side. Human whole blood monocytes were isolated from buffy-coats provided by the blood donation service SRK Bern as described by Lehmann et al. (2010), the only adaptation was the use of CD14 MicroBeads (Miltenyi Biotec) for monocyte isolation, which greatly increases the purity of the isolated monocytes.

2.2. Test vehicle and exposure system

An Opel Astra X20DTL (registration in 1998, Switzerland, mileage in course of the experiments: 60–70,000 km) was operated on a dynamometer at constant velocity of 35 km h^{-1} (engine speed 2180 rpm, a force of 66 N at the wheel) with standard low-sulfur (<10 mg sulfur kg^{-1}) fossil diesel (B0, Greenergy SA), a blend of 80% fossil diesel and 20% RME and pure RME (B20 and B100). Exhaust was sampled at the tailpipe, diluted ten-fold with filtered ambient air (air temperature: 120 °C. OptiFilter XL Type H, MSA. In the filtered air, no particles could be measured), and reached the cells with a delay of less than 30 s (temperature adjusted to 37 °C). All parts of the original exhaust after-treatment system were removed from the test vehicle. Before each experiment, the engine ran for at least 1 h for equilibration and to minimize possible fuel memory effects. The used cell exposure system has previously been described in detail (Morin et al. 2008; Muller et al. 2010). Diluted exhausts were brought to the exposure chambers with volumes of 4 l per chamber at a constant volume flow of 2 l min^{-1}. Throughout the exposure, conditions within the exposure chambers were maintained at 37 °C, 5% carbon dioxide (CO_2) and 80% relative humidity.

2.3. Fuel analysis

All analyses were performed by qualified personal at the Swiss Federal Institute for Materials Science and Technology, EMPA, in Dübendorf according to the test procedures required by the council directive 70/220/EEC for reference fuel analysis.

2.4. Exhaust characterization

The particle size-number distribution was measured in the ten-fold diluted exhaust using a scanning mobility particle sizer (differential mobility analyzer: TSI 3081, condensation particle counter: TSI 3772 A). Furthermore, the concentrations of carbon monoxide (CO), total gaseous hydrocarbons (HC), nitrogen oxides (NO_x) and nitric oxide (NO) were measured using the Horiba MEXA-9400H exhaust gas measuring system. Concentrations of nitrogen dioxide (NO_2) were estimated based on the assumption that NO_x consists of NO and NO_2 only.

By constant volume sampling, samples of the exhaust particles were collected on 47 mm PallFlex filters (Teflon-coated borosilicate, Pall Life Sciences, product no. 7212). The chemical structure of the particles was analyzed by Fourier Transform Infrared (FTIR) spectroscopy (IRPrestige-21, Shimadzu).

2.5. Exposure conditions

The cell cultures were exposed at the air-liquid interface for 2 or 6 h in order to reflect a low and a high dose exposure. All samples were then post-incubated for 6 h at 37 °C, 5% CO_2 and 80% relative humidity. These exposure durations were based on previous studies (Muller et al. 2010), in which 2 and 6 h yielded the best results. On each sampling day, identical cell cultures were exposed either to diluted diesel exhaust or to filtered ambient air for control. To test for the quality/health of the cell cultures, untreated controls were kept at the air–liquid interface in the incubator during exhaust and filtered air exposures and post-incubations.

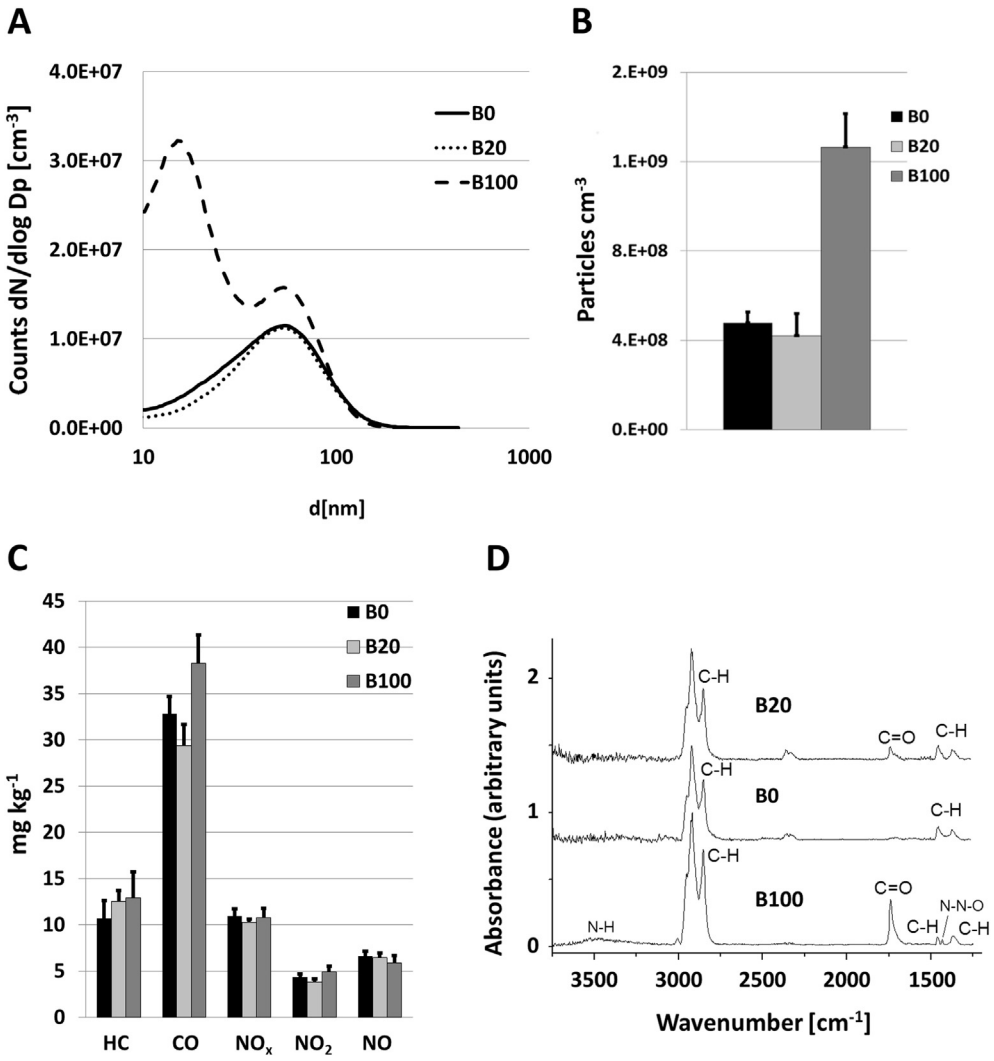

Fig. 1. On-line exhaust characterization. A) Size-number distributions of particles in the diluted exhausts. B) Total particle concentrations in the diluted exhausts C) Composition of the gaseous exhaust fractions. D) Representative FTIR spectra of B0, B20 and B100 particles. Error bars indicate standard deviations.

2.6. Laser scanning microscopy

Cell cultures were fixed with paraformaldehyde (3% in PBS, pH 7.4). After permeabilization with 0.2% Triton X-100, the samples were stained with phalloidin-rhodamine to label the F-actin cytoskeleton and with 4′,6′-diamidino-2-phenylindole (DAPI) to label the cell nuclei as previously described (Muller et al. 2010) and subsequently imaged by laser scanning microscopy.

2.7. Cytotoxicity

The release of the cytosolic protein lactate dehydrogenase (LDH) into the culture medium was used to estimate cell membrane integrity and hence cytotoxicity. The total amount of extracellular LDH was quantified using an LDH detection kit (Cytotoxicity Detection Kit, Roche).

2.8. Quantification of total reduced glutathione

The total amount of reduced glutathione (GSH) in the cell cultures was quantified with a glutathione assay kit (Cayman Chemical). Detected concentrations of reduced GSH are reported relative to the total protein concentrations in the samples, which were quantified using the Pierce BCA Protein Assay kit (Pierce).

2.9. Quantification of extracellular TNF-α and IL-8

The extracellular concentration of the pro-inflammatory cytokine tumor necrosis factor (TNF) α in the cell culture medium (basal side for cultures exposed to complete exhaust, basal and apical side for cultures exposed to organic extracts) was measured using the human TNF-alpha DuoSet (R&D Systems).

2.10. Real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR)

After post incubation, cells fixed in RNA protect buffer (Qiagen) and stored at 4 °C until further use. RNA isolation was done with the RNeasy plus kit (Qiagen). Reverse transcriptase reactions were performed in 10 μl volumes with an RNA concentration of 25 ng μl^{-1} (Omniscript RT, Qiagen) and Oligo dT primers (Qiagen). 0.25 μl RNase inhibitor (RNasin Plus RNase Inhibitor, Promega) was added to the reverse transcriptase reactions. A total of 2 μl of the ten-fold diluted cDNA was used for real-time PCR in reaction volumes of 10 μl with SYBR Green as reporter dye (Fast SYBR Green

master mix, 7500 fast real-time PCR system, Applied Biosystems). Relative expression levels of heme-oxygenase (*HMOX1*), tumor necrosis factor (*TNF*), interleukin-8 (*IL-8*), caspase7 (*CASP7*) and TNF receptor superfamily member 6 (*FAS*), were calculated using the $\Delta\Delta C_t$ method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard gene. Primer sequences and database accession numbers are listed in Supplemental Material Table S1.

2.11. Data analysis and statistics

Because for each experimental realization human blood-derived monocytes were isolated from a different buffy-coat and therefore from a different blood donor, variations in the absolute values measured for the background (filtered air exposure) between different sets of cell cultures were expected and observed. Because of this and because we expected only exhaust related effects to be relevant, we normalized all measurements to the filtered air exposure (raw data are listed in Supplemental Material Table S2).

In a single series of experiments (exposure to either filtered air, B0, B20 or B100 for 2 and 6 h) identical cell cultures (*i.e.* produced with macrophages and dendritic cells obtained from the same buffy-coat) were used as negative control, for filtered air exposure and for exhaust exposure. Differences in biological responses between the treatments were therefore considered as dependent variables and a *t*-test on the average of the observed differences was performed.

Differences between independent variables (*e.g.* the responses to exposures to B0- and B100 exhaust) were analyzed with the independent two-sample *t*-test. In both cases, *P*-values below 5% were considered statistically significant. The reported statistical significances are not Bonferroni-corrected. When this correction is included, none of the observed effects reach statistically significance.

3. Results

3.1. Fuel analysis and exhaust characterization

3.1.1. Fuel analysis

The results are summarized in Supplemental Material Table S3. RME is rich in fatty-acid esters, glycerides, glycerol and water, whereas these species could not be detected in fossil diesel. Aromatic hydrocarbons were only found in fossil diesel. RME is of higher density, viscosity and in particular flash point than fossil diesel, whereas the cetane numbers are comparable. These findings are in line with the literature (Basha et al. 2009; Lin et al. 2011).

3.1.2. Exhaust characterization

The size-number distributions of B0- and B20 exhausts are comparable (Fig. 1A), even though the number of emitted particles with diameters in the range from 10 to 30 nm is lower by 10–50% when using the B20 blend. Combustion of B100 resulted in the formation of three times more particles than B0 and B20 (Fig. 1B). Highest particle numbers were observed for B0 at around 50 nm diameter (1.14×10^7 particles cm^{-3}). These particles are also detected for B100 at 1.4-fold higher concentrations (1.57×10^7 particles cm^{-3}). Additionally, a very high particle number was observed at smallest particle diameters around 15 nm (3.2×10^7 particles cm^{-3}). With increasing biodiesel proportion HC emissions slightly increased (B0 < B20 < B100, Fig. 1C). CO emissions were also affected, but did not change as a function of the biodiesel proportion (B20 < B0 < B100). NO_2, NO_x and NO emissions were affected only weakly.

3.1.3. FTIR analysis of sampled exhaust particles

FTIR spectra of B0-, B20- and B100 exhaust particles are shown in Fig. 1D. Surface functionalities are dominated by bands of saturated aliphatic (alkane) C–C–H vibrations of asymmetric/symmetric stretches of methylene groups in the range of 2935–2915/2865–2845 cm^{-1}, and of asymmetric/symmetric bends of methyl groups in the range of 1470–1430/1380–1370 cm^{-1}. C=O groups are prominent in the range of 1700–1720 cm^{-1} for B0 and 1720–1740 cm^{-1} for B20 and B100 particles, with the most intense ones being detected for biofuel exhaust particles. Comparison of the relative concentration of C=O to C–C–H functional groups reveals an increased extent of the surface hydrophilicity from hydrophobic for B0 to the most hydrophilic for B100. Finally, B100 chemistry differs from B0 and B20 by prominent bands of N-containing group vibrations.

3.2. Biological responses

The general observation was made that mRNA extraction from cells that had been exposed to B20 or B100 did not work as well as from cells exposed to B0 exhaust. Furthermore, mRNA integrity appeared to be affected, resulting in a higher incidence of aberrant real-time PCR results for the biodiesel samples. We cannot exclude that this effect was exhaust-related. As a consequence, the size of the data set for gene expression analyses for B100 and B20 was diminished from originally five experimental repetitions to two in the worst case, which renders data interpretation difficult, increases standard deviations and hence decreases statistical significance.

3.2.1. Cellular morphology

No changes in cellular morphology or ruptures in the layer of epithelial cells could be detected for any of the samples by laser scanning microscopy (Fig. 2A). These findings are independent on the exhaust type and the exposure duration.

3.2.2. Cytotoxicity

The highest cytotoxic effect was observed upon 6 h exposure to B100 exhaust. Extracellular LDH levels were 1.3 ± 0.5-fold increased relative to filtered air exposure in these samples. This result, though statistically significant, is not considered to be biologically relevant (Fig. 2B).

3.2.3. Pro-apoptotic effects

No significant induction of the pro-apoptotic genes *CASP7* and *FAS* was detected, independent of exhaust type or exposure duration (Supplemental Material Fig. S1A and S1B). It has to be mentioned that the C_t values for the expression of both genes were high (C_t close to or >30). This indicates that the genes are basically silent that because of background and noise, these data should not be considered quantitative.

3.2.4. Induction of oxidative stress

Exhaust emissions of all three fuel types decreased the levels of total reduced GSH by 80–95% (Fig. 3A). Upon exposure to B0 exhaust, the detectable amount of reduced GSH was decreased to 0.08 ± 0.1- (2 h) and 0.07 ± 0.1-fold (6 h) of what was measured upon exposure to filtered air. The according values for B20 are 0.19 ± 0.21 (2 h) and 0.17 ± 0.18 (6 h) and 0.17 ± 0.11 (2 h) and 0.06 ± 0.16 (6 h) for B100. The difference between B0 and B100 (2 h) reached statistical significance. The exposure duration played a minor role, even though the dose response observed with B100 was statistically significant.

3.2.5. Transcriptional response to oxidative stress

HMOX1 expression was strongly induced by all treatments (Fig. 3B). B0 resulted in 24 ± 15 (2 h) and 41 ± 30 (6 h), B20 in

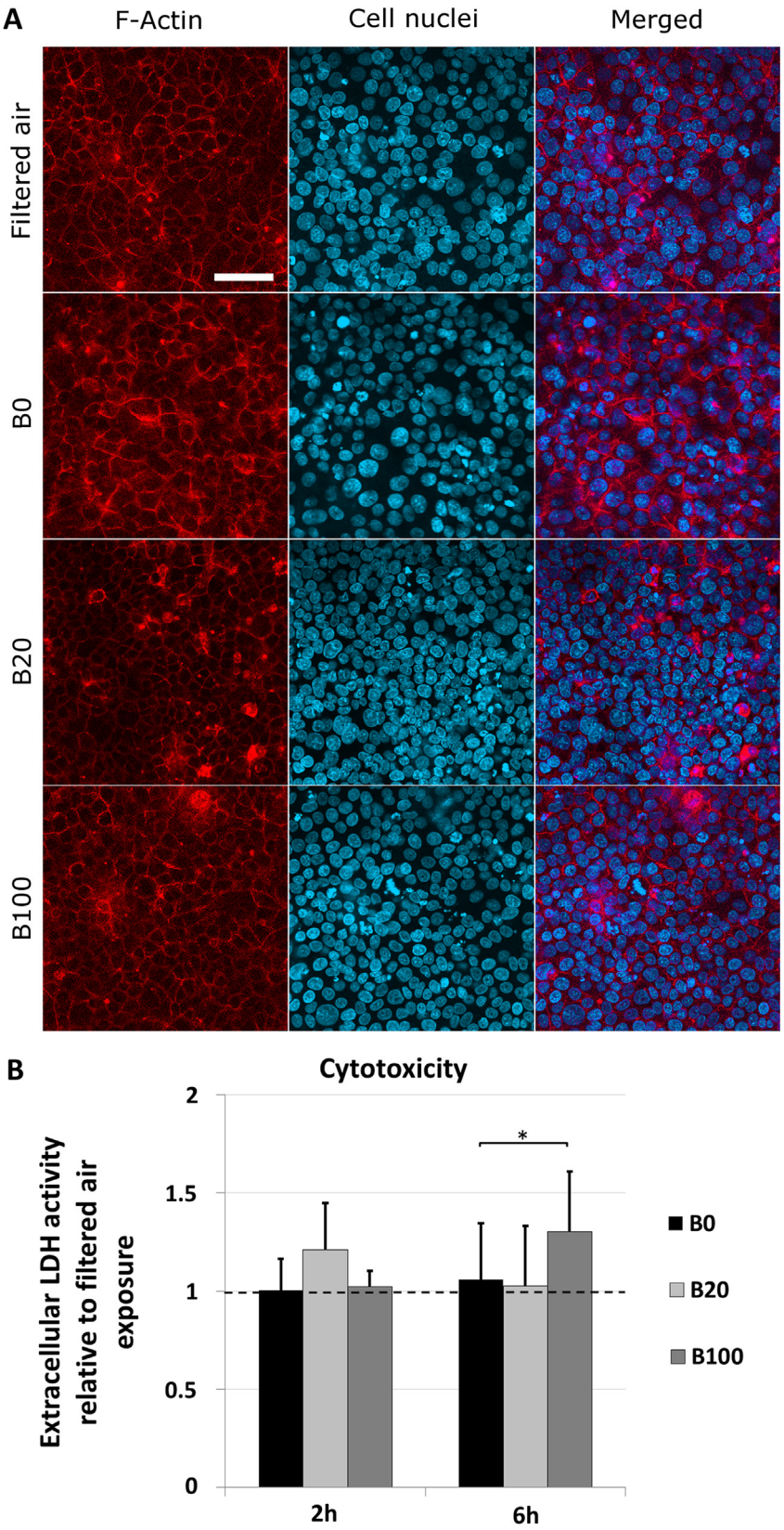

Fig. 2. Cell morphology and viability. A) Confocal laser scanning micrographs of cell cultures that were exposed for 6 h. Cell cultures were stained for F-actin (red) and nucleic acids (blue). The size bar corresponds to 50 μm. B) Induction of necrotic cell death, estimated by quantification of extracellular LDH activity, normalized to the filtered air exposure (dashed line). Error bars indicate standard deviations. The * indicates statistical significance ($p < 0.05$) between independent results as indicated by the bracket.

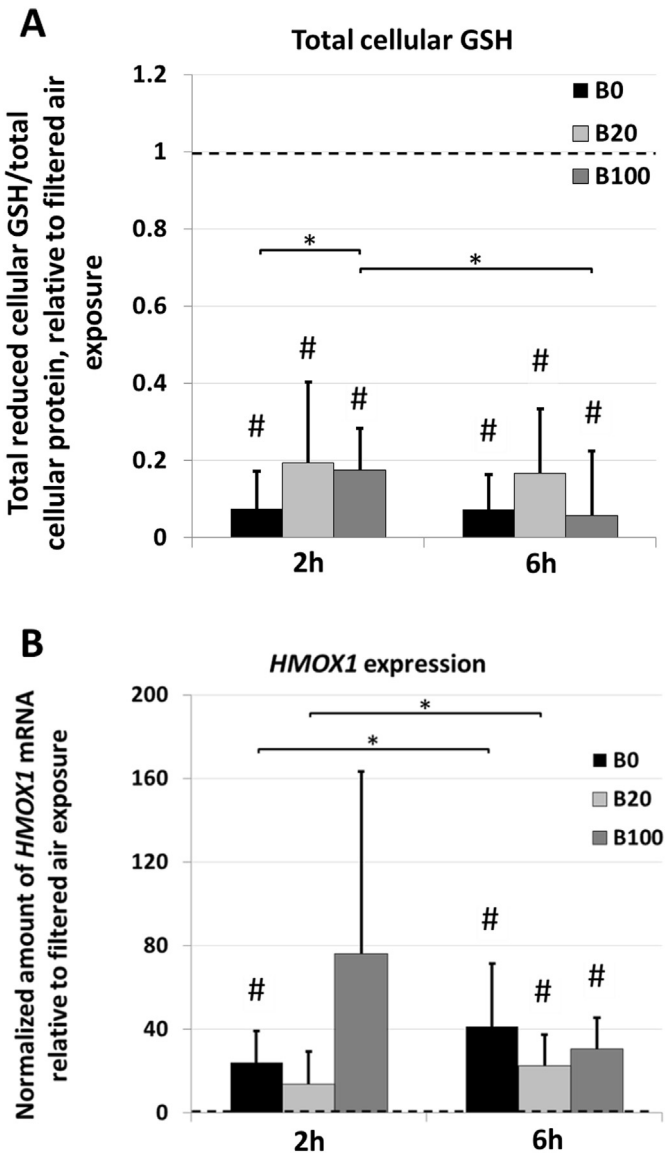

Fig. 3. Oxidative stress. A) Quantification of the total amount of the antioxidant molecule GSH. B) Transcriptional activity of the oxidative-stress responsive gene *HMOX1*. All results are normalized to the filtered air exposure (dashed lines). Error bars indicate standard deviations. * indicate statistical significance ($p < 0.05$) between independent results (different doses/exhaust types) as indicated by the brackets, # between exhaust- and filtered air exposure.

76 ± 86 (2 h) and 31 ± 15 (6 h), B100 in 14 ± 16 (2 h) and 22 ± 14 (6 h) fold increased gene expression upon exhaust exposure. Except for B20 (2 h) and B100 (2 h), all these results reached statistical significance. Responses were dose dependent, with a higher induction of gene expression upon 6 h than upon 2 h exposure for B0 and B20 and the other way round for B100. Differences between the three exhaust types did not reach statistical significance but show a weaker impact of B20 and B100 (6 h) compared to B0. Note that for B100 (2 h) $n = 2$ and that data interpretation may therefore be biased.

3.2.6. Pro-inflammatory responses

The transcriptional activity of the pro-inflammatory gene *TNF* (Fig. 4A) was increased 1.6 ± 1.0-fold and 2.2 ± 1.7-fold upon 2 and 6 h exposure to B0 exhaust. Exposure to B20- and B100 exhaust had no effect after 2 h, whereas after 6 h exposure a 1.2 ± 0.5-fold and a 2.5 ± 2.3-fold increase in gene expression was observed for B20- and B100 exhaust. The difference between 2 h exposure to B20- and B0 exhausts was statistically significant. TNF-α secretion was found to be 1.6 ± 0.7-fold (2 h) and 1.3 ± 0.5-fold (6 h) increased upon exposure to B0-exhaust (Fig. 4C). The according values for B20- and B100-exhuast are: 1.3 ± 0.6 and 1.3 ± 1.4 (B20) and 1.6 ± 0.4 and 2 ± 1.4 (B100).

IL-8 gene expression was increased by all treatments. B0 exhaust caused 4.1 ± 5.0-fold (2 h) and 3.8 ± 2.2-fold (6 h), B20 exhaust 1.7 ± 0.8-fold (2 h) and 2.5 ± 1.5-fold (6 h) and B100 exhaust 2.0 ± 0.5-fold (2 h) and 2.5 ± 2.3-fold (6 h) increased gene expression relative to filtered air (Fig. 4B). The response observed upon 6 h exposure to B0 exhaust was statistically significant. On the protein level (Fig. 4D), we found 1.2 ± 0.5 (2 h) and 1.2 ± 0.4-fold (6 h) increases upon exposure to B0 exhaust. The according values for B20- and B100 exhaust are 1.2 ± 0.4 and 1.0 ± 0.1 (B20) and 1.0 ± 0.1 and 1.0 ± 0.1 (B100).

4. Discussion

A look over the literature reveals that in the context of biodiesel exhaust toxicity, emphasis is put on primary genotoxic effects, *i.e.* the effects directly induced by particles and/or mutagenic chemicals in the exhaust. However, it is widely accepted that secondary genotoxicity may arise via the more persistent effect of exhaust induced oxidative stress and local inflammation (Schins, 2002). This is why we were mainly interested in how biodiesel affects these two biological endpoints which are not only involved in the induction of secondary genotoxicity, but also a variety of other adverse respiratory health effects (Donaldson et al. 2005; Xiao et al. 2003). The basic prerequisite for this purpose is the ability of the used biological test system to give insight into pro-inflammatory processes, which is why we chose a complex triple cell co-culture model containing the two most important types of lung-resident immune cells: dendritic cells and macrophages. Furthermore, we decided to use primary immune cells since in this way the inter-individual variation between the cell cultures (MDMs and MDDCs were obtained from a different blood donor for each experimental repetition) derives from – and hence describes – the variation in a human population. Even though this clearly decreased statistical significance of our results, we consider the introduction of this additional variation preferable over the risk that is taken when using only cell lines, the responses of which may not be representative for *in vivo* responses and do not reflect real-world inter-individual variation. That the observed variation does not result from low reproducibility but rather is an intrinsic property of the obtained data set is confirmed by the fact that after 4–5 experimental repetitions, further repetitions did not reduce the standard deviation, whereas the averages remained stable.

Because freshly produced complete exhaust was used, the present study is one among very few as performed by *e.g.* Brito et al. (2010) or Finch et al. (2002) that are able to account for the combined effects of volatile and non-volatile exhaust constituents and do not suffer from biased sample generation. Together with the presence of primary cells in the biological test system and the exposure at the air–liquid interface, this greatly increases the ability of the used approach to simulate *in vivo* responses to diesel exhaust exposure and further types of biodiesel, blending ratios and engine operation modes could and should be investigated in this way.

The literature generally reports decreased particle mass and number emissions from biodiesel combustion compared to fossil diesel (*e.g.* Dwivedi et al. 2006; Bakeas et al. 2011). A small reduction in particle number was indeed observed for B20, at least in the range of particle diameters between 10 and 30 nm, but for B100

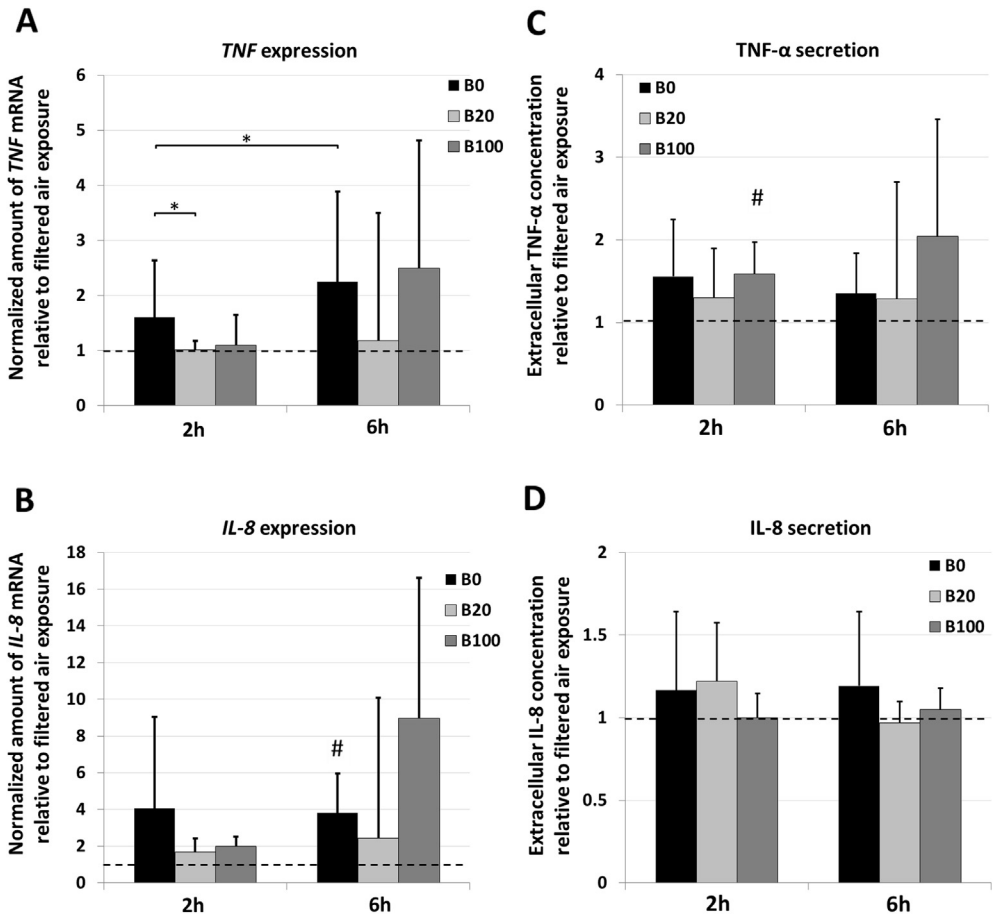

Fig. 4. Pro-inflammatory response. A) Transcriptional activity of the gene *TNF*. B) Transcriptional activity of the gene *IL-8*. C and D) Extracellular concentrations of the according gene products (cytokines) TNF-α and IL-8. All results are normalized to the filtered air exposure (dashed lines). Error bars indicate standard deviations. * indicate statistical significance ($p < 0.05$) between independent results (different doses/exhaust types) as indicated by the brackets, # between exhaust- and filtered air exposure..

particle number emissions increased 3-fold. Most notably, a distinct peak at smaller particle sizes (10–30 nm) was detected, which according to Bunger (Bunger et al. 2000) must be assumed to be made up of semi-volatile nucleation mode particles. Placing transmission electron microscopy (TEM)-grids next to the cell cultures in the exposure system and counting the deposited particles by TEM did not reveal higher numbers of particles for B100 exhaust, which supports this assumption because semi-volatile particles evaporate in the electron beam (data not shown).

The gaseous HC content of the exhausts was hardly affected by the different fuels. Only a slight increase with increasing biodiesel proportions was observed. However, together with the large number of semi-volatile nucleation mode particles, this implies that an overall higher load of (volatile and semi-volatile) HCs was delivered to the cell cultures by B100 exhaust compared to B0- and B20 exhaust.

FTIR analysis of the particle's surface chemistry showed that independently on the fuel type, saturated aliphatic functionalities dominated the surface chemistry. B20- and B100-particles contained higher amounts of C=O groups and it is reasonable to assume that the differences between hydrocarbons bound to B0-, B20- and-B100 particle surfaces also apply for the non-particle bound fraction. C=O groups are part of aldehydes and ketones (both known for their active participation in redox-chemistry (Cadenas, 1989; Shinyashiki et al. 2009) as well as of carbonic acids, esters and lactones, all of them with low redox-activity. A detailed analysis of the C=O containing chemical species was not performed and we therefore do not know which of the mentioned species were preferably formed. However, the fact that no strong differences in the induction of oxidative stress or anti-oxidant responses could be observed between the different treatments points toward the formation of compounds with low redox-activity. In particular for B100, where an overall higher load of HC has been delivered to the cells, a considerably increased formation of redox-active carbonyl compounds should have resulted in increased GSH oxidation compared to B0 exhaust (Cadenas, 1989; Stevens and Maier, 2008). However upon 2 h exposure the contrary was observed and no effect was found upon 6 h.

We expected increased emissions of NO_x and decreased emissions of HC and CO compared to B0 (Zhang et al. 2011). But we observed only very weak effects on NO_x, an increase for HC and, in the case of B100, for CO. Higher amounts of nitrogen-containing particle surface functionalities were detected by FTIR however, and even though NO_x levels in the exhaust were not affected, higher rates of nitration reactions during the combustion cannot be ruled out. As for particle emissions, a dependency of the emission of gaseous components on the engine operation mode and the feedstock is documented (Karavalakis et al. 2011; Macor et al. 2011) and we assume this to be one reason for our deviating findings. Whereas the changes in the emission of NO_x, NO_2 and NO are too small to be considered biologically relevant, the increased concentration of CO in B100 exhaust could be expected to have a certain effect on the induction of oxidative stress and the according responses (Piantadosi, 2008).

Assessed endpoints other than the ones related to oxidative stress revealed that in general, the biological responses of the human lung cell cultures to exposures with the different exhaust types were similar.

We detected no changes in cellular morphology upon exposure, neither between the different exhaust types nor between filtered air and exhaust exposure. A certain increase in the extracellular LDH activity was observed upon 6 h exposure to B100 exhaust, but we do not consider this increase sufficient to indicate the occurrence of cytotoxicity. This indicates that the acute cytotoxic potential of diesel exhaust is low and does not depend on whether RME or fossil diesel is combusted, a finding which is in agreement with other research groups (Bunger et al. 1998; Swanson et al. 2009). The expression levels of *CASP7* and *FAS* showed a trend towards lower gene activity upon exposure to B20- and B100 exhaust compared to B0 exhaust. Even though the data for pro-apoptotic gene expression should not be considered quantitative this may be interpreted as lower pro-apoptotic or even an anti-apoptotic potential of biodiesel, as also observed by Ackland et al. (2007).

Pro-inflammatory responses were found to be induced by all exhaust types. The results show however, that B100-exhaust (after 6 h exposure) has the highest pro-inflammatory potential, as demonstrated by the relative to B0- and B20-exhaust higher expression of *TNF*, the increased concentration of TNF-α and the increased expression of *IL-8*. The absence of increased IL-8 concentrations upon exposure to any of the exhausts must be interpreted as the result of the lag period between induction of gene expression and the presence of the protein. The best performance in terms of pro-inflammatory stimulation was observed for B20-exhaust, for which only very weak effects were observed upon all treatments.

The most likely cause for these results appears to be the different small-diameter particle concentrations in the three exhaust types. In particular the high amount of nucleation mode particles in B100-exhaust could be expected to have a profound effect, since various hydrocarbons are known to have a strong effect on pro-inflammatory responses (Bonvallot et al. 2001; Holder et al. 2007). HC-mediated ROS formation appears to play a major role in this context (Donaldson et al. 2005; Shinyashiki et al. 2009; Xiao et al. 2003) and in fact, in the case of *TNF* expression and to a certain degree in the case of TNF-α secretion and *IL-8* expression, the measured pro-inflammatory effects can well be correlated with the detected levels of GSH oxidation. As mentioned beforehand however, irrespective of the treatment, GSH oxidation is extensive and differences in the levels of residual reduced GSH are too small to be biologically relevant. We therefore do not consider the qualitative correlation between GSH oxidation and pro-inflammatory stimulation sufficient to imply a direct relation between the two, and assume another effect to be responsible for the increased pro-inflammatory stimulation by B100-exhaust.

Even though apparently without profound effect on the induction of oxidative stress, the increased load of HCs delivered by B100-exhaust are of concern. On one hand because the nucleation mode particles in B100-exhaust represent the most striking difference to B0- and B20-exhaust, and are therefore still the most likely reason – the nature of which can only be hypothesized at this point – for the different pro-inflammatory responses. On the other hand, they offer an explanation for the increased mutagenic potential of biodiesel-exhausts observed in other studies, *e.g.* by Kooter et al. (2011).

Regardless of the role of oxidative stress, our data indicate that the induction of pro-inflammatory responses depends on the ratio to which bio- and fossil diesel are blended. Compared to B0, the pro-inflammatory effect of B20 exhaust is lower whereas the opposite appears to be true for B100.

A general statement on the influence of biodiesel on exhaust toxicity is so far not possible neither based on our results nor on the findings reported in the literature. As reported by other research groups (Bakeas et al. 2011; Correa and Arbilla, 2006; He et al. 2010), the exhaust composition does not change as a linear function of the blending ratio, which obviously also applies for the exhaust toxicity. Exhaust toxicity therefore needs to be assessed separately for each blending ratio to be used – and as the literature implies, also for biodiesel produced from different feedstocks, for different engines and engine operation conditions. At least under the settings applied in this study, we can say that compared to fossil diesel, RME mainly affects the composition of the particulate exhaust fraction in strong dependency on the blending ratio but also the chemical structure of the particle surfaces and most likely of the non-particle bound volatile and semi-volatile hydrocarbon fraction. However, in relation to these changes in exhaust composition, the changes in exhaust toxicity brought by the use of RME were small. The performance of B100 was worse than the one of B20 and in certain aspects also worse than fossil diesel, which argues against the use of pure biodiesel. The overall minor effect of biodiesel (in particular the slightly beneficial effects of B20) on exhaust toxicity however, imply that given the compared to fossil fuels superior ecological sustainability, the widespread use of biodiesel – at least RME – is a reasonable alternative to pure fossil fuels.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atmosenv.2013.08.059.

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