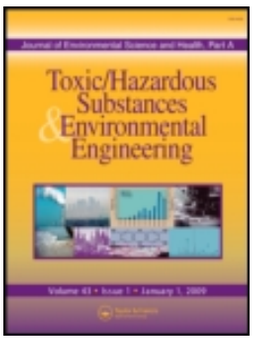

Taylor & Francis
Taylor & Francis Group

Journal of Environmental Science and Health, Part A
Toxic/Hazardous Substances and Environmental Engineering

ISSN: 1093-4529 (Print) 1532-4117 (Online) Journal homepage: http://www.tandfonline.com/loi/lesa20

The occurrence of polycyclic aromatic hydrocarbons and their derivatives and the proinflammatory potential of fractionated extracts of diesel exhaust and wood smoke particles

Annike I. Totlandsdal , Johan Øvrevik , Richard E. Cochran , Jan-Inge Herseth , Anette Kocbach Bølling , Marit Låg , Per Schwarze , Edel Lilleaas , Jørn A. Holme & Alena Kubátová

To cite this article: Annike I. Totlandsdal , Johan Øvrevik , Richard E. Cochran , Jan-Inge Herseth , Anette Kocbach Bølling , Marit Låg , Per Schwarze , Edel Lilleaas , Jørn A. Holme & Alena Kubátová (2014) The occurrence of polycyclic aromatic hydrocarbons and their derivatives and the proinflammatory potential of fractionated extracts of diesel exhaust and wood smoke particles, Journal of Environmental Science and Health, Part A, 49:4, 383-396, DOI: 10.1080/10934529.2014.854586

To link to this article: http://dx.doi.org/10.1080/10934529.2014.854586

Journal of Environmental Science and Health, Part A (2014) **49**, 383–396

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2014.854586

Taylor & Francis
Taylor & Francis Group

The occurrence of polycyclic aromatic hydrocarbons and their derivatives and the proinflammatory potential of fractionated extracts of diesel exhaust and wood smoke particles

ANNIKE I. TOTLANDSDAL[1], JOHAN ØVREVIK[1], RICHARD E. COCHRAN[3], JAN-INGE HERSETH[2], ANETTE KOCBACH BØLLING[1], MARIT LÅG[1], PER SCHWARZE[1], EDEL LILLEAAS[1], JØRN A. HOLME[1] and ALENA KUBÁTOVÁ[3]

[1]*Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway*
[2]*Faculty of Health Sciences, Oslo and Akershus University College of Applied Sciences, Oslo, Norway*
[3]*Chemistry Department, University of North Dakota, Grand Forks, North Dakota, USA*

Exposure to combustion emissions, including diesel engine exhaust and wood smoke particles (DEPs and WSPs), has been associated with inflammatory responses. To investigate the possible role of polycyclic aromatic hydrocarbons (PAHs) and PAH-derivatives, the DEPs and WSPs methanol extracts were fractionated by solid phase extraction (SPE), and the fractions were analyzed for more than ∼120 compounds. The pro-inflammatory effects of the fractionated extracts were characterized by exposure of bronchial epithelial lung cells (BEAS-2B). Both native DEPs and WSPs caused a concentration-dependent increase in IL-6 and IL-8 release and cytotoxicity. This is consistent with the finding of a rather similar total content of PAHs and PAH-derivatives. Yet, the samples differed in specific components, suggesting that different species contribute to the toxicological response in these two types of particles. The majority of the IL-6 release and cytotoxicity was induced upon exposure to the most polar (methanol) SPE fraction of extracts from both samples. In these fractions hydroxy-PAHs, carboxy-PAHs were observed along with nitro-amino-PAHs in DEP. However, the biological effects induced by the polar fractions could not be attributed only to the occurrence of PAH-derivatives. The present findings indicate a need for further characterization of organic extracts, beyond an extensive analysis of commonly suspected PAH and PAH-derivatives.

Supplemental materials are available for this article. Go to the publisher's online edition of *Journal of Environmental Science and Health, Part A,* to view the supplemental file.

Keywords: PAHs, PAHs derivatives, combustion particles, cytokines, inflammation.

Introduction

Exposure to particulate matter (PM) from combustion sources, including diesel engine exhaust particles (DEPs) and wood smoke particles (WSPs), has been associated with several adverse health outcomes, in which inflammation appears to play an important role.[1,2] To further characterize mutagenic and inflammatory effects of complex and variable PM-mixtures, it is of interest to identify specific components which may be responsible for such effects.[3] This knowledge may contribute to the development of more targeted strategies to improve air quality.

The complex composition of combustion PM and other airborne particles is a challenge from the toxicological point of view. For instance, DEPs typically consist of agglomerates of soot particles (with individual diameters >10 nm) exhibiting a carbonaceous core with embedded traces of metallic ashes and condensed organic compounds and sulphate on the surface, in addition to nucleation mode particles (<10 nm) of condensed hydrocarbons and sulphate.[4] The organic fraction may contain a wide range of hydrocarbons, the majority of which are not yet identified or quantified.[5] For both DEPs and WSPs, variations in the organic fraction composition have been reported, depending on the fuel type and combustion conditions.[6,7]

Address correspondence to Alena Kubátová, Chemistry Department, University of North Dakota, Grand Forks, ND 58202, USA; E-mail: alena.kubatova@UND.edu
Received May 28, 2013.

Several studies have indicated that the organic fraction of combustion PM is responsible for the toxicological effects.[8–15] We recently compared the potency of native DEPs, their corresponding methanol extracts and residual particles to induce expression of multiple pro-inflammatory linked genes in human bronchial epithelial cells. The extractable fraction, as opposed to the particle core, seemed to be responsible for the particle-induced cell death as well as the expression and release of pro-inflammatory mediators.[11] On a similar token, the extractable organic fraction has been reported to account for the majority of the pro-inflammatory potential of WSPs.[10]

Fractionation of PM extracts, combined with biological experiments, allows for investigation of the influence of specific groups of organic compounds. A combined *in vivo* and *in-vitro* study reported that the *n*-hexane-insoluble fraction of dichloromethane DEP-extract, containing compounds with many hydroxyl- and carbonyl groups, was most potent in inducing inflammatory responses.[16] Furthermore, the mid-polar fraction with oxygenated organics was most potent in depleting glutathione levels in macrophages when comparing WSP-extracts of different polarity.[17]

With respect to combustion emissions, polycyclic aromatic hydrocarbons (PAHs) represent a group of compounds that have gained particular attention due to their genotoxic and carcinogenic properties.[1,18] However, exposure to high concentrations of various single PAHs, as well as nitro- and amino-PAH derivatives, has been reported to induce additional pro-inflammatory responses, both *in vivo* and *in vitro*.[19,20] Such effects have been linked to both ligand binding and activation of the aryl hydrocarbon receptor, antagonist properties, as well as to the formation of reactive electrophilic metabolites during the PAH metabolism.[21,22] Although this indicates that PAHs may be important for inflammatory responses, their contribution to the pro-inflammatory effects of DEPs and other combustion particles, to our knowledge, has not yet been clarified.

The present study was initiated to further explore which constituents are responsible for the bulk of pro-inflammatory effects of DEP and WSP, with a particular focus on a potential role of PAHs and their derivatives. Methanol (MeOH)-extracts of DEP and WSP were fractionated by solid phase extraction (SPE) with a sequential use of solvents of increasing polarity, with the aim of separating PAHs and their polar derivatives.[23] The organic carbon (OC) content was determined by thermal optical analysis (TOA) and the occurrence of PAHs and their derivatives was determined by gas chromatography-mass spectrometry (GC-MS). The potency of the characterized fractions to induce cytotoxicity and release of interleukin (IL)-6 and IL-8 was examined in a human bronchial epithelial cell line (BEAS-2B). To evaluate the completeness of the applied extraction procedure, Soxhlet extracts were also investigated with respect to chemistry and pro-inflammatory effects.

Materials and methods

Chemicals and reagents

LHC-9 cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA) and PureCol from Inamed Biomaterials (Fremont, CA, USA). Solvents including methanol (MeOH, LC-MS Optima grade) and DCM (99.9%, LC-MS grade) were purchased from Fisher Scientific (Waltham, MA, USA) while *n*-hexane (HPLC grade, 95%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The analytical standards used for characterization are reported elsewhere.[23] All other chemicals were purchased from commercial sources at the highest purity available.

Collection and preparation of particle suspensions and extracts

The DEP sample, which has previously been chemically characterized, was generated by an unloaded diesel engine (Deutz, 4 cylinder, 2.2 L, 500 rpm) using gas oil (Petroplus Refining Teesside Ltd., Stockton-On-Tees, UK).[24] The DEP was collected from the inside of the main exhaust pipe after the diesel engine had been running for 8 weeks, in conjunction with an on-going clinical chamber study. The WSP sample was collected from the interior of a chimney that vented an airtight wood stove burning a mix of hardwoods.[25] To investigate the cellular responses to these samples, particles were suspended in a cell culture medium (2 mg/mL) and stirred overnight before administration to cells.

Organic extracts of the particles were prepared as previously described.[11] In brief, native particles (~30 mg) were suspended in MeOH (~60 mL), and subsequently sonicated in a water bath (30 min), prior to overnight incubation at 4°C. The suspensions were centrifuged (10 min, 8000 *g*) in order to separate the particles from the extract, and then the extracts were evaporated to dryness under a gentle stream of nitrogen gas. Subsequently, the extracts were fractionated based on polarity (with the aim of separating PAHs and their polar derivatives), using the previously described SPE protocol. Briefly, an amino propyl SPE cartridge (Sep-Pak, Waters, Milford, MA, USA) was preconditioned with 6 mL of DCM followed by 6 mL of *n*-hexane. Three fractions were eluted sequentially with *n*-hexane (5 mL), 20% DCM in *n*-hexane (5 mL), and MeOH (5 mL).

Following the first sonication, two additional extractions were performed on the residual particles to investigate if the first extraction was efficient, i.e., a second sonication (each with 60 mL of MeOH) and then a Soxhlet extraction [11] (with 90 mL of MeOH). The Soxhlet extracts of both samples contained 3–4 times more OC than the second sonication extracts (Table 1). The Soxhlet extracts were therefore chosen to be included in the cellular experiments to further confirm that all the biologically active organics were removed in the first extraction. To prepare the extracts for

Table 1. Percentages of OC in DEP and WSP methanol-extracts after the first and second sonication steps and of Soxhlet extraction of residual DEP and WSP.

Extraction	*DEP*	*WSP*
After first sonication	54 ± 3	16.8 ± 0.5
After second sonication	1.2 ± 0.3	2 ± 1
Soxhlet	4.7 ± 0.7	7 ± 1
Total	60 ± 3	23 ± 2

The results are presented as % OC of total mass of native particles submitted to extraction. The results are reported as averages of triplicate experiments with one standard deviation. The OC content was determined using TOA.

Table 2. Percentages of OC in the SPE-fractions (*n*-hexane, 20% DCM in *n*-hexane and methanol) of DEP and WSP methanol-extracts after first sonication step.

SPE-fraction	*DEP*	*WSP*
n-hexane (non-polar)	18 ± 2	0.22 ± 0.07
20% DCM in *n*-hexane (mid-polar)	4.9 ± 0.2	0.43 ± 0.02
MeOH (polar)	5.9 ± 0.7	10.8 ± 0.6
Total fractionation	30 ± 2	11 ± 1

The results are presented as % OC of total mass of native particles submitted to extraction, and are reported as averages of triplicate experiments with one standard deviation. The OC content was determined using TOA.

in vitro experiments, the fractionated SPE-extracts and the Soxhlet extracts were dried under nitrogen gas and stored at −20°C until further processing (see Table 2 for OC distribution). Later, these extracts were thawed, re-suspended in dimethyl sulfoxide (DMSO), and aliquoted in glass tubes before storage at −20°C until the day of exposure. On the day of exposure, aliquots of each extract fraction were further suspended in a cell culture medium at a concentration corresponding to 2 mg/mL of native particles.

Chemical characterisation of the particle extracts

The OC and elemental carbon (EC) determinations of all extracts (after 1st and 2nd sonication, SPE-fractions and Soxhlet) were conducted using a thermal optical OC/EC analyzer (Sunset Laboratories, Tigard, OR, USA). The representative chromatograms of the obtained fractions are shown in Fig. 1. The temperature program began with four steps under a helium atmosphere, starting at 300°C for 75 s, 500°C for 75 s, 600°C for 75 s, 700°C for 75 s, 870°C for 120 s; then the instrument was cooled down (35 s) to 550°C and helium with 5% oxygen was introduced. Five additional temperature stages were then used: 550°C for 45 s, 625°C for 45 s, 700°C for 45 s, 775°C for 45 s, 890°C for 120 s. Quantification was based on daily calibration curves using potassium hydrogen phthalate. OC/EC determinations of all samples were performed in the transmittance mode. For this analysis, the extracts were concentrated under nitrogen gas to 10 mL and were then used without further concentration. The aliquots of extract/fractions (10 μL) were spiked onto a 1.5 cm^2 punch from a quartz filter (prebaked at 1500°C overnight to remove any carbon residue). Solvents were evaporated from the filter for 4 min on a hot plate at 40°C, prior to inserting it into the thermal optical analysis (TOA) oven. The data for these six extracts are presented

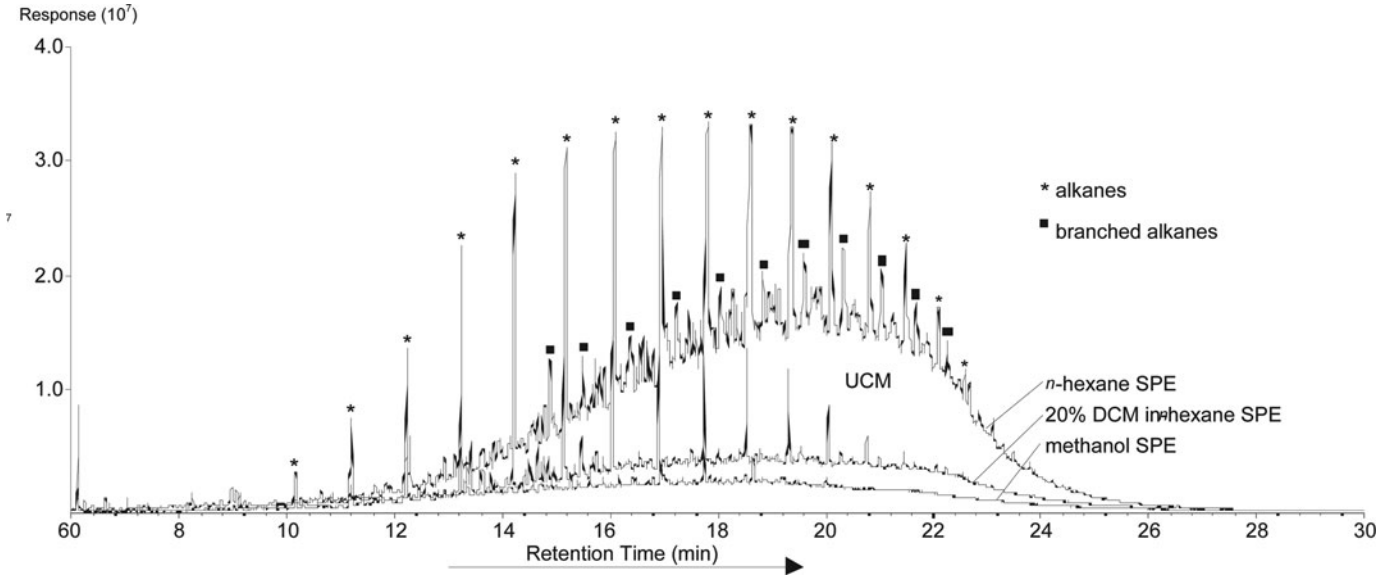

Fig. 1. Overlayed gas chromatography-mass spectrometry chromatograms of solid phase extraction (SPE) fractions of the first sonication extract of diesel exhaust particles. All profiles are shown as total ion current responses. Alkanes, branched alkanes, and the shoulder corresponding to the "unresolved complex mixture" (UCM) are labelled for clarity.

as % OC content relative to the particle mass they were extracted from (~30 mg).

A more detailed chemical characterization of the five extracts was performed by GC-MS analysis, using a 6890 GC with an 5975C MS (Agilent Technologies, Inc., Wilmington, DE, USA), as described previously.[11,23] A broad range of PAHs and PAH-derivatives were analysed, of which the complete list of individual organic species is provided in Figures 2 and 3, and Tables 1S and 2S in Supplementary data. Species for which pure analytical standards were not available were tentatively identified by matching MS spectra (at least 90% matching quality) with those obtained from the NIST 2005 MS Spectral Database. In addition, the sum of the individual organics analysed in each group of PAHs/PAH-derivatives is presented in Table 3 as μg/g of native particles.

Culture of cells

BEAS-2B, a SV40-transformed human bronchial epithelial cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in LHC-9 medium in collagen (PureCol)-coated flasks in a humidified atmosphere at 37°C with 5% CO_2, with a refreshment of medium on every second day. One day prior to exposure, BEAS-2B cells (passages 26–38) were plated into collagen (PureCol)-coated 35 mm 6-well culture dishes (460,000 cells/well).

Exposure of cells

Cells were exposed to either suspensions of native particles or to the various extracts in the concentration-range corresponding to 0–400 μg/mL of native particles. The final concentration of DMSO was equal in all cell culture wells (including the non-exposed control cells), and did not exceed 0.4%. The total exposure volume was 1.5 mL in 6-well 35 mm cell culture dishes. From this, it may be calculated that an applied particle concentration of 100 μg/mL corresponds to a concentration of 16 μg/cm^2, if all the suspended particles deposit on the cells present on the surface of the culture dishes.

Cells were also treated with a selection of the oxy- and hydroxy-PAHs detected in the fractionated extracts, in order to investigate their contribution to the effects induced by the fractions. The components tested were 9-fluorenone, xanthone, phenalenone, 9,10-anthraquinone, benz[*a*]anthracene-7,12-dione and 2-hydroxy-9-fluorenone, both alone (at concentrations ranging from 1 nM–30 μM) and in combination. When tested in combination, the applied concentrations of each compound were based on the corresponding concentrations detected in the high polar SPE fraction of the DEP-extract. The resulting mixture of PAH derivatives was tested at concentrations corresponding to 1, 10 and 100 times the concentrations in the high polar methanol fraction of the DEP-extract.

Examination of cytotoxicity

After a 24-h exposure, cells were stained with propidium iodide (PI; 10 μg/mL) and Hoechst 33342 (5 μg/mL) and evaluated using a Nikon Eclipse E 400 (Nikon Gmbh., Düsseldorf, Germany) fluorescence microscope, as previously described.[24] The percentage of apoptotic and necrotic cells were determined as a fraction of the total number of counted cells.

Quantification of cytokine release

After a 24-h exposure, cell culture supernatants were collected and centrifuged for the removal of dead cells (300 × *g*) and, if necessary, for removal of particles (8000 × *g*) and stored at −70°C until cytokine analysis. Concentrations of IL-6 and IL-8 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis MN, USA), according to the manufacturer's manual. The increase in colour intensity was measured and quantified using a plate reader with Magellan V 1.10 software (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA). Cytokine concentrations in cell culture supernatants are expressed in pg/mL.

Calculations and statistical analysis

All chemical characterization data are based on triplicate extraction/fractionation experiments analysis and are reported as average plus/minus one standard deviation. The results from cell culture experiments represent the mean values of three independent experiments. Statistical analysis of the data was performed by application of a two-way ANOVA with Bonferroni post-tests. The data in Figures 3 and 4 were log-transformed prior to the analysis to fulfill the assumptions for ANOVA. Statistical analyses were performed using GraphPad Prism software (version 4.03, Inc., San Diego, CA, USA).

Linear regression analysis was performed to investigate the influence of the contents of identified PAHs and PAH derivatives on cytokine release and toxicity. Analyses were performed for the total PAH content, and the contents of PAHs and hydroxy-, oxy- and nitro-PAHs. For all the statistical analyses, P values < 0.05 were considered to reflect statistically significant differences.

Results

Chemical composition of particle extracts

The OC content in the extracts obtained from three methanol extractions (first, second, and Soxhlet) is presented in Table 1 as mass percent compared to the amount

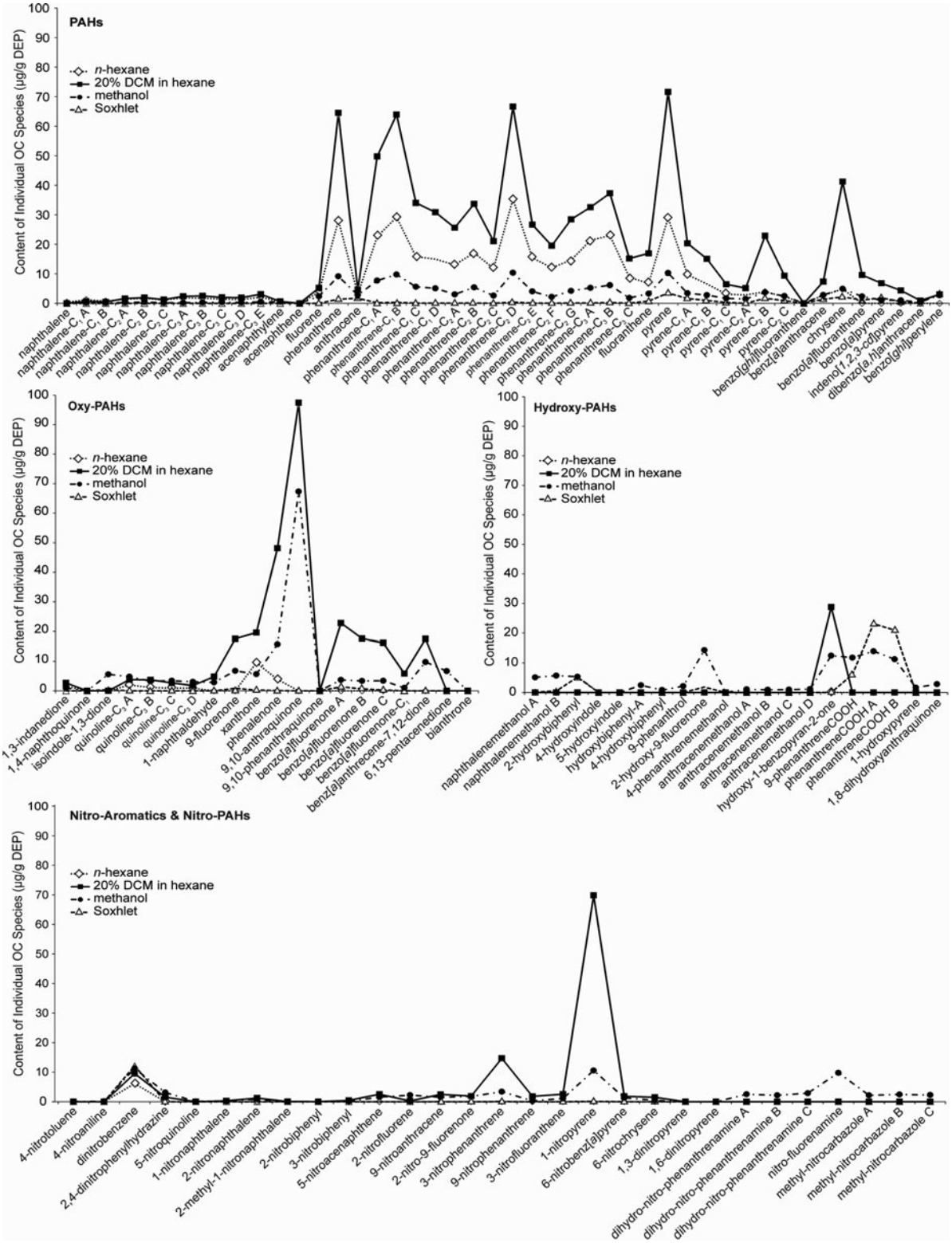

Fig. 2. The distribution of identified polycyclic aromatic hydrocarbons (PAHs) and PAH-derivatives in fractionated methanol diesel exhaust particle (DEP)-extracts of increasing polarity (*n*-hexane, 20% dichloromethane (DCM) in *n*-hexane, methanol), and in Soxhlet extracts of residual DEPs. The results are presented as average concentrations (µg/g of native particles) detected in three replicate extractions. Methanol particle extracts were fractionated using SPE, and the characterization of both methanol particle extracts and the Soxhlet extract was performed using gas chromatography-mass spectrometric analysis.

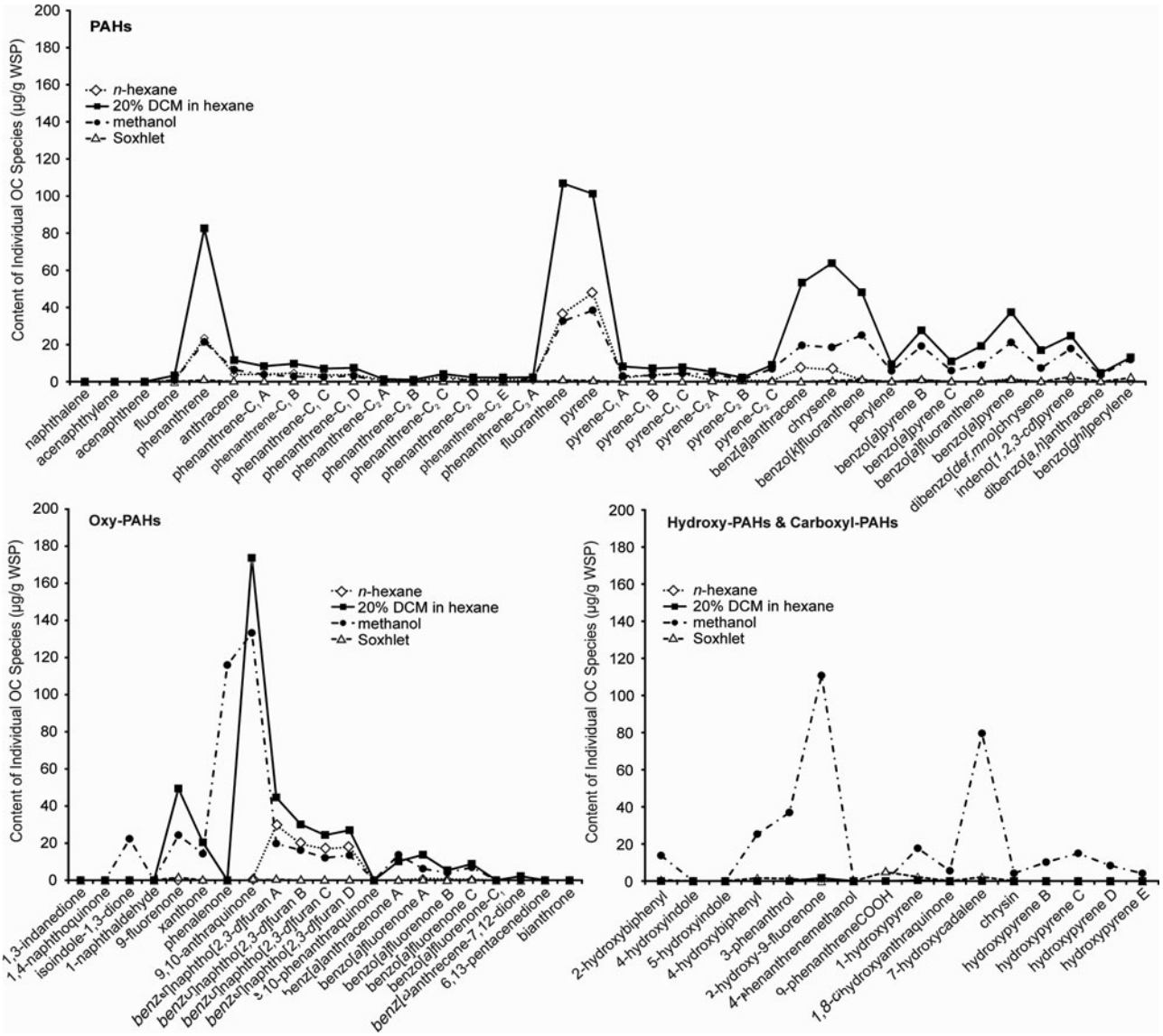

Fig. 3. The distribution of identified polycyclic aromatic hydrocarbons (PAHs) and PAH-derivatives in fractionated methanol wood smoke particle (WSP)-extracts of increasing polarity (*n*-hexane, 20% dichloromethane (DCM) in *n*-hexane, methanol), and in Soxhlet extracts of residual WSPs. The results are presented as average concentrations (µg/g of native particles) detected in three replicate extractions. Methanol particle extracts were fractionated using solid phase extraction (SPE), and the characterization of both methanol particle extracts and the Soxhlet extract was performed using gas chromatography-mass spectrometric analysis.

of native particles submitted to extraction. The TOA revealed that DEP contained a considerably higher fraction of methanol extractable OC of native particles (60%) than WSP (23%). A significant proportion of the extractable material was extracted within the 1st sonication step (54% and 17% for DEP and WSP, respectively), accounting for 90 and 70%, respectively, of the total OC extractable in methanol. Although this observation suggested that the 1st extraction was relatively successful, a considerable portion was also extracted by the following Soxhlet extraction. Therefore, the Soxhlet extracts, in addition to the three SPE fractions of the extract obtained after the 1st sonication step, were included in the biological experiments.

The three SPE-fractions of the 1st sonication extract differed for DEP and WSP with respect to which fractions contained the highest amount of OC. For DEP, the highest OC level was detected in the non-polar *n*-hexane fraction whereas the polar methanol fraction contained the highest OC level of the WSP extracts. It should be noted that a significant portion of the OC spiked onto the SPE column was not eluted (ca. 50% for both DEP and WSP) and was thus retained on the column, even upon addition of a more polar solvent, such as water. As shown below, a significant toxicological response reported for unfractionated extracts in our previous work [11] was also observed in this study in the obtained SPE fractions.

Table 3. Content of PAHs, oxy-PAHs, hydroxy-PAHs, carboxy-PAHs and nitro-PAHs in the SPE-fractionated DEP and WSP methanol-extracts after the 1st sonication step, in the extract after the 2nd sonication step and in soxhlet extracts of residual DEP and WSP.

	DEP						*WSP*					
	1st sonication extract SPE fractions						*1st sonication extract SPE fractions*					
Class	*n-hexane*	*20% DCM in n-hexane*	*MeOH*	*2nd sonication extract*	*Soxhlet extract*	*Total from all extracts*	*n-hexane*	*20% DCM in n-hexane*	*MeOH*	*2nd sonication extract*	*Soxhlet extract*	*Total from all extracts*
PAHs	377 ± 34	819 ± 46	135 ± 2	131 ± 7	26 ± 3	1488 ± 64	121 ± 13	720 ± 44	303 ± 36	100 ± 2	11 ± 2	1255 ± 62
oxy-PAHs	20 ± 9	282 ± 24	147 ± 24	51 ± 8	4 ± 1	478 ± 50	86 ± 25	409 ± 25	402 ± 18	77 ± 3	2.0 ± 0.4	976 ± 70
hydroxy-PAHs and carboxy-PAHs	ND	29 ± 5	94 ± 7	10 ± 1	58 ± 2	191 ± 12	0.5 ± 0.1	2.1 ± 0.3	331 ± 13	48 ± 2	13 ± 1	394 ± 19
nitro-PAHs	ND	102 ± 16	50 ± 31	6 ± 5	ND	158 ± 38	ND	ND	ND	ND	ND	ND
Total	397 ± 35	1232 ± 54	426 ± 40	197 ± 12	88 ± 4	2315 ± 90	207 ± 28	1131 ± 50	1036 ± 43	225 ± 4	25 ± 2	2625 ± 95

The data are average of triplicate experiments and are presented as the sum of amounts of identified species in each group of compounds compared to total amounts of native particles (μg/g). The complete data set is presented in supplements in Table S1 and S2. The characterization was performed using GC-MS analysis.

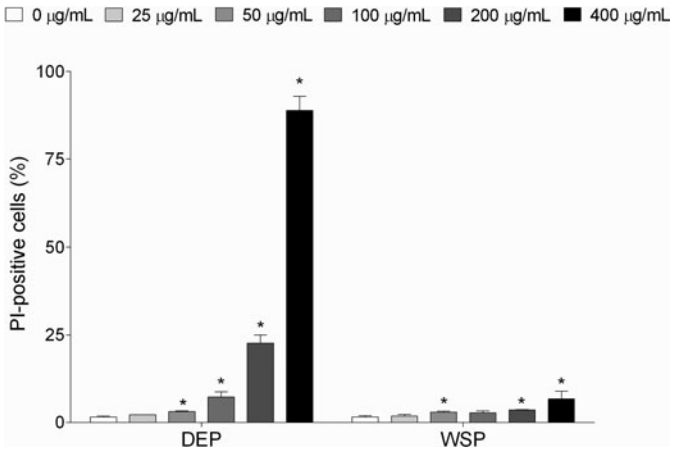

Fig. 4. Cytotoxicity induced by native diesel exhaust and wood smoke particles (DEP and WSP). Human bronchial epithelial BEAS-2B cells were exposed to native diesel and wood smoke particles in the concentration range corresponding to 0–400 μg/mL for 24 h, before they were stained with Hoechst 33342 and PI and analysed by fluorescence microscopy. Bars represent the means ± SEM of the percentage of PI-positive cells counted in separate experiments ($n = 3$). * $P < 0.05$; exposed vs. control.

The GC-MS analysis targeted the quantification of PAHs and their derivatives (+120 species), providing trends in the distribution of major classes of compounds. However, alkanes and branched alkanes are typically the most abundant organic species in DEP.[26] These non-polar compounds occur in GC chromatograms as an unresolved complex mixture (UCM). Presently, as shown in Figure 1, the SPE fractionation enabled the separation of the majority of non-polar species (i.e., alkanes and UCM) in the *n*-hexane fraction.

An overview of the total content of PAHs and PAH-derivatives in the SPE fractionated extracts, the extracts obtained after the second sonication step and the Soxhlet extracts is given in Table 3 for both DEP and WSP (for a full dataset including quantification of individual compounds see Table 1S and 2S). Although the OC levels were almost three times higher in DEP than in WSP (Table 1), the total contents of PAHs and PAH-derivatives were relatively similar, 2315 and 2625 μg/g, respectively. However, the distribution of PAHs and PAH-derivatives varied between the three DEP and WSP fractions. The mid-polar 20% DCM/*n*-hexane extract contained the highest total amount of identified PAHs and PAH-derivatives for both samples, but the polar MeOH fraction from WSP also contained high levels of identified species in contrast to DEP (Table 3, last row).

The total levels of oxy-, hydroxy- and carboxy-PAHs were significantly higher in WSP than in DEP, with approximate sums of 1400 and 700 μg/g, respectively (Table 3). Nevertheless, for both particle samples the total content of identified PAHs and PAH-derivatives constituted only a minor fraction (<1%) of the total extractable organic carbon (EOC) content. As for the total OC content, the SPE fractions (from the 1st sonication) contained the majority of the total PAHs and PAH-derivatives for each sample, compared to the extracts obtained within the second sonication step and the Soxhlet extract, which contained approximately 10% of the identified species (Table 3). However, the extract from the 2nd sonication step contained considerably higher amounts of PAHs and PAH-derivatives than the Soxhlet extract.

The purpose of SPE fractionation was to separate the majority of PAHs and their derivatives into several fractions (Figs. 2 and 3) allowing for a toxicological assessment of the fractions obtained. Figures 2 and 3 show the distribution of identified PAHs and PAH-derivatives in the SPE fractionated extracts as well as the Soxhlet extracts of the residual particles. The unsubstituted PAHs were detected mainly in the mid-polar fraction (20% DCM in *n*-hexane) of both DEP and WSP. The majority of high molecular weight PAHs, oxy-PAHs, and nitro-PAH were observed mainly in the mid-polar 20% DCM in *n*-hexane of both DEP and WSP (Figs. 2 and 3).

The only exception was phenalone, which was abundant also in the MeOH fraction. For WSP, the hydroxy-PAHs and carboxy-PAHs were detected mainly in the polar methanol fraction (Fig. 3), whereas for DEP different hydroxy-PAHs were detected in two higher polarity SPE and Soxhlet fractions in comparable amounts. In WSP, the most abundant species were 2-hydroxy-9-fluorenone, 7-hydroxycadelene, 2-hydroxybiphenyl and isomers of hydroxypyrene. In DEP, 2-hydroxy-9-fluorenone was also abundant; but, in addition, hydroxy-*n*-benzopyran-*n*-one (tentatively identified by MS library match) was observed. However, it is important to note that concentrations in DEP were approximately 5 times lower than those in WSP. Besides the hydroxy-PAHs, 3-ring carboxy-PAHs (tentatively identified) were also found in the methanol SPE DEP-fraction and Soxhlet extract of the sonication residue. Moreover, nitroamine-PAH species (which to our knowledge have not been previously observed) eluted in the methanol SPE DEP-fraction (Table 1S). These species included dihydro-nitro-phenanthrenamine isomers, nitrofluorenamine, as well as methyl-nitrocarbazole isomers (all tentatively identified with 80–85% match to the MS database).

The DEP sample, but not the WSP sample, also contained nitro-PAHs, which were detected mainly in the mid-polar 20% DCM in *n*-hexane fraction, where 1-nitropyrene was the dominating species. In the WSP methanol SPE-fraction, organic species other than PAH and PAH-derivatives were also observed following the derivatization. These species were previously identified WSP components including levoglucosan, mono- and dicarboxylic acids, and syringol and guaiacol derivatives.[27] As expected, none of these compounds were observed in the DEP extracts.

The results from the GC-MS analysis show that the SPE fractionation successfully separated the non-polar alkanes and branched alkanes into the appropriate polarity extracts. For the PAH and PAH derivatives, there was a

higher extent of overlap between the various fractions; however, the majority of PAHs and oxy-, nitro-PAHs were recovered in the 20% DCM in *n*-hexane whereas hydroxy- and carboxy- and nitroamine-PAHs were recovered in the methanol SPE fractions.

Cytotoxicity induced by native particles and SPE fractionated particle extracts

Both DEP- and WSP-samples reduced the number of viable cells. As judged by visual examination of cell number under light microscopy, combined with fluorescence microscopy of cells stained with PI/Hoechst, the cells died by necrosis. Both PM samples induced PI-positive cells, with DEP clearly more cytotoxic than the WSP (Fig. 4). At a concentration of 400 μg/mL, DEP induced approximately 90% necrotic cells (PI-positive) versus 10% in the cell cultures exposed to WSP.

Cells were exposed to three consecutive SPE fractions or the Soxhlet extract in the amounts corresponding to the concentrations of native particles they were extracted from, with 400 μg/mL of native particles as the maximum. Only the polar methanol fraction of both DEP and WSP induced a significant increase in necrotic cell death (Fig. 5). In accordance with the effects induced by the native particles, the polar methanol fraction of the DEP appeared to be more potent than the corresponding WSP fraction, with maximum responses of similar magnitudes as the respective native particles (Fig. 5). The Soxhlet extracts did not significantly increase the cytotoxicity.

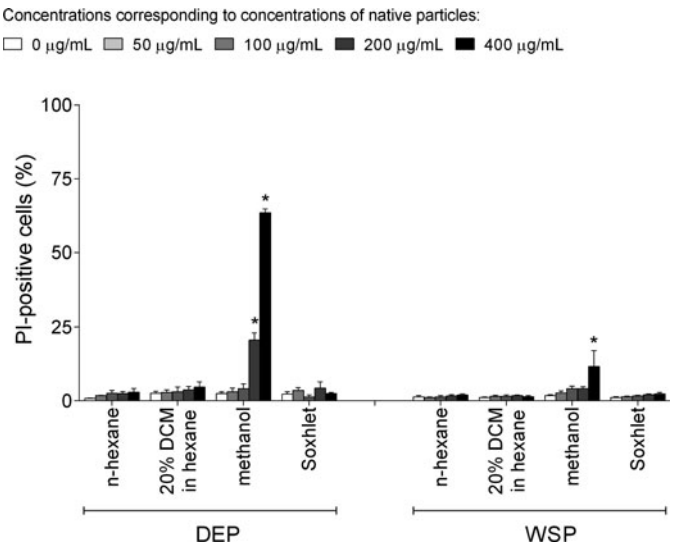

Fig. 5. Cytotoxicity induced by fractionated extracts of diesel exhaust and wood smoke particles (DEP and WSP). Human bronchial epithelial BEAS-2B cells were exposed to SPE-fractionated DEP and WSP methanol-extracts of increasing polarity (*n*-hexane, 20% DCM in *n*-hexane, methanol), and to Soxhlet extracts of residual DEP and WSP in the concentration range corresponding to 0–400 μg/mL of native particles for 24 h, before they were stained with Hoechst 33342 and PI and analysed by fluorescence microscopy. Bars represent the means ± SEM of the percentage of PI-positive cells counted in separate experiments ($n = 3$). * $P < 0.05$; exposed vs. control.

Cellular release of IL-6 and IL-8 induced by native particles and SPE fractionated particle extracts

The 24-h exposure to DEP induced concentration-dependent increases in both IL-6 and IL-8 release, which reached statistical significance at 50 μg/mL, before the levels declined at concentrations above 100 μg/mL (Fig. 6). WSP did not induce any statistical significant increase in the release of these mediators, although the levels tended to increase with increasing particle concentrations, with maximum levels at 400 μg/mL (Fig. 6).

The IL-6 release induced by the three SPE extracts showed a similar trend as the cytotoxicity; namely, only the polar methanol-fraction from both particle types increased the IL-6 release (Fig. 7A). Concentrations corresponding to 100 μg/mL of native DEP and 200 μg/mL of native WSP increased the IL-6 release significantly, in contrast to the responses induced by the native particles, of which only DEP induced a significant increase. The IL-8 release did however show a different response pattern, since the high-polar methanol-fractions from both DEP and WSP as well as the DEP Soxhlet extract reduced the release of IL-8 significantly (Fig. 7B).

Thus, the polar methanol SPE fraction exhibited the most severe effect on both the IL-6 and IL-8 release, but caused increases in IL-6 release and decreases in IL-8 release. The reduced IL-8 release induced by the DEP Soxhlet extract suggests that some biologically active organic compounds remained in the residual particles after the 1st and 2nd extraction procedures.

Cells were also treated with a selection of commercially available oxy- and hydroxy-PAHs detected in the fractionated extracts, in order to investigate their contribution to the observed responses. This included 2-hydroxy-9-fluorenone, which was detected in higher concentrations in the polar methanol SPE fraction, as compared to the other fractions. Concentrations from 1 nM-30 μM of 9-fluorenone, xanthone, phenalenone, 9,10-anthraquinone, benz[*a*]anthracene-7,12-dione and 2-hydroxy-9-fluorenone did not cause significant cellular effects; neither did the mixture of these compounds corresponding to 1, 10 and 100 times the concentrations of the compounds detected in the polar fraction of the DEP methanol extract (Figs. 1S and 2S, in the online supplementary files). Notably, the occurrence and abundance of these and other derivatives differed in extracts of the two studied PM samples, making it difficult to estimate the contribution of individual species.

Influence of the content of identified PAHs and PAH derivatives on the biological responses

To what extent the presence of the identified PAHs and PAH derivatives could explain the differential cellular responses to the different various extracts, was also

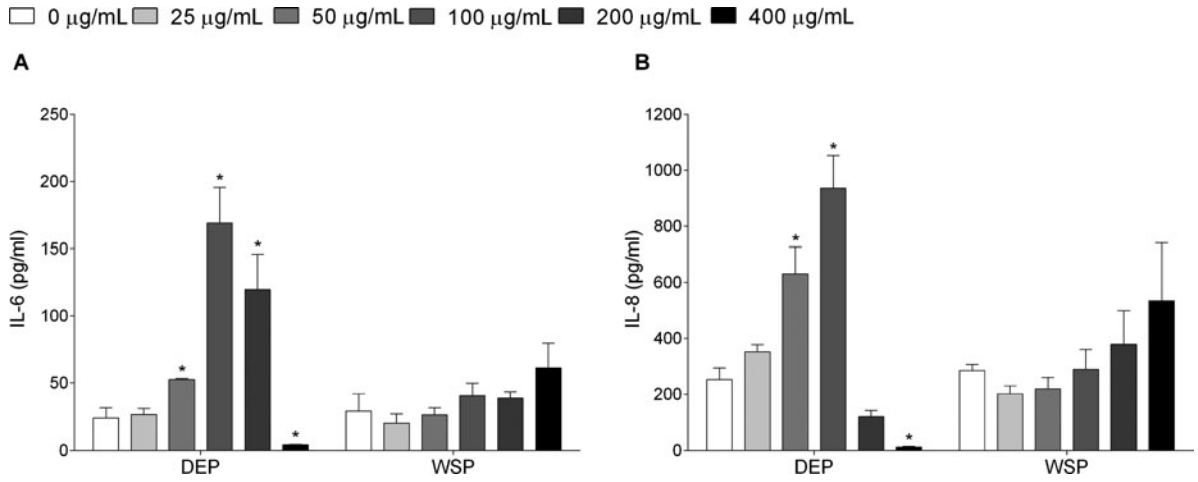

Fig. 6. Cellular release of IL-6 and IL-8 induced by native diesel exhaust and wood smoke particles (DEP and WSP). Human bronchial epithelial BEAS-2B cells were exposed to native diesel and wood smoke particles in the concentration range of 0–400 μg/mL for 24 h, before IL-6 (A) and IL-8 (B) concentrations in cell culture supernatants were determined by ELISA. Bars represent means ± SEM of separate experiments ($n = 3$). * $P < 0.05$; exposed vs. control.

investigated by linear regression analysis. With two exceptions, the overall analysis indicated that the content of the identified PAHs and PAH derivatives had either limited influence or no influence on the biological effects induced by the fractionated extracts. However, the presence of hydroxy-PAHs was positively correlated with IL-6 ($r^2 = 0.32$) and negatively correlated with IL-8 ($r^2 = 0.40$). Further, the content of PAHs (not total PAHs) was positively correlated with IL-8 ($r^2 = 0.36$). However, the r^2 values of about 0.4 indicated that only 40% of the variability could be explained by the content of hydroxy-PAHs and PAHs. Moreover, the slopes of the corresponding regression lines were relatively low and therefore had limited biological relevance. For instance, IL-6 versus hydroxy-PAHs resulted in a positive slope of 22 pg/mL per 100 μg/g PM. As a reference, the levels of hydroxy-PAHs varied from 0 to 330 μg/g in the different DEP and WSP extracts, and the general IL-6 levels varied between 50–190 pg/mL.

Discussion

We and others previously reported that the organic fraction of combustion PM accounts for the majority of their biological reactivity, ranging from genotoxic and mutagenic effects to induction of pro-inflammatory mediators.[8,10,11,28–32] In the present study we have specifically characterized SPE fractionated organic extracts of DEP and WSP with regard to their specific content of OC, PAHs and PAH-derivatives and evaluated their cytotoxicity and cytokine responses in BEAS-2B cells. In particular, the aim was to explore any possible role of PAHs and their oxidation products in the pro-inflammatory effects of DEP and WSP. For both of the two distinct PM samples it was demonstrated that the most polar (methanol) SPE fraction was the most potent in inducing cytotoxicity and cellular release of IL-6. However, the chemical compounds accounting for these toxicological effects could not easily be identified among the >120 PAHs and PAH-derivatives quantified. The present findings indicate a need for further characterisation of PM organic extracts, also beyond an extensive analysis of PAH and PAH-derivatives, in order to enhance our understanding of the driving effects behind the toxicity of PM.

Role of alkanes, PAHs and their mildly polar derivatives

The majority of abundant alkanes and components of the unresolved complex mixture were successfully separated from PAHs and other species in the non-polar *n*-hexane SPE fraction of DEP (Fig. 1). This enabled us to confirm that these species do not have significant effects on cytoxicity and cytokine release induced by the investigated DEP and WSP samples.

With respect to combustion emissions, PAHs represent a group of compounds that gained a particular attention due to their carcinogenic properties.[1] In this work, PAHs, nitro-PAHs and also oxy-PAHs were recovered primarily in the mid-polar SPE fractions, with individual concentrations in the low nM-range. These concentrations are about 1000-fold less than those previously used to demonstrate cytokine responses to single PAHs.[19,20]

Moreover, these mid-polar (20% DCM in *n*-hexane) SPE fractions did not induce significant cellular effects (Figs. 5 and 7), and accordingly the content of these groups of compounds did not correlate with the toxicological effects in the linear regression analysis. This is in line with previous findings for one of the most carcinogenic PAHs, B[*a*]P, which did not induce cytokine responses in the BEAS-2B cells.[19] Thus, the present findings seem to support the previous

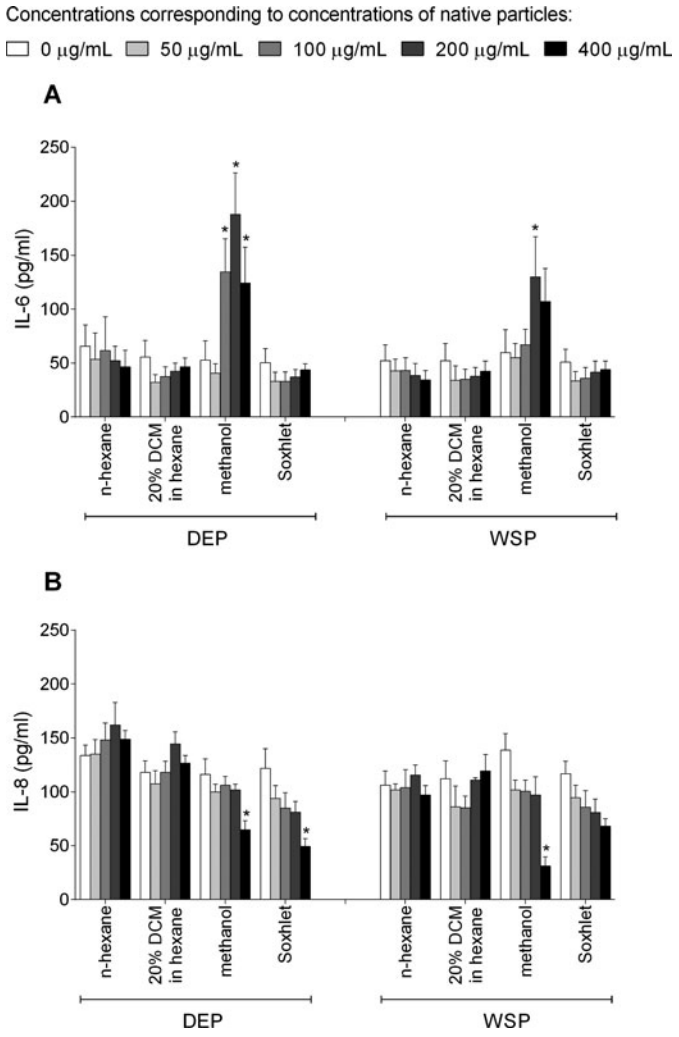

Fig. 7. Cellular release of IL-6 and IL-8 induced by fractionated extracts of diesel exhaust and wood smoke particles (DEP and WSP). Human bronchial epithelial BEAS-2B cells were exposed to SPE-fractionated DEP and WSP methanol-extracts of increasing polarity (*n*-hexane, 20% dichloromethane (DCM) in *n*-hexane, methanol), and to Soxhlet extracts of residual DEP and WSP in the concentration range corresponding to 0–400 μg/mL of native particles for 24 h, before IL-6 (A) and IL-8 (B) concentrations in cell culture supernatants were determined by ELISA. Bars represent the means ± SEM of separate experiments (n = 3). * $P < 0.05$; exposed vs. control.

suggestions that unsubstituted PAHs and other organic compounds with high affinity for the aryl hydrocarbon receptor are not the primary drivers of the pro-inflammatory effects induced by DEP.[19]

We previously reported that certain nitro-PAHs may induce strong increases in cytokine responses in BEAS-2B cells, but that these effects required high concentrations above 1 μM.[19] Previous studies also suggested that quinones and resins may be involved in the cytotoxic effects,[30] while DEP-induced suppression of NO-production in macrophages has been attributed to resins only.[33] In this study, the content of oxy-PAHs (including quinonic species) did not correlate with the toxicological responses.

In addition, exposure to a selection of the identified oxy-PAHs (9-fluorenone, xanthone, phenalenone, 9,10-anthraquinone and benz[*a*]anthracene-7,12-dione) did not induce any significant IL-6 and IL-8 responses or cytotoxicity in the BEAS-2B cells. In accordance with our data, Shima and colleagues were also unable to emulate the effects of the *n*-hexane insoluble fraction of DEP DCM-extracts when using selected quinones (1,2-/1,4-naphthoquinone, 9,10-anthraquinone and 9,10-phenanthraquinone).[16] Thus, similarly as for PAHs and nitro-PAHs, the pro-inflammatory and cytotoxic effects of DEP and WSP could not easily be explained by the presence of quinonic species here identified (i.e., oxy-PAHs).

Contribution of polar fractions, toxicity vs. chemical characterisation

In contrast to the non- and mid-polar SPE fractions, the polar methanol SPE fractions of both DEP and WSP induced significant increases in IL-6 release and cytotoxicity in BEAS-2B cells. Notably, DEP-induced mitochondrial dysfunction was previously attributed to the polar fraction,[30] and was suggested to be the main contributor to the pro-inflammatory and cytotoxic effects of PM2.5 from Puerto Rico.[32] Moreover, the mid- and high-polar fractions of DEP DCM-extract (*n*-hexane insoluble) have been reported to induce pulmonary inflammation in mice.[16] Additionally, it was reported that the non-polar (*n*-hexane soluble) fraction did not induce pulmonary inflammation response.[16] However, studies on immune responses in mice suggest that different fractions of DEP organic extracts differentially regulate Th1 and Th2 responses.[34]

Thus, the contribution of different organic compounds to the DEP-effects is complex and different organic compounds might affect different biological endpoints. Shima and colleagues previously reported that the polar *n*-hexane insoluble fraction of DEP DCM-extracts displayed the highest oxidative capacity and induced the strongest inflammatory response in mice.[16] The authors noted that this polar fraction was mainly composed of PAHs with functional groups related to oxygenation, such as hydroxyl- and carbonyl groups.[16] In the present study, the chemical analysis revealed that the polar methanol fraction contained higher amounts of hydroxy-PAHs.

Moreover, the hydroxy-PAH abundances slightly correlated to the toxicological effects in the linear regression analysis, partly supporting the suggestion that these PAH oxidation products might be involved in the observed pro-inflammatory and cytotoxic effects. In contrast, the oxy-PAHs (e.g., carbonyls) did not appear to influence the toxicological endpoints measured. We did not detect any significant cytotoxicity and/ or pro-inflammatory effects with the most abundant hydroxy-PAH detected in DEP and WSP polar SPE fractions (Supplementary Information, Fig. 1S). The occurrence and abundance of other hydroxy

species differed drastically in the two PM samples, making it difficult to estimate the contribution of individual species.

Other species observed in the polar fraction of DEP were tentatively identified nitro-amino-PAHs. These species were not previously reported for PM, possibly due to problems of their isolation from PM samples. A previous comparison of some amino-PAH derivatives to their nitro-counterparts showed a decreased or altered cytokine and chemokine response for the amino-derivative.[19] However, these effects were observed with concentrations several orders of magnitude higher than those occurring in DEP-exposed cells. Thus, the nitro-PAH species identified in this work do not readily account for the DEP-induced effects in the BEAS-2B cells. It is of note, we identified PAH-derivatives containing both nitro and amino groups, but to our knowledge, the pro-inflammatory responses to such PAH-species have yet to be determined.

Differential effects on IL-6 and IL-8 release and mechanisms involved

Whereas both native WSP and DEP increased the release of IL-6 and IL-8 (Fig. 6), a contrasting trend was observed for the SPE fractions for these two endpoints. Although the polar methanol fractions increased the IL-6 release, the IL-8 release was reduced (Fig. 7). Similarly, polar organic extracts from PM2.5 have been found to increase IL-6 and reduce IL-8 release from BEAS-2B cells.[32] Additionally, a reduced IL-8 response was observed for the Soxhlet DEP extract (obtained following the sonication) (Fig. 7). Interestingly, both the methanol SPE fraction and Soxhlet extracts of DEP contained carboxy-PAHs that could contribute to a reduced IL-8 response, although no association could be detected in the regression analysis.

A possible explanation of the discrepancy between the IL-8 responses to the native particles and the fractionated extracts is that the organics inhibiting IL-8 release could be less available when bound to the particles, while others may be more efficiently delivered. Alternatively, it may be related to some specific PM-properties of functional groups necessary for receptor-interactions.

Contribution of other organic species

In addition to the suggested influence of hydroxy-PAHs on the IL-6 release and cytotoxicity, other organic compounds are also likely to have contributed to the observed toxicological effects. Besides the need for identification of the groups of organic species responsible for the toxicological effects, the exact cellular mechanisms involved also remain to be elucidated. Suggested novel mechanisms for DEP-induced inflammation include calcium signalling and increased matrix metalloproteinase-1 release, activation of TRPV1 receptors (transient receptor potential cation channel subfamily V member 1), and activation of the receptor for advanced glycation end products.[35–37] Interestingly, these mechanisms involve other groups of compounds than PAHs and PAH-derivatives, including carbonyls.

Relevance of the applied PM samples

The potential of PM to induce biological effects seems to strongly depend on its physical and chemical properties such as size, structure and surface area of the particles, and components adsorbed on the particle surface.[38] Notably, the physicochemical properties of combustion PM, including the content of organic compounds such as PAHs, may be rather diverse, depending on the properties of the fuel and emitting source, as well as post-formation processes.[6] The chemical analysis revealed that the DEP used in the present study contained about 60% EOC. This is a relatively high amount, but within the range of EOC levels reported by others.[27,39] Both samples used in the present study were collected from either an exhaust pipe or a chimney and may therefore be less representative for the DEPs and WSPs emitted to ambient air. In addition, ambient particles are also subjected to atmospheric aging, causing further changes in their organic chemistry. Thus, studies on combustion PM collected from ambient air are necessary in order to confirm and further explore the present findings, also including application of other models like primary human lung epithelial cells.

Conclusion

In this study we have characterized the SPE fractionated organic extracts of DEP and WSP with regard to their specific content of OC, PAHs and PAH-derivatives; and evaluated their cytotoxicity and cytokine responses in BEAS-2B cells. We report that the most polar fractions of the combustion PMs were most potent in inducing both cytotoxicity and cellular release of IL-6. However, the chemical compounds accounting for these toxicological effects could not be readily identified among the >120 quantified compounds. To totally exclude a possible role of PAHs and PAHs derivatives in inflammatory responses to combustion PM, more work is needed. Future work may include testing of pro-inflammatory responses of a wider range of individual PAHs and PAHs-derivatives alone or in combination as well as application of other model systems such as primary human lung epithelial cells. Finally, the present findings suggest a need for further characterisation of organic PM extracts, beyond extensive analyses of PAH and PAH-derivatives.

Acknowledgment

This work was funded by the Research Council of Norway through the "Environment, Genetics and Health" program (grant no. 185620/V40). Additional funding was provided by the National Science Foundation (NSF) Grant No.

ATM-0747349. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the NSF. At the Department of Air Pollution and Noise at the Norwegian Institute of Public Health (NIPH) H. J. Dahlman is thanked for assistance with ELISA-analysis. Furthermore, F.R. Cassee at the National Institute for Public Health and the Environment in the Netherlands, is thanked for providing us with the DEPs. We also appreciate feedback on the manuscript from Dr. Kozliak at the Chemistry Department, University of North Dakota, Grand Forks, USA.

References

[1] Lewtas, J. Air pollution combustion emissions: characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat. Res. **2007**, *636*, 95–133.

[2] Anderson, J.O.; Thundiyil, J.G.; Stolbach, A. Clearing the air: A review of the effects of particulate matter air pollution on human health. J. Med.Toxicol. **2012**, *8*, 166–175.

[3] Dergham, M.; Lepers, C.; Verdin, A.; Billet, S.; Cazier, F.; Courcot, D.; Shirali, P.; Garçon, G. Prooxidant and proinflammatory potency of air pollution particulate matter (PM2.5–0.3) produced in rural, urban, or industrial surroundings in human bronchial epithelial cells (BEAS-2B). Chem. Res. Toxicol. **2012**, *25*, 904–919.

[4] Matti Maricq, M. Chemical characterization of particulate emissions from diesel engines: A review. J. Aerosol Sci. **2007**, *38*, 1079–1118.

[5] Turpin, B.J.; Saxena, P.; Andrews, E. Measuring and simulating particulate organics in the atmosphere: Problems and prospects. Atmos. Environ. **2000**, *34*, 2983–3013.

[6] Bølling, A.K.; Pagels, J.; Yttri, K.E.; Barregard, L.; Sallsten, G.; Schwarze, P.E.; Boman, C. Health effects of residential wood smoke particles: The importance of combustion conditions and physicochemical particle properties. Part. Fibre Toxicol. **2009**, *6*, 29.

[7] Hesterberg, T.W.; Long, C.M.; Bunn, W.B.; Lapin, C.A.; McClellan, R.O.; Valberg, P.A. Health effects research and regulation of diesel exhaust: An historical overview focused on lung cancer risk. Inhal. Toxicol. **2012**, *24*, 1–45.

[8] Bonvallot, V.; Baeza-Squiban, A.; Baulig, A.; Brulant, S.; Boland, S.; Muzeau, F.; Barouki, R.; Marano, F. Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. Am. J. Respir. Cell Mol. Biol. **2001**, *25*, 515–521.

[9] Takano, H.; Yanagisawa, R.; Inoue, K.-I. Components of diesel exhaust particles diversely enhance a variety of respiratory diseases related to infection or allergy: Extracted organic chemicals and the residual particles after extraction differently affect respiratory diseases. J. Clin. Biochem. Nutr. **2007**, *40*, 101–107.

[10] Bølling, A.K.; Herseth, J.I.; Låg, M.; Refsnes, M.; Schwarze, P.E. Particles from wood smoke and traffic induce differential proinflammatory response patterns in co-cultures. Toxicol. Appl. Pharmacol. **2008**, *232*, 317–326.

[11] Totlandsdal, A.I.; Herseth, J.I.; Bølling, A.K.; Kubátová, A.; Braun, A.; Cochran, R.E.; Refsnes, M.; Øvrevik, J.; Låg, M. Differential effects of the particle core and organic extract of diesel exhaust particles. Toxicol. Lett. **2012**, *208*, 262–268.

[12] Zielinska, B. Atmospheric transformation of diesel emissions. Exp. Toxicol. Pathol. **2005**, *57*, 31–42.

[13] Zielinska, B.; Arey, J.; Atkinson, R.; Ramdahl, T.; Winer, A.M.; Pitts, J.N. Reaction of dinitrogen pentoxide with fluoranthene. J. Am. Chem. Soc. **1986**, *108*, 4126–4132.

[14] DeMarini, D.M.; Brooks, L.R.; Warren, S.H.; Kobayashi, T.; Gilmour, M.I.; Singh, P. Bioassay-directed fractionation and salmonella mutagenicity of automobile and forklift diesel exhaust particles. Environ. Health Perspec. **2004**, *112*, 814–819.

[15] Lewtas, J.; Claxton, L.; Mumford, J.; Lofroth, G. Bioassay of Complex mixtures of indoor air pollutants. IARC Sci. Pub. **1993**, 85–95.

[16] Shima, H.; Koike, E.; Shinohara, R.; Kobayashi, T. Oxidative ability and toxicity of n-hexane insoluble fraction of diesel exhaust particles. Toxicol. Sci. **2006**, *91*, 218–226.

[17] Kubátová, A.; Dronen, L.C.; Picklo, M.J.; Hawthorne, S.B. Midpolarity and nonpolar wood smoke particulate matter fractions deplete glutathione in RAW 264.7 macrophages. Chem. Res. Toxicol. **2006**, *19*, 255–261.

[18] James, K.; Farrell, R.E.; Siciliano, S.D. Comparison of Human exposure pathways in an urban brownfield: reduced risk from paving roads. Environ. Toxicol. Chem. **2012**, *31*, 2423–2430.

[19] Øvrevik, J.; Arlt, V.M.; Oya, E.; Nagy, E.; Mollerup, S.; Phillips, D.H.; Låg, M.; Holme, J.A. Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial BEAS-2B cells. Toxicol. Appl. Pharmacol. **2010**, *242*, 270–280.

[20] Podechard, N.; Lecureur, V.; Le Ferrec, E.; Guenon, I.; Sparfel, L.; Gilot, D.; Gordon, J.R.; Lagente, V.; Fardel, O. Interleukin-8 induction by the environmental contaminant benzo(a)pyrene is aryl hydrocarbon receptor-dependent and leads to lung inflammation. Toxicol. Lett. **2008**, *177*, 130–137.

[21] Vondracek, J.; Umannova, L.; Machala, M. Interactions of the Aryl hydrocarbon receptor with inflammatory mediators: Beyond CYP1A regulation. Curr. Drug Metab. **2011**, *12*, 89–103.

[22] Esser, C.; Rannug, A.; Stockinger, B. The Aryl hydrocarbon receptor in immunity. Trends Immunol. **2009**, *30*, 447–454.

[23] Cochran, R.E.; Dongari, N.; Jeong, H.; Beránek, J.; Haddadi, S.; Shipp, J.; Kubátová, A. Determination of polycyclic aromatic hydrocarbons and their oxy-, nitro-, and hydroxy-oxidation products. Anal. Chim. Acta **2012**, *740*, 93–103.

[24] Totlandsdal, A.I.; Cassee, F.R.; Schwarze, P.; Refsnes, M.; Låg, M. Diesel exhaust particles induce CYP1A1 and pro-inflammatory responses via differential pathways in human bronchial epithelial cells. Part. Fibre Toxicol. **2010**, *7*, Article 41.

[25] Hawthorne, S.B.; Krieger, M.S.; Miller, D.J.; Mathiason, M.B. Collection and quantitation of methoxylated phenol tracers for atmospheric pollution from residential wood stoves. Environ. Sci. Technol. **1989**, *23*, 470–475.

[26] Schauer, J.J.; Kleeman, M.J.; Cass, G.R.; Simoneit, B.R.T. Measurement of emissions from air pollution sources. 2. C1 through C30 organic compounds from medium duty diesel trucks. Environ. Sci. Technol. **1999**, *33*, 1578–1587.

[27] Kubátová, A.; Lahren, T.; Beranek, J.; Smoliakova, I.; Braun, A.; Huggins, F. Extractable organic carbon and its differentiation by polarity in diesel exhaust, wood smoke, and urban particulate matter. Aerosol Sci. Technol. **2009**, *43*, 714–729.

[28] Yang, H.-M.; Ma, J.Y.C.; Castranova, V.; Ma, J.K.H. Effects of diesel exhaust particles on the release of interleukin-1 and tumor necrosis factor-alpha from rat alveolar macrophages. Exp. Lung Res. **1997**, *23*, 269–284.

[29] Kawasaki, S.; Takizawa, H.; Takami, K.; Desaki, M.; Okazaki, H.; Kasama, T.; Kobayashi, K.; Yamamoto, K.; Nakahara, K.; Tanaka, M.; Sagai, M.; Ohtoshi, T. Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. **2001**, *24*, 419–426.

[30] Xia, T.; Korge, P.; Weiss, J.N.; Li, N.; Venkatesen, M.I.; Sioutas, C.; Nel, A. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity. Environ. Health Perspec. **2004**, *112*, 1347–1358.

[31] Holder, A.L.; Lucas, D.; Goth-Goldstein, R.; Koshland, C.P. Inflammatory response of lung cells exposed to whole, filtered, and hydrocarbon denuded diesel exhaust. Chemosphere **2007**, *70*, 13–19.

[32] Fuentes-Mattei, E.; Rivera, E.; Gioda, A.; Sanchez-Rivera, D.; Roman-Velazquez, F.R.; Jimenez-Velez, B.D. Use of human bronchial epithelial cells (beas-2b) to study immunological markers resulting from exposure to PM(2.5) organic extract from Puerto Rico. Toxicol. Appl. Pharmacol. **2010**, *243*, 381–389.

[33] Saxena, Q.B.; Saxena, R.K.; Siegel, P.D.; Lewis, D.M. Identification of organic fractions of diesel exhaust particulate (DEP) which inhibit nitric oxide (NO) production from a murine macrophage cell line. Toxicol. Lett. **2003**, *143*, 317–322.

[34] Yoshino, S.; Hayashi, H.; Taneda, S.; Takano, H.; Sagai, M.; Mori, Y. Effects of diesel exhaust particle extracts on Th1 and Th2 immune responses in mice. Int. J. Immunopath. Ph. **2002**, *15*, 13–18.

[35] Li, J.; Kanju, P.; Patterson, M.; Chew, W.-L.; Cho, S.-H.; Gilmour, I.; Oliver, T.; Yasuda, R.; Ghio, A.; Simon, S.A.; Liedtke, W. TRPV4-mediated calcium influx into human bronchial epithelia upon exposure to diesel exhaust particles. Environ. Health Perspect. **2011**, *119*, 784–793.

[36] Deering-Rice, C.E.; Romero, E.G.; Shapiro, D.; Hughen, R.; Light, A.R.; Yost, G.S.; Veranth, J.M.; Reilly, C.A. Electrophilic components of diesel exhaust particles (DEP) activate Transient Receptor Potential Ankyrin-1 (TRPA1): A probable mechanism ofacute pulmonary toxicity for DEP. Chem. Res. Toxicol. **2011**, *24*, 950–959.

[37] Reynolds, P.R.; Wasley, K.M.; Allison, C.H. Diesel particulate matter induces receptor for advanced glycation end-products (RAGE) expression in pulmonary epithelial cells, and RAGE signaling influences NF-κB-Mediated inflammation. Environ. Health Perspec. **2011**, *119*, 332–336.

[38] Schwarze, P.E.; Ovrevik, J.; Låg, M.; Refsnes, M.; Nafstad, P.; Hetland, R.B.; Dybing, E. Particulate matter properties and health effects:Consistency of epidemiological and toxicological studies. Hum. Exp. Toxicol. **2006**, *25*, 559–579.

[39] Stevens, T.; Cho, S.-H.; Linak, W.P.; Gilmour, M.I. Differential potentiation of allergic lung disease in mice exposed to chemically distinct diesel samples. Toxicol. Sci. **2009**, *107*, 522–534.