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

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27 **Abstract**

28 Exposure to combustion emissions, including diesel engine exhaust and wood smoke particles
29 (DEPs and WSPs), has been associated with inflammatory responses. To investigate the
30 possible role of polycyclic aromatic hydrocarbons (PAHs) and PAH-derivatives, the DEPs
31 and WSPs methanol extracts were fractionated by solid phase extraction (SPE), and the
32 fractions were analyzed for more than ~120 compounds. The pro-inflammatory effects of the
33 fractionated extracts were characterized by exposure of bronchial epithelial lung cells (BEAS-
34 2B). Both native DEPs and WSPs caused a concentration-dependent increase in IL-6 and IL-8
35 release and cytotoxicity. This is consistent with the finding of a rather similar total content of
36 PAHs and PAH-derivatives. Yet, the samples differed in specific components, suggesting that
37 different species contribute to the toxicological response in these two types of particles. The
38 majority of the IL-6 release and cytotoxicity was induced upon exposure to the most polar
39 (methanol) SPE fraction of extracts from both samples. In these fractions hydroxy-PAHs,
40 carboxy-PAHs were observed along with nitro-amino-PAHs in DEP. However, the biological
41 effects induced by the polar fractions could not be attributed only to the occurrence of PAH-
42 derivatives. The present findings indicate a need for further characterization of organic
43 extracts, beyond an extensive analysis of commonly suspected PAH and PAH-derivatives.

44

45 **Keywords:** PAHs, PAHs derivatives, combustion particles, cytokines, inflammation

46

47 **Introduction**

48 Exposure to particulate matter (PM) from combustion sources, including diesel engine
49 exhaust particles (DEPs) and wood smoke particles (WSPs), has been associated with several
50 adverse health outcomes, in which inflammation appears to play an important role.[1,2] To
51 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]

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,

77 containing compounds with many hydroxyl- and carbonyl groups, was most potent in
78 inducing inflammatory responses.[16] Furthermore, the mid-polar fraction with oxygenated
79 organics was most potent in depleting glutathione levels in macrophages when comparing
80 WSP-extracts of different polarity.[17]
81
82 With respect to combustion emissions, polycyclic aromatic hydrocarbons (PAHs) represent a
83 group of compounds that have gained particular attention due to their genotoxic and
84 carcinogenic properties.[1,18] However, exposure to high concentrations of various single
85 PAHs, as well as nitro- and amino-PAH derivatives, has been reported to induce additional
86 pro-inflammatory responses, both *in vivo* and *in vitro*.[19,20] Such effects have been linked to
87 both ligand binding and activation of the aryl hydrocarbon receptor, antagonist properties, as
88 well as to the formation of reactive electrophilic metabolites during the PAH metabolism.[21,22]
89 Although this indicates that PAHs may be important for inflammatory responses, their
90 contribution to the pro-inflammatory effects of DEPs and other combustion particles, to our
91 knowledge, has not yet been clarified.
92
93 The present study was initiated to further explore which constituents are responsible for the
94 bulk of pro-inflammatory effects of DEP and WSP, with a particular focus on a potential role
95 of PAHs and their derivatives. Methanol (MeOH)-extracts of DEP and WSP were fractionated
96 by solid phase extraction (SPE) with a sequential use of solvents of increasing polarity, with
97 the aim of separating PAHs and their polar derivatives.[23] The organic carbon (OC) content
98 was determined by thermal optical analysis (TOA) and the occurrence of PAHs and their
99 derivatives was determined by gas chromatography-mass spectrometry (GC-MS). The
100 potency of the characterized fractions to induce cytotoxicity and release of interleukin (IL)-6
101 and IL-8 was examined in a human bronchial epithelial cell line (BEAS-2B). To evaluate the

102 completeness of the applied extraction procedure, Soxhlet extracts were also investigated with
103 respect to chemistry and pro-inflammatory effects.
104
105 **Materials and methods**
106 ***Chemicals and reagents***
107 LHC-9 cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA) and
108 PureCol™ from Inamed Biomaterials (Fremont, CA, USA). Solvents including methanol
109 (MeOH, LC-MS Optima grade) and DCM (99.9%, LC-MS grade) were purchased from
110 Fisher Scientific (Waltham, MA, USA) while *n*-hexane (HPLC grade, 95%) was obtained
111 from Sigma-Aldrich (St. Louis, MO, USA). The analytical standards used for characterization
112 are reported elsewhere.[23] All other chemicals were purchased from commercial sources at
113 the highest purity available.
114
115 ***Collection and preparation of particle suspensions and extracts***
116 The DEP sample, which has previously been chemically characterized, was generated by an
117 unloaded diesel engine (Deutz, 4 cylinder, 2.2 L, 500 rpm) using gas oil (Petroplus Refining
118 Teesside Ltd., United Kingdom).[24] The DEP was collected from the inside of the main
119 exhaust pipe after the diesel engine had been running for 8 weeks, in conjunction with an on-
120 going clinical chamber study. The WSP sample was collected from the interior of a chimney
121 that vented an airtight wood stove burning a mix of hardwoods.[25] To investigate the cellular
122 responses to these samples, particles were suspended in a cell culture medium (2 mg/mL) and
123 stirred overnight before administration to cells.
124
125 Organic extracts of the particles were prepared as previously described.[11] In brief, native
126 particles (~30 mg) were suspended in MeOH (~60 mL), and subsequently sonicated in a water

127 bath (30 min), prior to overnight incubation at 4 °C. The suspensions were centrifuged (10
128 min, 8000 g) in order to separate the particles from the extract, and then the extracts were
129 evaporated to dryness under a gentle stream of nitrogen gas. Subsequently, the extracts were
130 fractionated based on polarity (with the aim of separating PAHs and their polar derivatives),
131 using the previously described SPE protocol. Briefly, an amino propyl SPE cartridge (Sep-
132 Pak, Waters, Milford, MA, USA) was preconditioned with 6 mL of DCM followed by 6 mL
133 of *n*-hexane. Three fractions were eluted sequentially with *n*-hexane (5 mL), 20% DCM in *n*-
134 hexane (5 mL), and MeOH (5 mL). Following the 1st sonication, two additional extractions
135 were performed on the residual particles to investigate if the 1st extraction was efficient, i.e., a
136 2nd sonication (each with 60 mL of MeOH) and then a Soxhlet extraction [11] (with 90 mL of
137 MeOH). The Soxhlet extracts of both samples contained 3–4 times more OC than the 2nd
138 sonication extracts (Table 1). The Soxhlet extracts were therefore chosen to be included in the
139 cellular experiments to further confirm that all the biologically active organics were removed
140 in the 1st extraction. To prepare the extracts for *in vitro* experiments, the fractionated SPE-
141 extracts and the Soxhlet extracts were dried under nitrogen gas and stored at -20 °C until
142 further processing. Later, these extracts were thawed, re-suspended in dimethyl sulfoxide
143 (DMSO), and aliquoted in glass tubes before storage at -20 °C until the day of exposure. On
144 the day of exposure, aliquots of each extract fraction were further suspended in a cell culture
145 medium at a concentration corresponding to 2 mg/mL of native particles.
146
147 ***Chemical characterisation of the particle extracts***
148 The OC and elemental carbon (EC) determinations of all extracts (after 1st and 2nd sonication,
149 SPE-fractions and Soxhlet) were conducted using a thermal optical OC/EC analyzer (Sunset
150 Laboratories, Tigard, OR, USA). The temperature program began with four steps under a
151 helium atmosphere, starting at 300 °C for 75 s, 500 °C for 75 s, 600 °C for 75 s, 700 °C for

152 75 s, 870 °C for 120 s; then the instrument was cooled down (35s) to 550 °C and helium with
153 5% oxygen was introduced. Five additional temperature stages were then used: 550 °C for 45
154 s, 625 °C for 45 s, 700 °C for 45 s, 775 °C for 45 s, 890 °C for 120 s. Quantification was
155 based on daily calibration curves using potassium hydrogen phthalate. OC/EC determinations
156 of all samples were performed in the transmittance mode. For this analysis, the extracts were
157 concentrated under nitrogen gas to 10 mL and were then used without further concentration.
158 The aliquots of extract/fractions (10 µL) were spiked onto a 1.5 cm^2 punch from a quartz filter
159 (pre-baked at 1500 °C overnight to remove any carbon residue). Solvents were evaporated
160 from the filter for 4 min on a hot plate at 40 °C, prior to inserting it into the thermal optical
161 analysis (TOA) oven. The data for these six extracts are presented as % OC content relative to
162 the particle mass they were extracted from (~30 mg).
163
164 A more detailed chemical characterization of the five extracts was performed by GC-MS
165 analysis, using a 6890 GC with an 5975C MS (Agilent Technologies, Inc., Wilmington, DE,
166 USA), as described previously.[11,23] A broad range of PAHs and PAH-derivatives were
167 analysed, of which the complete list of individual organic species is provided in Figures 2 and
168 3, and Tables 1S and 2S in Supplementary data. Species for which pure analytical standards
169 were not available were tentatively identified by matching MS spectra (at least 90% matching
170 quality) with those obtained from the NIST 2005 MS Spectral Database. In addition, the sum
171 of the individual organics analysed in each group of PAHs/PAH-derivatives is presented in
172 Table 3 as µg/g of native particles.
173
174 ***Culture of cells***
175 BEAS-2B, a SV40-transformed human bronchial epithelial cell line was purchased from the
176 European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were maintained in

177 LHC-9 medium in collagen (PureCol™)-coated flasks in a humidified atmosphere at 37 °C

178 with 5% CO_2, with a refreshment of medium on every second day. One day prior to exposure,

179 BEAS-2B cells (passages 26–38) were plated into collagen (PureCol™)-coated 35 mm 6-well

180 culture dishes (460,000 cells/well).

181

182 ***Exposure of cells***

183 Cells were exposed to either suspensions of native particles or to the various extracts in the

184 concentration-range corresponding to 0–400 µg/mL of native particles. The final

185 concentration of DMSO was equal in all cell culture wells (including the non-exposed control

186 cells), and did not exceed 0.4 %. The total exposure volume was 1.5 mL in 6-well 35 mm cell

187 culture dishes. From this, it may be calculated that an applied particle concentration of

188 100 µg/mL corresponds to a concentration of 16 µg/cm^2, if all the suspended particles deposit

189 on the cells present on the surface of the culture dishes.

190

191 Cells were also treated with a selection of the oxy- and hydroxy-PAHs detected in the

192 fractionated extracts, in order to investigate their contribution to the effects induced by the

193 fractions. The components tested were 9-fluorenone, xanthone, phenalenone, 9,10-

194 anthraquinone, benz[*a*]anthracene-7,12-dione and 2-hydroxy-9-fluorenone, both alone (at

195 concentrations ranging from 1 nM–30 µM) and in combination. When tested in combination,

196 the applied concentrations of each compound were based on the corresponding concentrations

197 detected in the high polar SPE fraction of the DEP-extract. The resulting mixture of PAH

198 derivatives was tested at concentrations corresponding to 1, 10 and 100 times the

199 concentrations in the high polar methanol fraction of the DEP-extract.

200

201 ***Examination of cytotoxicity***

202 After a 24 h exposure, cells were stained with propidium iodide (PI; 10 µg/mL) and Hoechst
203 33342 (5 µg/mL) and evaluated using a Nikon Eclipse E 400 (Nikon Gmbh., Düsseldorf,
204 Germany) fluorescence microscope, as previously described.[24] The percentage of apoptotic
205 and necrotic cells were determined as a fraction of the total number of counted cells.
206
207 ***Quantification of cytokine release***
208 After a 24 h exposure, cell culture supernatants were collected and centrifuged for the
209 removal of dead cells (300 x g) and, if necessary, for removal of particles (8000 x g) and
210 stored at -70 °C until cytokine analysis. Concentrations of IL-6 and IL-8 in cell culture
211 supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (R&D
212 Systems, Minneapolis MN, USA), according to the manufacturer's manual. The increase in
213 colour intensity was measured and quantified using a plate reader (TECAN Sunrise, Phoenix
214 Research Products, Hayward, CA, USA) with software (Magellan V 1.10). Cytokine
215 concentrations in cell culture supernatants are expressed in pg/mL.
216
217 ***Calculations and statistical analysis***
218 All chemical characterization data are based on triplicate extraction/fractionation experiments
219 analysis and are reported as average plus/minus one standard deviation. The results from cell
220 culture experiments represent the mean values of three independent experiments. Statistical
221 analysis of the data was performed by application of a two-way ANOVA with Bonferroni
222 post tests. The data in Figures 3 and 4 were log-transformed prior to the analysis to fulfill the
223 assumptions for ANOVA. Statistical analyses were performed using GraphPad Prism
224 software (version 4.03, Inc., San Diego, CA).
225

226 Linear regression analysis was performed to investigate the influence of the contents of
227 identified PAHs and PAH derivatives on cytokine release and toxicity. Analyses were
228 performed for the total PAH content, and the contents of PAHs and hydroxy-, oxy- and nitro-
229 PAHs.

230

231 For all the statistical analyses, p values < 0.05 were considered to reflect statistically
232 significant differences.

233

234 **Results**

235 ***Chemical composition of particle extracts***

236 The OC content in the extracts obtained from three methanol extractions (1st, 2nd and Soxhlet)
237 is presented in Table 1 as mass percent compared to the amount of native particles submitted
238 to extraction. The TOA revealed that DEP contained a considerably higher fraction of
239 methanol extractable OC of native particles (60 %) than WSP (23 %). A significant
240 proportion of the extractable material was extracted within the 1st sonication step (54% and
241 17% for DEP and WSP, respectively), accounting for 90 and 70 %, respectively, of the total
242 OC extractable in methanol. Although this observation suggested that the 1st extraction was
243 relatively successful, a considerable portion was also extracted by the following Soxhlet
244 extraction. Therefore, the Soxhlet extracts, in addition to the three SPE fractions of the extract
245 obtained after the 1st sonication step, were included in the biological experiments.

246

247 The three SPE-fractions of the 1st sonication extract differed for DEP and WSP with respect to
248 which fractions contained the highest amount of OC. For DEP, the highest OC level was
249 detected in the non polar n-hexane fraction whereas the polar methanol fraction contained the
250 highest OC level of the WSP extracts. It should be noted that a significant portion of the OC

251 spiked onto the SPE column was not eluted (ca. 50% for both DEP and WSP) and was thus
252 retained on the column, even upon addition of a more polar solvent, such as water. As shown
253 below, a significant toxicological response reported for unfractionated extracts in our previous
254 work[11] was also observed in this study in the obtained SPE fractions.
255
256 The GC-MS analysis targeted the quantification of PAHs and their derivatives (+120 species),
257 providing trends in the distribution of major classes of compounds. However, alkanes and
258 branched alkanes are typically the most abundant organic species in DEP.[26] These non-polar
259 compounds occur in GC chromatograms as an unresolved complex mixture (UCM).
260 Presently, as shown in Figure 1, the SPE fractionation enabled the separation of the majority
261 of non-polar species (i.e., alkanes and UCM) in the *n*-hexane fraction.
262
263 An overview of the total content of PAHs and PAH-derivatives in the SPE fractionated
264 extracts, the extracts obtained after the 2nd sonication step and the Soxhlet extracts is given in
265 Table 3 for both DEP and WSP (for a full dataset including quantification of individual
266 compounds see Table 1S and 2S). Although the OC levels were almost three times higher in
267 DEP than in WSP (Table 1), the total contents of PAHs and PAH-derivatives were relatively
268 similar, 2315 and 2625 µg/g respectively. However, the distribution of PAHs and PAH-
269 derivatives varied between the three DEP and WSP fractions. The mid-polar DCM/*n*-hexane
270 extract contained the highest total amount of identified PAHs and PAH-derivatives for both
271 samples, but the polar methanol extract from WSP also contained high levels of identified
272 species in contrast to DEP (Table 3, last row). The total levels of oxy-, hydroxy- and carboxy-
273 PAHs were significantly higher in WSP than in DEP, with approximate sums of 1400 and 700
274 µg/g, respectively (Table 3). Nevertheless, for both particle samples the total content of
275 identified PAHs and PAH-derivatives constituted only a minor fraction (< 1%) of the total

276 extractable organic carbon (EOC) content. As for the total OC content, the SPE fractions
277 (from the 1st sonication) contained the majority of the total PAHs and PAH-derivatives for
278 each sample, compared to the extracts obtained within the 2nd sonication step and the Soxhlet
279 extract, which contained approximately 10 % of the identified species (Table 3). However, the
280 extract from the 2nd sonication step contained considerably higher amounts of PAHs and
281 PAH-derivatives than the Soxhlet extract.
282
283 The purpose of SPE fractionation was to separate the majority of PAHs and their derivatives
284 into several fractions (Fig. 2 and 3) allowing for a toxicological assessment of the fractions
285 obtained. Figures 2 and 3 show the distribution of identified PAHs and PAH-derivatives in
286 the SPE fractionated extracts as well as the Soxhlet extracts of the residual particles. The
287 unsubstituted PAHs were detected mainly in the mid-polar fraction (20 % DCM in *n*-hexane)
288 of both DEP and WSP. The majority of high molecular weight PAHs, oxy-PAHs, and nitro-
289 PAH were observed mainly in the mid-polar 20 % DCM in *n*-hexane of both DEP and WSP
290 (Figs. 2 and 3). The only exception was phenalone, which was abundant also in the MeOH
291 fraction. For WSP, the hydroxy-PAHs and carboxy-PAHs were detected mainly in the polar
292 methanol fraction (Fig. 3), whereas for DEP different hydroxy-PAHs were detected in two
293 higher polarity SPE and Soxhlet fractions in comparable amounts. In WSP, the most abundant
294 species were 2-hydroxy-9-fluorenone, 7-hydroxycadelene, 2-hydroxybiphenyl and isomers of
295 hydroxypyrene. In DEP, 2-hydroxy-9-fluorenone was also abundant; but, in addition,
296 hydroxy-*n*-benzopyran-*n*-one (tentatively identified by MS library match) was observed.
297 However, it is important to note that concentrations in DEP were approximately 5 times lower
298 than those in WSP. Besides the hydroxy-PAHs, 3-ring carboxy-PAHs (tentatively identified)
299 were also found in the methanol SPE DEP-fraction and Soxhlet extract of the sonication
300 residue. Moreover, nitroamine-PAH species (which to our knowledge have not been

301 previously observed) eluted in the methanol SPE DEP-fraction (Table 1S). These species
302 included dihydro-nitro-phenanthrenamine isomers, nitro-fluorenamine, as well as methyl-
303 nitrocarbazole isomers (all tentatively identified with 80–85 % match to the MS database).
304
305 The DEP sample, but not the WSP sample, also contained nitro-PAHs, which were detected
306 mainly in the mid-polar 20% DCM in *n*-hexane fraction, where 1-nitropyrene was the
307 dominating species. In the WSP methanol SPE-fraction, organic species other than PAH and
308 PAH-derivatives were also observed following the derivatization. These species were
309 previously identified WSP components including levoglucosan, mono- and dicarboxylic
310 acids, and syringol and guaiacol derivatives.[27] As expected, none of these compounds were
311 observed in the DEP extracts.
312
313 The results from the GC-MS analysis show that the SPE fractionation successfully separated
314 the non-polar alkanes and branched alkanes into the appropriate polarity extracts. For the
315 PAH and PAH derivatives, there was a higher extent of overlap between the various fractions;
316 however, the majority of PAHs and oxy-, nitro-PAHs were recovered in the 20% DCM in *n*-
317 hexane whereas hydroxy- and carboxy- and nitroamine-PAHs were recovered in the methanol
318 SPE fractions.
319
320 ***Cytotoxicity induced by native particles and SPE fractionated particle extracts***
321 Both DEP- and WSP-samples reduced the number of viable cells. As judged by visual
322 examination of cell number under light microscopy, combined with fluorescence microscopy
323 of cells stained with PI/ Hoechst, the cells died by necrosis. Both PM samples induced PI-
324 positive cells, with DEP clearly more cytotoxic than the WSP (Figure 4). At a concentration

325 of 400 µg/mL, DEP induced approximately 90 % necrotic cells (PI-positive) versus 10 % in
326 the cell cultures exposed to WSP.
327
328 Cells were exposed to three consecutive SPE fractions or the Soxhlet extract in the amounts
329 corresponding to the concentrations of native particles they were extracted from, with 400
330 µg/mL of native particles as the maximum. Only the polar methanol fraction of both DEP and
331 WSP induced a significant increase in necrotic cell death (Figure 5). In accordance with the
332 effects induced by the native particles, the polar methanol fraction of the DEP appeared to be
333 more potent than the corresponding WSP fraction, with maximum responses of similar
334 magnitudes as the respective native particles (Figure 5). The Soxhlet extracts did not
335 significantly increase the cytotoxicity.
336
337 ***Cellular release of IL-6 and IL-8 induced by native particles and SPE fractionated particle***
338 ***extracts***
339 The 24 h exposure to DEP induced concentration-dependent increases in both IL-6 and IL-8
340 release, which reached statistical significance at 50 µg/mL, before the levels declined at
341 concentrations above 100 µg/mL (Figure 6). WSP did not induce any statistical significant
342 increase in the release of these mediators, although the levels tended to increase with
343 increasing particle concentrations, with maximum levels at 400 µg/mL (Figure 6).
344
345 The IL-6 release induced by the three SPE extracts showed a similar trend as the cytotoxicity;
346 namely, only the polar methanol-fraction from both particle types increased the IL-6 release
347 (Figure 7A). Concentrations corresponding to 100 µg/mL of native DEP and 200 µg/mL of
348 native WSP increased the IL-6 release significantly, in contrast to the responses induced by
349 the native particles, of which only DEP induced a significant increase. The IL-8 release did

350 however show a different response pattern, since the high-polar methanol-fractions from both
351 DEP and WSP as well as the DEP Soxhlet extract reduced the release of IL-8 significantly
352 (Figure 7B).
353
354 Thus, the polar methanol SPE fraction exhibited the most severe effect on both the IL-6 and
355 IL-8 release, but caused increases in IL-6 release and decreases in IL-8 release. The reduced
356 IL-8 release induced by the DEP Soxhlet extract suggests that some biologically active
357 organic compounds remained in the residual particles after the 1st and 2nd extraction
358 procedures.
359
360 Cells were also treated with a selection of commercially available oxy- and hydroxy-PAHs
361 detected in the fractionated extracts, in order to investigate their contribution to the observed
362 responses. This included 2-hydroxy-9-fluorenone, which was detected in higher
363 concentrations in the polar methanol SPE fraction, as compared to the other fractions.
364 Concentrations from 1 nM-30 µM of 9-fluorenone, xanthone, phenalenone, 9,10-
365 anthraquinone, benz[*a*]anthracene-7,12-dione and 2-hydroxy-9-fluorenone did not cause
366 significant cellular effects; neither did the mixture of these compounds corresponding to 1, 10
367 and 100 times the concentrations of the compounds detected in the polar fraction of the DEP
368 methanol extract (Figures 1S and 2S, Supplementary Data). Notably, the occurrence and
369 abundance of these and other derivatives differed in extracts of the two studied PM samples,
370 making it difficult to estimate the contribution of individual species.
371
372 ***Influence of the content of identified PAHs and PAH derivatives on the biological***
373 ***responses***

374 To what extent the presence of the identified PAHs and PAH derivatives could explain the
375 differential cellular responses to the different various extracts, was also investigated by linear
376 regression analysis. With two exceptions, the overall analysis indicated that the content of the
377 identified PAHs and PAH derivatives had either limited influence or no influence on the
378 biological effects induced by the particles. However, the presence of hydroxy-PAHs was
379 positively correlated with IL-6 (r^2=0.32) and negatively correlated with IL-8 (r^2=0.40).
380 Further, the content of PAHs (not total PAHs) was positively correlated with IL-8 (r^2= 0.36).
381 However, the r^2 values of about 0.4 indicated that only 40 % of the variability could be
382 explained by the content of hydroxy-PAHs and PAHs. Moreover, the slopes of the
383 corresponding regression lines were relatively low and therefore had limited biological
384 relevance. For instance, IL-6 versus hydroxy-PAHs resulted in a positive slope of 22 pg/mL
385 per 100 µg/g PM. As a reference, the levels of hydroxy-PAHs varied from 0 to 290 µg/g in
386 the different DEP and WSP extracts, and the general IL-6 levels varied between 20–170
387 pg/mL.
388

389 **Discussion**

390 We and others previously reported that the organic fraction of combustion PM accounts for
391 the majority of their biological reactivity, ranging from genotoxic and mutagenic effects to
392 induction of pro-inflammatory mediators.[8,10,11,28–32] In the present study we have specifically
393 characterized SPE fractionated organic extracts of DEP and WSP with regard to their specific
394 content of OC, PAHs and PAH-derivatives and evaluated their cytotoxicity and cytokine
395 responses in BEAS-2B cells. In particular, the aim was to explore any possible role of PAHs
396 and their oxidation products in the pro-inflammatory effects of DEP and WSP. For both of the
397 two distinct PM samples it was demonstrated that the most polar (methanol) SPE fraction was
398 the most potent in inducing cytotoxicity and cellular release of IL-6. However, the chemical

399 compounds accounting for these toxicological effects could not easily be identified among the
400 >120 PAHs and PAH-derivatives quantified. The present findings indicate a need for further
401 characterisation of PM organic extracts, also beyond an extensive analysis of PAH and PAH-
402 derivatives, in order to enhance our understanding of the driving effects behind the toxicity of
403 PM.
404
405 ***Role of alkanes, PAHs and their mildly polar derivatives***
406 The majority of abundant alkanes and components of the unresolved complex mixture were
407 successfully separated from PAHs and other species in the non-polar *n*-hexane SPE fraction
408 of DEP (Fig. 1). This enabled us to confirm that these species do not have significant effects
409 on cytoxicity and cytokine release induced by the investigated DEP and WSP samples.
410 With respect to combustion emissions, PAHs represent a group of compounds that gained a
411 particular attention due to their carcinogenic properties.[1] In this work, PAHs, nitro-PAHs
412 and also oxy-PAHs were recovered primarily in the mid-polar SPE fractions, with individual
413 concentrations in the low nM-range. These concentrations are about 1000-fold less than those
414 previously used to demonstrate cytokine responses to single PAHs.[19,20] Moreover, these mid-
415 polar (20% DCM in *n*-hexane) SPE fractions did not induce significant cellular effects
416 (Figures 5 and 7), and accordingly the content of these groups of compounds did not correlate
417 with the toxicological effects in the linear regression analysis. This is in line with previous
418 findings for one of the most carcinogenic PAHs, B[*a*]P, which did not induce cytokine
419 responses in the BEAS-2B cells.[19] Thus, the present findings seem to support the previous
420 suggestions that unsubstituted PAHs and other organic compounds with high affinity for the
421 aryl hydrocarbon receptor are not the primary drivers of the pro-inflammatory effects induced
422 by DEP.[19]
423

424 We previously reported that certain nitro-PAHs may induce strong increases in cytokine
425 responses in BEAS-2B cells, but that these effects required high concentrations above 1
426 μM.[19] Previous studies also suggested that quinones and resins may be involved in the
427 cytotoxic effects,[30] while DEP-induced suppression of NO-production in macrophages has
428 been attributed to resins only.[33] In this study, the content of oxy-PAHs (including quinonic
429 species) did not correlate with the toxicological responses. In addition, exposure to a selection
430 of the identified oxy-PAHs (9-fluorenone, xanthone, phenalenone, 9,10-anthraquinone and
431 benz[*a*]anthracene-7,12-dione) did not induce any significant IL-6 and IL-8 responses or
432 cytotoxicity in the BEAS-2B cells. In accordance with our data, Shima and colleagues were
433 also unable to emulate the effects of the *n*-hexane insoluble fraction of DEP DCM-extracts
434 when using selected quinones (1,2-/1,4-naphtoquinone, 9,10-anthraquinone and 9,10-
435 phenanthraquinone).[16] Thus, similarly as for PAHs and nitro-PAHs, the pro-inflammatory
436 and cytotoxic effects of DEP and WSP could not easily be explained by the presence of
437 quinonic species here identified (i.e., oxy-PAHs).
438

439 ***Contribution of polar fractions, toxicity vs. chemical characterisation***

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

449 regulate Th1 and Th2 responses.[34] Thus the contribution of different organic compounds to
450 the DEP-effects is complex and different organic compounds might affect different biological
451 endpoints.

452 Shima and colleagues previously reported that the polar *n*-hexane insoluble fraction of DEP
453 DCM-extracts displayed the highest oxidative capacity and induced the strongest
454 inflammatory response in mice.[16] The authors noted that this polar fraction was mainly
455 composed of PAHs with functional groups related to oxygenation, such as hydroxyl- and
456 carbonyl groups [16]. In the present study, the chemical analysis revealed that the polar
457 methanol fraction contained higher amounts of hydroxy-PAHs. Moreover, the hydroxy-PAH
458 abundances slightly correlated to the toxicological effects in the linear regression analysis,
459 partly supporting the suggestion that these PAH oxidation products might be involved in the
460 observed pro-inflammatory and cytotoxic effects. In contrast, the oxy-PAHs (e.g., carbonyls)
461 did not appear to influence the toxicological endpoints measured. We did not detect any
462 significant cytotoxicity and/ or pro-inflammatory effects with the most abundant hydroxy-
463 PAH detected in DEP and WSP polar SPE fractions (Supplementary Information, Figure 1S).
464 The occurrence and abundance of other hydroxy species differed drastically in the two PM
465 samples, making it difficult to estimate the contribution of individual species.

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

474 to our knowledge, the pro-inflammatory responses to such PAH-species have yet to be

475 determined.

476

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

478 Whereas both native WSP and DEP increased the release of IL-6 and IL-8 (Figure 6), a

479 contrasting trend was observed for the SPE fractions for these two endpoints. While the polar

480 methanol fractions increased the IL-6 release, the IL-8 release was reduced (Figure 7).

481 Similarly, polar organic extracts from PM2.5 have been found to increase IL-6 and reduce IL-

482 8 release from BEAS-2B cells.[32] Additionally, a reduced IL-8 response was observed for the

483 Soxhlet DEP extract (obtained following the sonication) (Fig. 7). Interestingly, both the

484 methanol SPE fraction and Soxhlet extracts of DEP contained carboxy-PAHs that could

485 contribute to a reduced IL-8 response, although no association could be detected in the

486 regression analysis.

487

488 A possible explanation of the discrepancy between the IL-8 responses to the native particles

489 and the fractionated extracts is that the organics inhibiting IL-8 release could be less available

490 when bound to the particles, while others may be more efficiently delivered. Alternatively, it

491 may be related to some specific PM-properties of functional groups necessary for receptor-

492 interactions.

493

494 ***Contribution of other organic species***

495 In addition to the suggested influence of hydroxy-PAHs on the IL-6 release and cytotoxicity,

496 other organic compounds are also likely to have contributed to the observed toxicological

497 effects. Besides the need for identification of the groups of organic species responsible for the

498 toxicological effects, the exact cellular mechanisms involved also remain to be elucidated.

499 Suggested novel mechanisms for DEP-induced inflammation include calcium signalling and
500 increased matrix metalloproteinase-1 release, activation of TRPV1 receptors (transient
501 receptor potential cation channel subfamily V member 1), and activation of the receptor for
502 advanced glycation end products.[35–37] Interestingly, these mechanisms involve other groups
503 of compounds than PAHs and PAH-derivatives, including carbonyls.
504

505 ***Relevance of the applied PM samples***

506 The potential of PM to induce biological effects seems to strongly depend on its physical and
507 chemical properties such as size, structure and surface area of the particles, and components
508 adsorbed on the particle surface.[38] Notably, the physicochemical properties of combustion
509 PM, including the content of organic compounds such as PAHs, may be rather diverse,
510 depending on the properties of the fuel and emitting source, as well as post-formation
511 processes.[6] The chemical analysis revealed that the DEP used in the present study contained
512 about 60% EOC. This is a relatively high amount, but within the range of EOC levels reported
513 by others.[27,39] Both samples used in the present study were collected from either an exhaust
514 pipe or a chimney and may therefore be less representative for the DEPs and WSPs emitted to
515 ambient air. In addition, ambient particles are also subjected to atmospheric aging, causing
516 further changes in their organic chemistry. Thus, studies on combustion PM collected from
517 ambient air are necessary in order to confirm and further explore the present findings, also
518 including application of other models like primary human lung epithelial cells.
519

520 **Conclusion**

521 In this study we have characterized the SPE fractionated organic extracts of DEP and WSP
522 with regard to their specific content of OC, PAHs and PAH-derivatives; and evaluated their
523 cytotoxicity and cytokine responses in BEAS-2B cells. We report that the most polar fractions

524 of the combustion PMs were most potent in inducing both cytotoxicity and cellular release of
525 IL-6. However, the chemical compounds accounting for these toxicological effects could not
526 be readily identified among the > 120 quantified compounds. To totally exclude a possible
527 role of PAHs and PAHs derivatives in inflammatory responses to combustion PM, more work
528 is needed. Future work may include testing of pro-inflammatory responses of a wider range of
529 individual PAHs and PAHs-derivatives alone or in combination as well as application of other
530 model systems such as primary human lung epithelial cells. Finally, the present findings
531 suggest a need for further characterisation of organic PM extracts, beyond extensive analyses
532 of PAH and PAH-derivatives.
533

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540

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546

547 **Supporting Information**

548 Detailed overview of the content of PAHs and PAH- derivatives in SPE-fractionated DEP and
549 WSP methanol-extracts and in soxhlet extracts of residual DEP and WSP, and the effects of a
550 selection of the detected PAH-derivatives, both in single and in combination, on cellular
551 release of IL-6 and IL-8.

552 **References**

553 [1] Lewtas, J. Air Pollution Combustion Emissions: Characterization of Causative Agents
554 and Mechanisms Associated With Cancer, Reproductive, and Cardiovascular Effects.
555 Mutat. Res. **2007**, *636*, 95–133.

556 [2] Anderson, J. O.; Thundiyil, J. G.; Stolbach, A. Clearing the Air: A Review of the
557 Effects of Particulate Matter Air Pollution on Human Health. J. Med.Toxicol. **2012**, *8*,
558 166–175.

559 [3] Dergham, M.; Lepers, C.; Verdin, A.; Billet, S.; Cazier, F.; Courcot, D.; Shirali, P.;
560 Garçon, G. Prooxidant and Proinflammatory Potency of Air Pollution Particulate
561 Matter (PM2.5-0.3) Produced in Rural, Urban, or Industrial Surroundings in Human
562 Bronchial Epithelial Cells (BEAS-2B). Chem. Res. Toxicol. **2012**, *25*, 904–919.

563 [4] Matti Maricq, M. Chemical Characterization of Particulate Emissions from Diesel
564 Engines: A Review. J. Aerosol Sci. **2007**, *38*, 1079–1118.

565 [5] Turpin, B. J.; Saxena, P.; Andrews, E. Measuring and Simulating Particulate Organics
566 in the Atmosphere: Problems and Prospects. Atmos. Environ. **2000**, *34*, 2983–3013.

567 [6] Bølling, A. K.; Pagels, J.; Yttri, K. E.; Barregard, L.; Sallsten, G.; Schwarze, P. E.;
568 Boman, C. Health Effects of Residential Wood Smoke Particles: The Importance of
569 Combustion Conditions and Physicochemical Particle Properties. Part. Fibre Toxicol.
570 **2009**, *6*, Article 29.

571 [7] Hesterberg, T. W.; Long, C. M.; Bunn, W. B.; Lapin, C. A.; McClellan, R. O.; Valberg,
572 P. A. Health Effects Research and Regulation of Diesel Exhaust: An Historical
573 Overview Focused on Lung Cancer Risk. Inhal. Toxicol. **2012**, *24*, 1–45.

574 [8] Bonvallot, V.; Baeza-Squiban, A.; Baulig, A.; Brulant, S.; Boland, S.; Muzeau, F.;
575 Barouki, R.; Marano, F. Organic Compounds From Diesel Exhaust Particles Elicit a
576 Proinflammatory Response in Human Airway Epithelial Cells and Induce Cytochrome
577 p450 1A1 Expression. Am. J. Respir. Cell Mol. Biol. **2001**, *25*, 515–521.

578 [9] Takano, H.; Yanagisawa, R.; Inoue, K.-I. Components of Diesel Exhaust Particles
579 Diversely Enhance a Variety of Respiratory Diseases Related to Infection or Allergy:
580 Extracted Organic Chemicals and the Residual Particles After Extraction Differently
581 Affect Respiratory Diseases. J. Clin. Biochem. Nutr. **2007**, *40*, 101–107.

582 [10] Bølling, A. K.; Herseth, J. I.; Låg, M.; Refsnes, M.; Schwarze, P. E. Particles From
583 Wood Smoke and Traffic Induce Differential Pro-Inflammatory Response Patterns in
584 Co-Cultures. Toxicol. Appl. Pharmacol. **2008**, *232*, 317–326.

585 [11] Totlandsdal, A. I.; Herseth, J. I.; Bølling, A. K.; Kubátová, A.; Braun, A.; Cochran, R.
586 E.; Refsnes, M.; Øvrevik, J.; Låg, M. Differential Effects of the Particle Core and
587 Organic Extract of Diesel Exhaust Particles. Toxicol. Lett. **2012**, *208*, 262–268.

588 [12] Zielinska, B. Atmospheric Transformation of Diesel Emissions. Exp. Toxicol. Pathol.
589 **2005**, *57*, 31–42.

590 [13] Zielinska, B.; Arey, J.; Atkinson, R.; Ramdahl, T.; Winer, A. M.; Pitts, J. N. Reaction
591 of Dinitrogen Pentoxide with Fluoranthene. J. Am. Chem. Soc. **1986**, *108*, 4126–4132.

592 [14] DeMarini, D. M.; Brooks, L. R.; Warren, S. H.; Kobayashi, T.; Gilmour, M. I.; Singh,
593 P. Bioassay-Directed Fractionation and Salmonella Mutagenicity of Automobile and
594 Forklift Diesel Exhaust Particles. Environ. Health Perspec. **2004**, *112*, 814–819.

595 [15] Lewtas, J.; Claxton, L.; Mumford, J.; Lofroth, G. Bioassay of Complex Mixtures of
596 Indoor Air Pollutants. IARC Sci. Pub. **1993**, 85–95.

597 [16] Shima, H.; Koike, E.; Shinohara, R.; Kobayashi, T. Oxidative Ability and Toxicity of
598 n-Hexane Insoluble Fraction of Diesel Exhaust Particles. Toxicol. Sci. **2006**, *91*, 218–
599 226.

600 [17] Kubátová, A.; Dronen, L. C.; Picklo, M. J.; Hawthorne, S. B. Midpolarity and
601 Nonpolar Wood Smoke Particulate Matter Fractions Deplete Glutathione in RAW
602 264.7 Macrophages. Chem. Res. Toxicol. **2006**, *19*, 255–261.

603 [18] James, K.; Farrell, R. E.; Siciliano, S. D. Comparison of Human Exposure Pathways in
604 an Urban Brownfield: Reduced Risk From Paving Roads. Environ. Toxicol. Chem.
605 **2012**, *31*, 2423–2430.

606 [19] Øvrevik, J.; Arlt, V. M.; Oya, E.; Nagy, E.; Mollerup, S.; Phillips, D. H.; Låg, M.;
607 Holme, J. A. Differential Effects of Nitro-PAHs and Amino-PAHs on Cytokine and
608 Chemokine Responses in Human Bronchial Epithelial BEAS-2B Cells. Toxicol. Appl.
609 Pharmacol. **2010**, *242*, 270–280.

610 [20] Podechard, N.; Lecureur, V.; Le Ferrec, E.; Guenon, I.; Sparfel, L.; Gilot, D.; Gordon,
611 J. R.; Lagente, V.; Fardel, O. Interleukin-8 Induction by the Environmental
612 Contaminant Benzo(a)pyrene is Aryl Hydrocarbon Receptor-Dependent and Leads to
613 Lung Inflammation. Toxicol. Lett. **2008**, *177*, 130–137.

614 [21] Vondracek, J.; Umannova, L.; Machala, M. Interactions of the Aryl Hydrocarbon
615 Receptor With Inflammatory Mediators: Beyond CYP1A Regulation. Curr. Drug
616 Metab. **2011**, *12*, 89–103.

617 [22] Esser, C.; Rannug, A.; Stockinger, B. The Aryl Hydrocarbon Receptor in Immunity.
618 Trends Immunol. **2009**, *30*, 447–454.

619 [23] Cochran, R. E.; Dongari, N.; Jeong, H.; Beránek, J.; Haddadi, S.; Shipp, J.; Kubátová,
620 A. Determination of Polycyclic Aromatic Hydrocarbons and Their Oxy-, Nitro-, and
621 Hydroxy-Oxidation Products. Anal. Chim. Acta **2012**, *740*, 93–103.

622 [24] Totlandsdal, A. I.; Cassee, F. R.; Schwarze, P.; Refsnes, M.; Låg, M. Diesel Exhaust
623 Particles Induce CYP1A1 and Pro-Inflammatory Responses via Differential Pathways
624 in Human Bronchial Epithelial Cells. Part. Fibre Toxicol. **2010**, *7*, Article 41.

625 [25] Hawthorne, S. B.; Krieger, M. S.; Miller, D. J.; Mathiason, M. B. Collection and
626 Quantitation of Methoxylated Phenol Tracers for Atmospheric Pollution from
627 Residential Wood Stoves. Environ. Sci. Technol. **1989**, *23*, 470–475.

628 [26] Schauer, J. J.; Kleeman, M. J.; Cass, G. R.; Simoneit, B. R. T. Measurement of
629 Emissions from Air Pollution Sources. 2 . C1 Through C30 Organic Compounds from
630 Medium Duty Diesel Trucks. Environ. Sci. Technol. **1999**, *33*, 1578–1587.

631 [27] Kubátová, A.; Lahren, T.; Beranek, J.; Smoliakova, I.; Braun, A.; Huggins, F.
632 Extractable Organic Carbon and its Differentiation by Polarity in Diesel Exhaust,
633 Wood Smoke, and Urban Particulate Matter. Aerosol Sci. Technol. **2009**, *43*, 714–729.

634 [28] Yang, H.-M.; Ma, J. Y. C.; Castranova, V.; Ma, J. K. H. Effects of Diesel Exhaust
635 Particles on the Release of Interleukin-1 and Tumor Necrosis Factor-Alpha from Rat
636 Alveolar Macrophages. Exp. Lung Res. **1997**, *23*, 269–284.

637 [29] Kawasaki, S.; Takizawa, H.; Takami, K.; Desaki, M.; Okazaki, H.; Kasama, T.;
638 Kobayashi, K.; Yamamoto, K.; Nakahara, K.; Tanaka, M.; Sagai, M.; Ohtoshi, T.
639 Benzene-Extracted Components Are Important for the Major Activity of Diesel
640 Exhaust Particles: Effect on Interleukin-8 gene Expression in Human Bronchial
641 Epithelial Cells. Am. J. Respir. Cell Mol. Biol. **2001**, *24*, 419–426.

642 [30] Xia, T.; Korge, P.; Weiss, J. N.; Li, N.; Venkatesen, M. I.; Sioutas, C.; Nel, A.
643 Quinones and Aromatic Chemical Compounds in Particulate Matter Induce
644 Mitochondrial Dysfunction: Implications for Ultrafine Particle Toxicity. Environ.
645 Health Perspec. **2004**, *112*, 1347–1358.

646 [31] Holder, A. L.; Lucas, D.; Goth-Goldstein, R.; Koshland, C. P. Inflammatory Response
647 of Lung Cells Exposed to Whole, Filtered, and Hydrocarbon Denuded Diesel Exhaust.
648 Chemosphere **2007**, *70*, 13–19.

649 [32] Fuentes-Mattei, E.; Rivera, E.; Gioda, A.; Sanchez-Rivera, D.; Roman-Velazquez, F.
650 R.; Jimenez-Velez, B. D. Use of Human Bronchial Epithelial Cells (BEAS-2B) to
651 Study Immunological Markers Resulting from Exposure to PM(2.5) Organic Extract
652 from Puerto Rico. Toxicol. Appl. Pharmacol. **2010**, *243*, 381–389.

653 [33] Saxena, Q. B.; Saxena, R. K.; Siegel, P. D.; Lewis, D. M. Identification of Organic
654 Fractions of Diesel Exhaust Particulate (DEP) Which Inhibit Nitric Oxide (NO)
655 Production from a Murine Macrophage Cell Line. Toxicol. Lett. **2003**, *143*, 317–322.

656 [34] Yoshino, S.; Hayashi, H.; Taneda, S.; Takano, H.; Sagai, M.; Mori, Y. Effects of Diesel
657 Exhaust Particle Extracts on Th1 and Th2 Immune Responses in Mice. Int. J.
658 Immunopath. Ph. **2002**, *15*, 13–18.

659 [35] Li, J.; Kanju, P.; Patterson, M.; Chew, W.-L.; Cho, S.-H.; Gilmour, I.; Oliver, T.;
660 Yasuda, R.; Ghio, A.; Simon, S. A.; Liedtke, W. TRPV4-Mediated Calcium Influx into
661 Human Bronchial Epithelia upon Exposure to Diesel Exhaust Particles. Environ.
662 Health Perspec. **2011**, *119*, 784–793.

663 [36] Deering-Rice, C. E.; Romero, E. G.; Shapiro, D.; Hughen, R. .; Light, A. R.; Yost, G.
664 S.; Veranth, J. M.; Reilly, C. a Electrophilic Components of Diesel Exhaust Particles
665 (DEP) Activate Transient Receptor Potential Ankyrin-1 (TRPA1): a Probable
666 Mechanism ofAcute Pulmonary Toxicity for DEP. Chem. Res. Toxicol. **2011**, *24*, 950–
667 959.

668 [37] Reynolds, P. R.; Wasley, K. M.; Allison, C. H. Diesel Particulate Matter Induces
669 Receptor for Advanced Glycation End-Products (RAGE) Expression in Pulmonary
670 Epithelial Cells, and RAGE Signaling Influences NF-κB-Mediated Inflammation.
671 Environ. Health Perspec. **2011**, *119*, 332–336.

672 [38] Schwarze, P. E.; Ovrevik, J.; Låg, M.; Refsnes, M.; Nafstad, P.; Hetland, R. B.;
673 Dybing, E. Particulate Matter Properties and Health Effects: Consistency of
674 Epidemiological and Toxicological Studies. Hum. Exp. Toxicol. **2006**, *25*, 559–579.

675 [39] Stevens, T.; Cho, S.-H.; Linak, W. P.; Gilmour, M. I. Differential Potentiation of
676 Allergic Lung Disease in Mice Exposed to Chemically Distinct Diesel Samples.
677 Toxicol. Sci. **2009**, *107*, 522–534.

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685 **Table 1**. Percentages of OC in DEP and WSP methanol-extracts after the 1st and 2nd
686 sonication steps and of soxhlet extraction of residual DEP and WSP. The results are presented
687 as % OC of total mass of native particles submitted to extraction. The results are reported as
688 averages of triplicate experiments with one standard deviation. The OC content was
689 determined using TOA.

Extraction	DEP			WSP		
After 1st sonication	54	±	3	16.8	±	0.5
After 2nd sonication	1.2	±	0.3	2	±	1
soxhlet	4.7	±	0.7	7	±	1
Total	**60**	±	**3**	**23**	±	**2**

690
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692
693 **Table 2**. Percentages of OC in the SPE-fractions (*n*-hexane, 20 % DCM in *n*-hexane and
694 methanol) of DEP and WSP methanol-extracts after 1st sonication step. The results are
695 presented as % OC of total mass of native particles submitted to extraction, and are reported
696 as averages of triplicate experiments with one standard deviation. The OC content was
697 determined using TOA.

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**

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699

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. 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.

	DEP						WSP					
	1st sonication extract			2nd sonication extract	Soxhlet extract	Total from all extracts	1st sonication extract			2nd sonication extract	Soxhlet extract	Total from all extracts
	SPE fractions						SPE fractions					
Class	*n*-hexane	20% DCM in *n*-hexane	MeOH				*n*-hexane	20% DCM in *n*-hexane	MeOH			
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 & 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

705 **Figure Legends**

706

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

711

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

719

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

728

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

735

736 **Figure 5.** Cytotoxicity induced by fractionated extracts of diesel exhaust and wood smoke
737 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.

Figure 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.

Figure 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.

762 **Figure 1**

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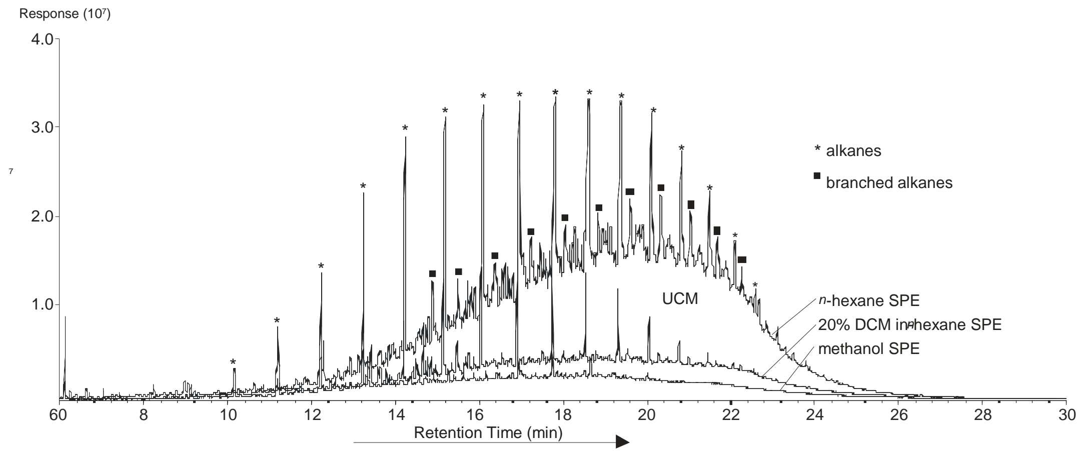

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768 **Figure 2**

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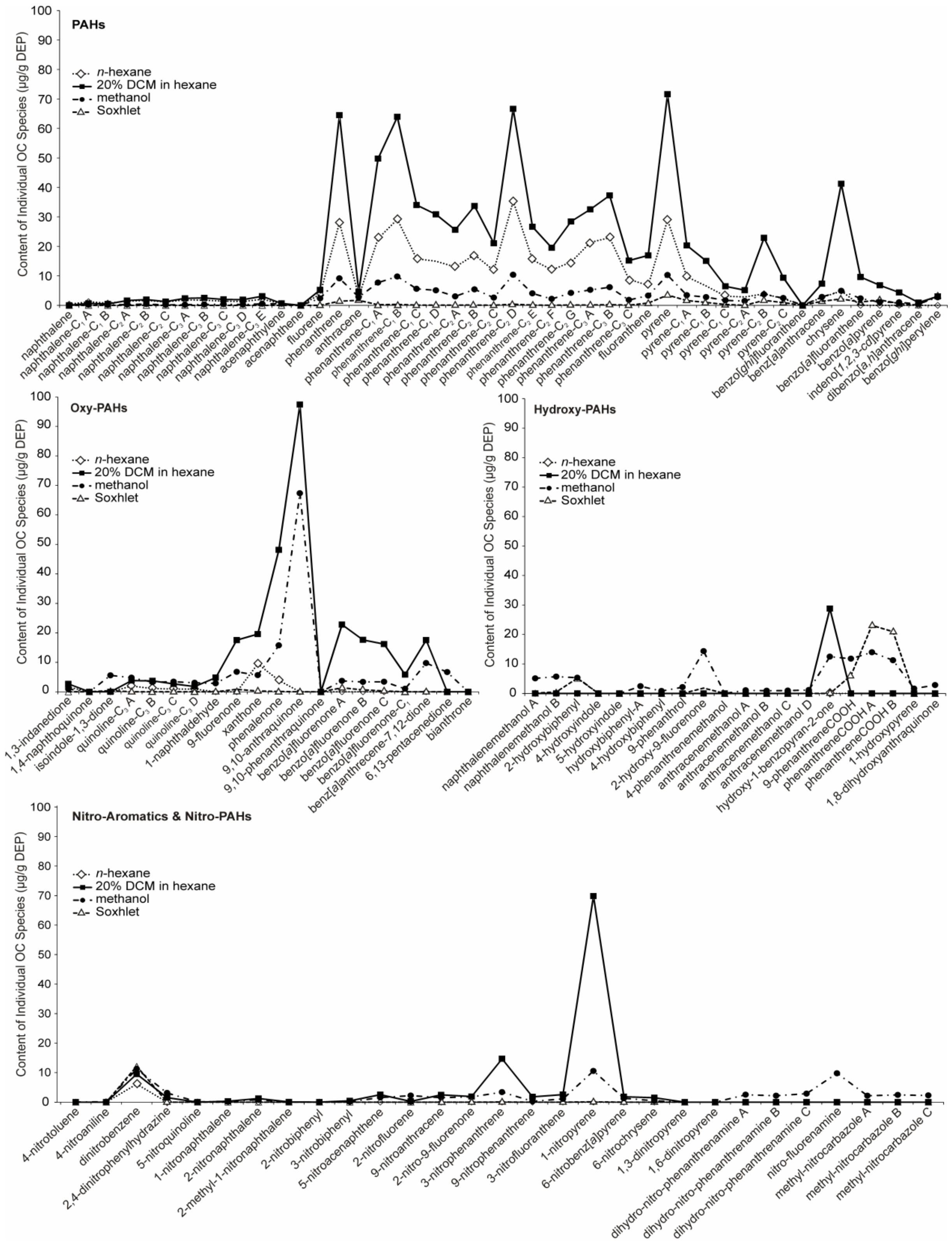

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772 **Figure 3**

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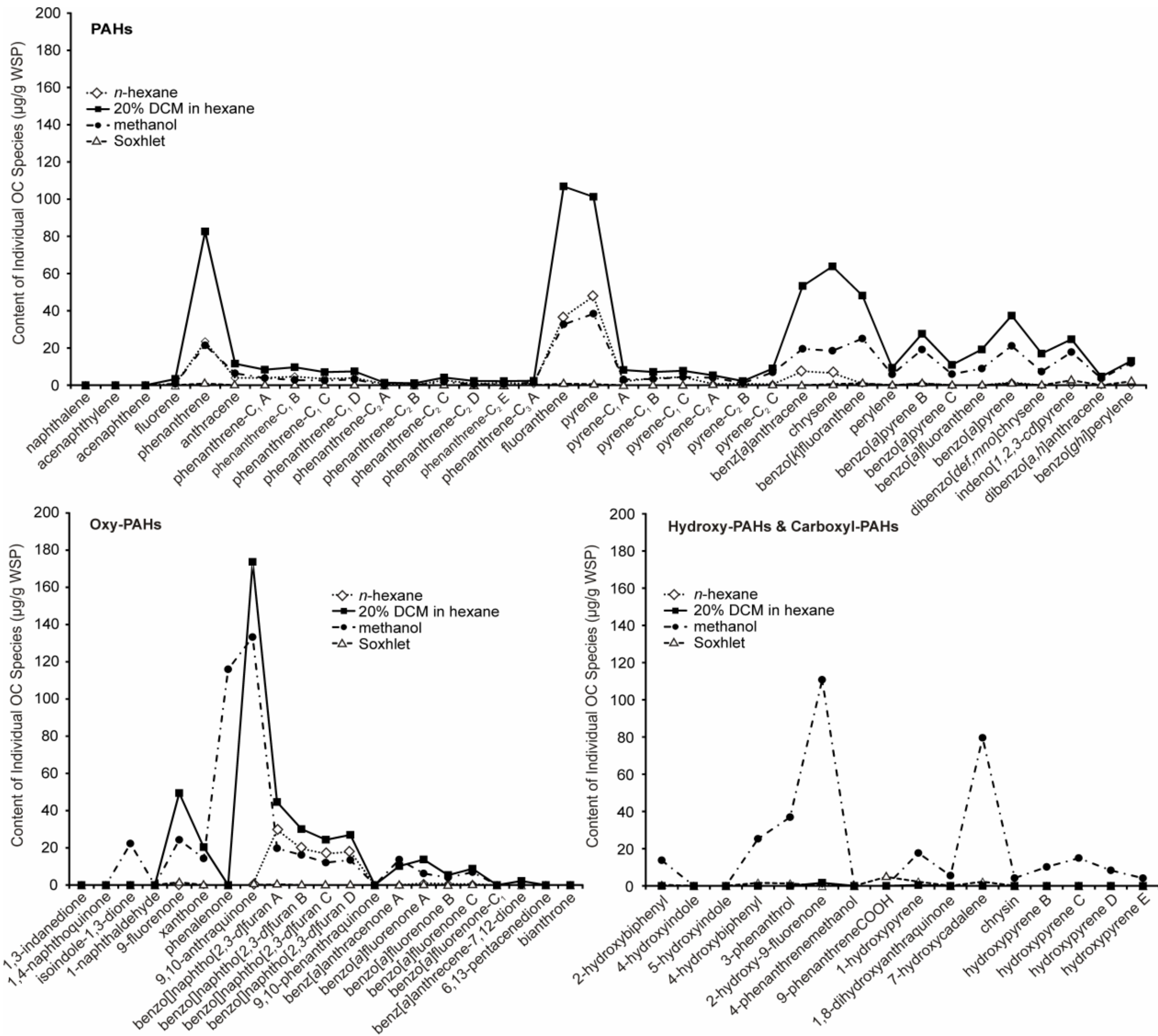

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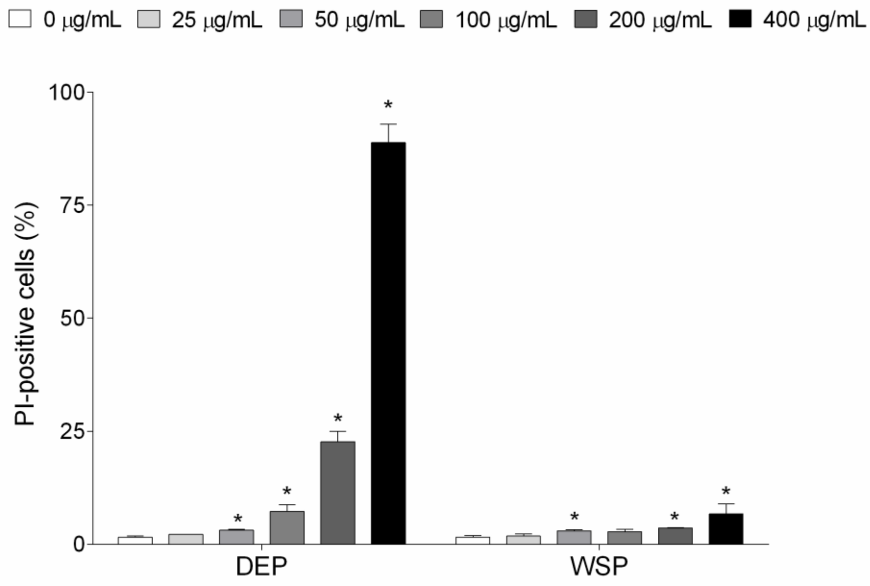
777 **Figure 4**



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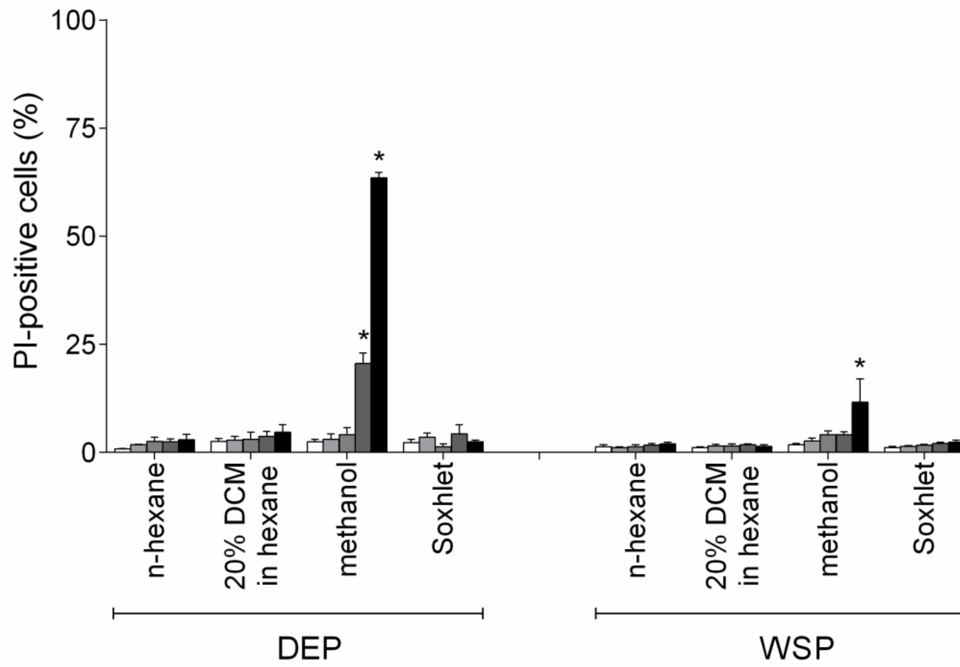
784 **Figure 5**



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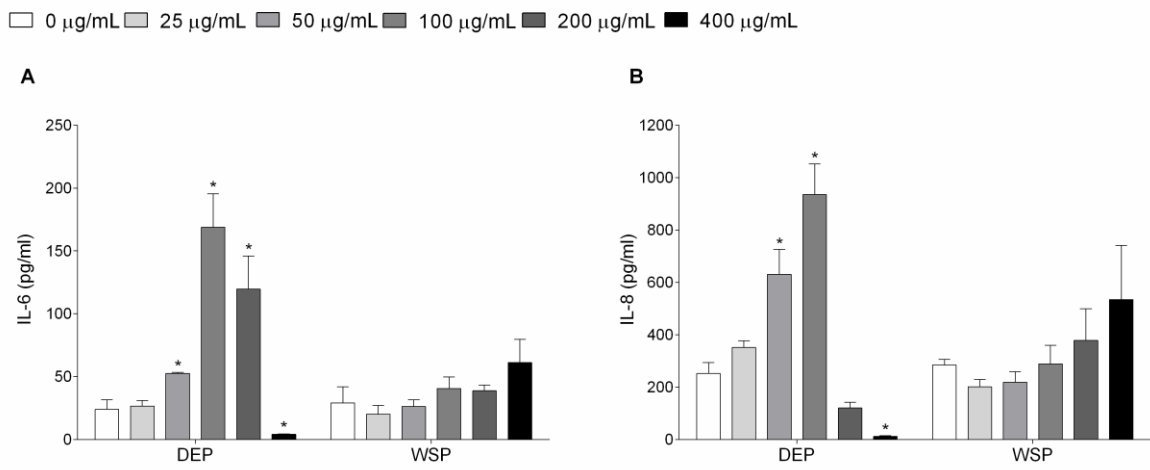
788 **Figure 6**



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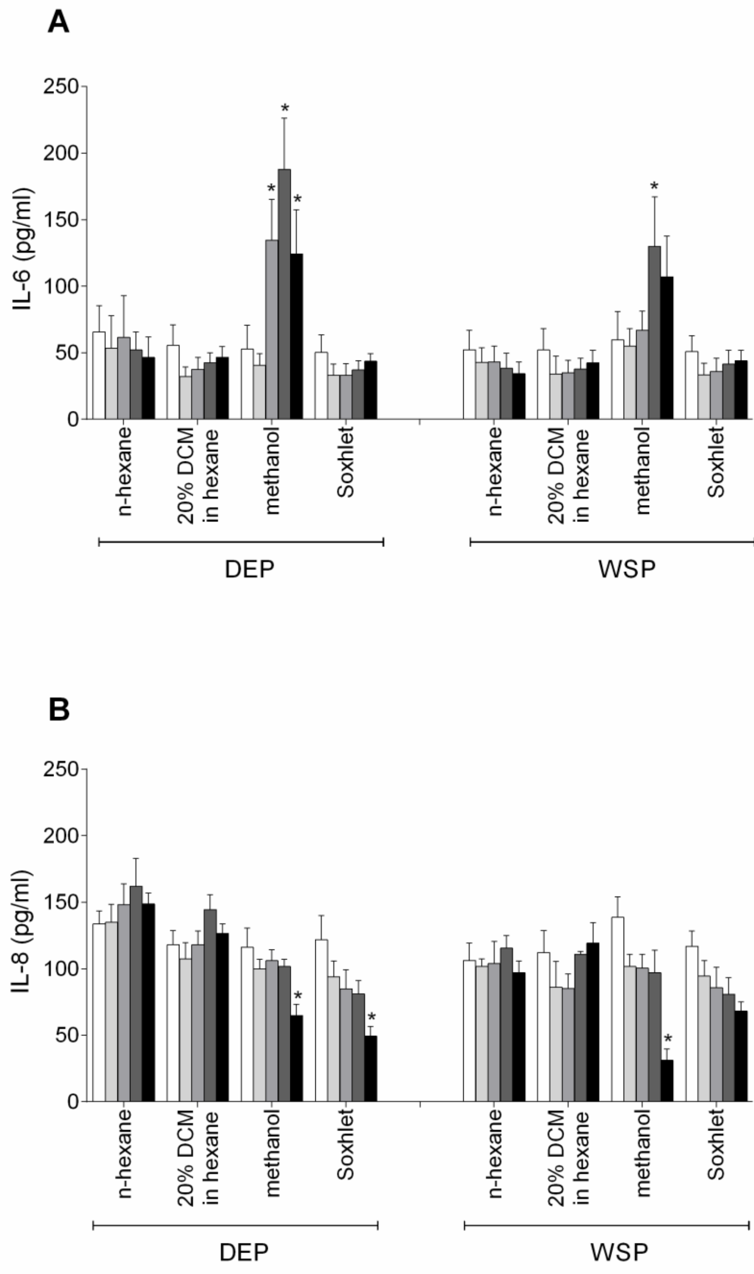
793 **Figure 7**



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