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Oxidative Stress, DNA Damage, and Inflammation Induced by Ambient Air and Wood Smoke Particulate Matter in Human A549 and THP-1 Cell Lines

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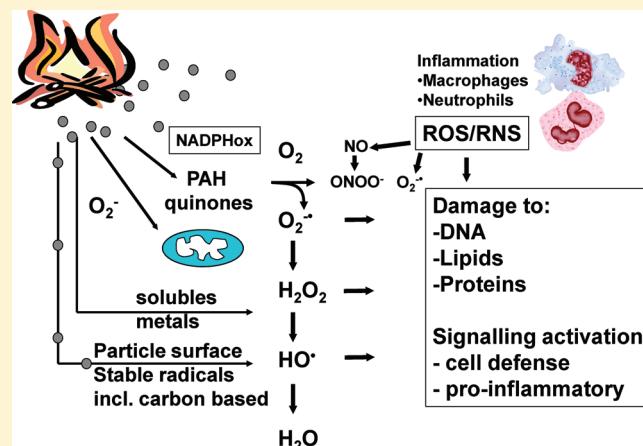
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ABSTRACT: Combustion of biomass and wood for residential heating and/or cooking contributes substantially to both ambient air and indoor levels of particulate matter (PM). Toxicological characterization of ambient air PM, especially related to traffic, is well advanced, whereas the toxicology of wood smoke PM (WSPM) is poorly assessed. We assessed a wide spectrum of toxicity end points in human A549 lung epithelial and THP-1 monocytic cell lines comparing WSPM from high or low oxygen combustion and ambient PM collected in a village with many operating wood stoves and from a rural background area. In both cell types, all extensively characterized PM samples (1.25–100 µg/mL) induced dose-dependent formation of reactive oxygen species and DNA damage in terms of strand breaks and formamidopyrimidine DNA glycosylase sites assessed by the comet assay with WSPM being most potent. The WSPM contained more polycyclic aromatic hydrocarbons (PAH), less soluble metals, and expectedly also had a smaller particle size than PM collected from ambient air. All four types of PM combined increased the levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine dose-dependently in A549 cells, whereas there was no change in the levels of etheno-adducts or bulky DNA adducts. Furthermore, mRNA expression of the proinflammatory genes monocyte chemoattractant protein-1, interleukin-8, and tumor necrosis factor-α as well as the oxidative stress gene heme oxygenase-1 was upregulated in the THP-1 cells especially by WSPM and ambient PM sampled from the wood stove area. Expression of oxoguanine glycosylase 1, lymphocyte function-associated antigen-1, and interleukin-6 did not change. We conclude that WSPM has small particle size, high level of PAH, low level of water-soluble metals, and produces high levels of free radicals, DNA damage as well as inflammatory and oxidative stress response gene expression in cultured human cells.



INTRODUCTION

Exposure to particulate matter (PM) from ambient air is associated with adverse effects on human health, such as respiratory and cardiovascular diseases and mortality.^{1–3} An extensive review concerning the health effects of biomass and wood smoke PM (WSPM) concluded that there is adequate evidence linking exposure with both acute and chronic illnesses. However, there is

still inadequate evidence to conclude whether biomass PM and WSPM are less or more damaging to health, compared to other types of air pollution particles.⁴ Furthermore, The International Agency for Research on Cancer has concluded that indoor

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exposure to biomass combustion (mainly wood) is probably carcinogenic for humans.⁵

The toxicological mechanisms of PM-induced health effects are thought to involve the generation of reactive oxygen species (ROS), oxidative stress, and inflammation.⁶ The mechanisms of oxidative stress induction include (i) direct generation of ROS caused by physicochemical surface properties of the particles, (ii) effects of soluble compounds such as transition metals or organic compounds, (iii) effects of particles on mitochondria or NADPH-oxidases, and (iv) activation of inflammatory cells capable of generating ROS and reactive nitrogen species (RNS). Oxidative stress can induce damage to DNA such as strand breaks (SB) and the mutagenic 8-oxo-7,8-dihydroguanine (8-oxoGua).^{7–10} The few available studies of the toxicological effects of some types of WSPM have indicated elevated generation of ROS, release of cytokines, increased levels of SB, and lipid peroxidation products in cultured cells.^{11–14} Recently, we have reported that a sample of WSPM collected from a Norwegian wood stove gave higher levels of DNA damage, including SB and purine oxidation, per unit mass than PM originating from traffic; especially the extractable organic fraction seemed to be detrimental.¹⁵ However, the chemical composition of WSPM depends on many factors, including the type of wood and combustion conditions.¹⁶ In ambient air, WSPM will admix and further react with other pollutants from local and distant sources, resulting in a complex and heterogeneous mixture of particles with variable biological effects. Accordingly, further understanding of the importance of WSPM in ambient air may be achieved by comparing biological effects as well as physicochemical characteristics of WSPM collected directly under different combustion conditions with those of ambient PM collected in areas with and without high emissions from wood combustion.

In this study, we collected PM material from ambient air in a village with high wood stove emission and from a rural area, and WSPM from wood stove combustion at high and low oxygen supplies. The PM samples were extensively characterized with respect to their size-distributions in stock solutions measured by photon correlation spectroscopy, particle characteristics by transmission electron microscopy (TEM), composition of polycyclic aromatic hydrocarbons (PAH) and water-soluble metals, and by their capacity to generate radicals and ROS. We measured the level of oxidatively damaged DNA and bulky DNA lesions as well as gene expression of the DNA repair protein oxoguanine glycosylase 1 (*OGG1*), proinflammatory genes (tumor necrosis factor- α (*TNF- α*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), lymphocyte function-associated antigen-1 (*LFA-1*) and monocyte chemoattractant protein-1 (*MCP-1*), and oxidative stress response gene heme oxygenase-1 (*HO-1*) in exposed human lung epithelial adenocarcinoma (A549) and mononuclear (THP-1) cell lines, which represent cell types of the pulmonary target tissue and circulating inflammatory mononuclear blood cells, respectively. Our hypothesis was that WSPM would be more toxic in terms of oxidative stress, DNA damage, and inflammatory signaling than would rural background PM due to the higher content of organic compounds.

MATERIALS AND METHODS

Collection of Particles. We collected fine PM with a modified commercially available electrostatic precipitator at a sampling volume flow of 175 m³/h.¹⁷ Briefly, the sampler was modified from an electrostatic office air cleaner by replacing the steel collecting plates with 17 plates of

massive Sterling silver and installing a custom-made F6 prefilter (filter class F6, Camfil Farr, Kokkedal, Denmark) to remove coarse particles. The modification resulted in a collection efficiency of 60–80% between 0.2 and 0.8 μ m and an upper cut-point of 4.2 μ m. The massive silver plates replaced the chemically less stable silver-coated steel plates produced for the original modification in Sharma et al.¹⁷ Measured by a TSI condensation particle counter (Model 3022A, TSI Inc., USA), the sample collection efficiency using the Sterling silver plates was similar to the efficiency using silver-coated plates. Inductively coupled plasma-mass spectrometry analysis of the Sterling silver plates showed that these plates only contained trace amounts of impurities (in ppm, mainly Au (1.4), Ca (4.3), Cu (38.1), Fe (50.9), Mg (2.8), Na (34.6), P (43.1), Pd (1.8), Si (8.0), Sn (1.0), and Zn (2.97)). The collection plates were kept at 4 °C after sampling. The PM material was extracted from the plates 1–4 days after sampling.

Ambient PM was collected at two sites: the first was inside Slagslunde, a village in North Zealand, Denmark. The village consists of 350 mainly one-family houses of which approximately 200 use wood stoves for supplemental heating. Previous investigations have shown a contribution of wood smoke to the local ambient air in similar villages.^{18,19} The collection site in the middle of the village represents PM including the contribution from the stoves (AAWS). The second site, was located approximately 500 m west of the village and was generally upwind of the village collection site. The second site represents PM from background ambient air in a rural area without significant contribution from wood stoves (AARU). The AAWS sample was collected between the 19/01 and 01/02–2007 (2 weeks) during anticyclone weather with subzero temperatures and stagnant air, whereas the AARU sample was collected between the 05/01 and 01/02–2007 (2 times, 2 weeks). The total particle recovery amount after extraction was approximately 129 mg for the AAWS sample and 115 mg for the AARU sample.

WSPM was collected from the exhaust from a wood stove (Model 7110, Morsø, Nykøbing Mors, Denmark) during the combustion of beech wood (water content 12–18%) in pieces of approximately 1 kg. The particle-sampler inlet was placed in a 1 m³ tank (height, 157 cm; width, 75 cm; depth, 91 cm). The flow of flue gas into the tank was 6–14 m³/h. Collection of PM was done during two combustion conditions: 4 h of combustion with high oxygen (HOWS) and 2 h with low oxygen (LOWS) in the stove. During the high oxygen combustion, the temperature was 100–120 °C just after the stove, and for low oxygen, the temperature was 30–60 °C, mostly below 50 °C. The PM concentration generated by the stove in the tank was about 5 mg/m³. The total particle recovery after extraction was approximately 58 mg for the HOWS sample and 1594 mg for the LOWS sample, which resembled a tar-like mixture.

PM was retrieved from the 17 collection plates in the sampler following the procedure explained in Sharma et al.¹⁷ with some modifications. Lyophilizing was done for 2 days at a pressure of 0.1 mbar (Heto Holten Power dry LLI 500, Heto Holten, Denmark). The particles were subsequently concentrated to the final samples over four times of pooling suspensions in Milli-Q-filtered water and lyophilized for one day. The final lyophilized PM samples were gently disaggregated in an agate mortar, weighed, and stored at –80 °C until the preparation of stock suspension as described later. The LOWS sample was extracted with 70% ethanol because the sample was a mixture of tar and particles. This sample was evaporated in a Rotavapor for about 30 min where the water temperature was 70–100 °C; the residue was extracted with 99.9% ethanol and evaporated in the Rotavapor three times for about 15–30 min. After evaporation in the Rotavapor, the sample was extracted with 99.9% ethanol, which was evaporated to remove the ethanol under a gentle stream of nitrogen at 40 °C for 2 h.

Printex 90 carbon black (CB) was used as an internal positive reference sample in the ROS measurements. The material was a gift from Degussa–Hüls, Frankfurt, Germany.

Photon Correlation Spectroscopy. The hydrodynamic size distribution of the PM suspended in F12 and RPMI cell culture media used for the bioassays was measured by photon correlation spectroscopy using a Dynamic Laser Scatter (DLS) Malvern Nano ZS (Malvern Inc., UK), following the analytical conditions as previously described.²⁰ Frozen PM stock suspensions were thawed and sonicated for at least 20 min at 47 hHz ± 6% and 185 W (Bransonic Ultrasonic Cleaner, Model Branson S210) followed by 5–10 s of vortex at 200 rpm using a Vortex VF2 (IKA Werke GmbH & Co. KG, Staufen, Germany). Laboratory tests have shown that particle suspensions can sustain this freeze–thaw treatment without further agglomeration. The DLS analysis was performed on unfiltered as well as 0.8 μm-filtered samples to identify the potential presence and size of smaller particles in the samples. The results were calculated using Dispersion Technology Software, version 5.0 (Malvern Instruments Ltd.). Six repeated analyses were performed to evaluate the size distribution spectra. Accepted spectra ($n = 3$ to 6) were averaged to obtain a final size distribution for each sample.

Electron Microscopy. Transmission (TEM) and Scanning Transmission Electron Microscopy (STEM) were performed using a 200 kV FEI Tecnai T20 TEM (FEI Company, Oregon, USA) mounted with a LaB6 filament and a Jeol STEM 1200 EX-II (Jeol Ltd., Japan) mounted with a W-filament and operated at 80 to 120 kV. Both instruments were equipped with online energy dispersive spectrometers, for *in situ* elemental analysis. Frozen stock suspensions (500 μg/mL) were thawed and sonicated for 20 min at 47 kHz ± 6% and 185 W (Bransonic Ultrasonic Cleaner, Model Branson S210). The preparation of the stock suspensions is explained in the Cell Cultures and PM Exposure section below. Then a sample was withdrawn using a pipet, and a few droplets were deposited onto holey carbon-film-coated Cu-grids, placed on filter paper. The TEM-grid samples were allowed to dry a few minutes covered by a glass Petri dish and stored in individual polymer capsules until analysis.

PAH, Levoglucosan, Mannosan, Endotoxin, and Water-Soluble Elements. PAH was extracted by shaking the sample with toluene. The extracts were analyzed for 18 selected PAH by GC/MS, as previously described.¹⁹ Levoglucosan and mannosan were analyzed by HPLC-MS.²¹ The level of endotoxin in the samples was assessed by the Pyrogen Gel Clot LAL assay with a sensitivity of 0.06 EU/mL according to the procedure described by the manufacturer (Lonza Copenhagen ApS, Vallensbak Strand, Denmark).

The concentration of water-soluble elements in the PM samples was determined after 24 h of dissolution in filtered ultrapure water (0.22 μm Millipore Millipak Gamma Gold filter) using a stirred atmosphere (clean atmospheric air with 5% CO₂) and a 37 °C temperature-controlled batch reactor. PM was rapidly brought into suspension using the Bransonic Ultrasonic Cleaner mentioned above. The samples were then diluted to 0.25 mg/mL and subjected to hydrous dissolution for 24 h in the stirred batch reactor (Pyrex; Radiometer Analytical, Lyon, France) wrapped in aluminum foil to avoid photocatalytic reactions. After 24 h, 40 mL of solution was centrifuged at 3856g for 99 min at 20 °C. Assuming a density of 2.2 g/cm³, these conditions settle particles larger than 30 nm. After centrifugation, the upper 6 mL of the suspensions were kept in darkness at room temperature until analysis. The concentrations of water-soluble elements were determined by Graphite-Furnace Atomic Absorption Spectrometry (GF-AAS; Perkin-Elmer SIMAA 6000) equipped with a AS-72 Autosampler (Perkin-Elmer Denmark A/S, Skovlunde, Denmark). Data were recorded and calculated using the Perkin-Elmer AA Winlab software. Analysis was conducted as either single (Fe, Cu, Zn), bi-, or multielement analysis (Mn + Cd + Pb, As + Se, Mo + V, and Cr + Ni). Four- (Cu) or five-point (all other elements) linear calibration curves ($R^2 = 0.9872$) were calculated using multielement standards from Perkin-Elmer. No further action against potential interference was performed.

Cell Cultures and PM Exposure. Human lung epithelial A549 and monocytic THP-1 cell lines were obtained from the American Type

Culture Collection (Manassas, VA, USA) and grown in F12 and RPMI cell media, respectively, with 10% serum as previously described.¹⁵ The A549 cell line is an adherent growing on the bottom of cell culture flasks, whereas the THP-1 cell line grows in suspension.

The PM stock suspensions (500 μg/mL) were made in the F12 and RPMI cell media using a Bransonic Sonifer mounted with a disruptor horn (Model S-450D, Branson Ultrasonics Corp., Danbury, CT, USA). The sonicator was operated with a 10 s pulse and a 10 s pause for a total of 8 min with an amplitude of 10%. The PM stock suspensions were kept frozen until use. The thawed PM stock suspensions were vortexed prior to dilution with the respective cell culture medium to the final concentrations, and residual stock solutions were instantly frozen at –80 °C for subsequent DLS-analysis (described above).

The cytotoxicity of the PM was measured as LDH activity in cell medium by Cytotoxicity Detection Kit from Roche Applied Science, Penzberg, Germany. The cell cultures were exposed to a 200 μL PM suspension for 24 h at the following concentrations: 0, 2.5, 25, and 100 μg/mL in 96 well-plates (0.3 cm²/well) corresponding to 0, 1.65, 16.5, and 66 μg/cm². The results are expressed as percent cytotoxicity, where 100% maximum LDH release is obtained by treatment of cell cultures with Triton X-100, whereas the baseline LDH release in untreated cell cultures is set at 0%. The cell proliferation was measured in A549 cells exposed to LOWS and CB for 3 h using the WST-1 assay from Roche Applied Science, Penzberg, Germany.

Detection of Radical and ROS Generation. The ROS production was measured in A549 and THP-1 cells as well as in a cell-free environment by the DCFH-DA assay.^{22,23} Fifty thousand A549 cells were seeded in a 96-well black fluorescence plate and incubated in cell culture medium for 24 h before the incubation with DCFH-DA (Cayman Chemical, USA). The cells were incubated with 2 μM DCFH-DA for 15 min and washed twice with Hank's balanced saline solution. The THP-1 cells were treated likewise but were spun down and washed twice before they were transferred into a 96-well black fluorescence plate. The final concentration of THP-1 cells was 5×10^5 cells/mL in a 200 μL total volume. The particle suspensions were added to the cells in the following concentrations: 1.56, 3.13, 6.25, 12.5, 25, and 50 μg/mL. For the acellular experiment, DCFH-DA was deacetylated in accordance with the method described by LeBel et al.²⁴ CB was used as a positive control as it previously has been shown that CB produces ROS in alveolar macrophages and a mouse epithelial cell line in a concentration-dependent manner.^{22,23} The DCF fluorescence was measured continuously on a fluorescence spectrophotometer at $\lambda_{ex} = 485$ and $\lambda_{em} = 538$ (Fluoreskan Ascent FL, Thermo Labsystems) at 37 °C for 3 h. All experiments were repeated 3 times, and the results are normalized and reported as fold-increase.

We also analyzed the PM-induced radical generation by ESR spectroscopy. Briefly, PM was sonicated in pure water (1 mg/mL) using a sonicator horn as described above and kept frozen at –20 °C until use. The samples were sonicated in combination with vortexing twice for resuspension, and 300 μL (300 μg) of the suspension was added to a filter piece (Quartz microfibre, Whatman, 0.5 × 4 cm²) and dried. Then 12.5 μM vitamin C and 100 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO-spin trap) in 40 mM Tris-HCl buffer were added, and samples were measured by ESR spectroscopy on a Bruker EMX 1273 spectrometer operating at X band frequencies and equipped with an ER 4119HS high sensitivity resonator as described before.²⁵ Each sample was prepared and measured in duplicate.

Detection of DNA Damage. Each set of cell culture experiments were carried out on three different days, each with exposures to the PM samples and concentrations in duplicates. The levels of SB and FPG sites in the DNA were measured by the comet assay as previously described.^{15,26} 2.25 × 10⁵ cells were seeded into 24 well culture plates (1.9 cm²/well). After 24 h, the cells were treated with 1 mL of PM stock solution diluted with cell culture medium to the following final concentrations: 0, 2.5, 25,

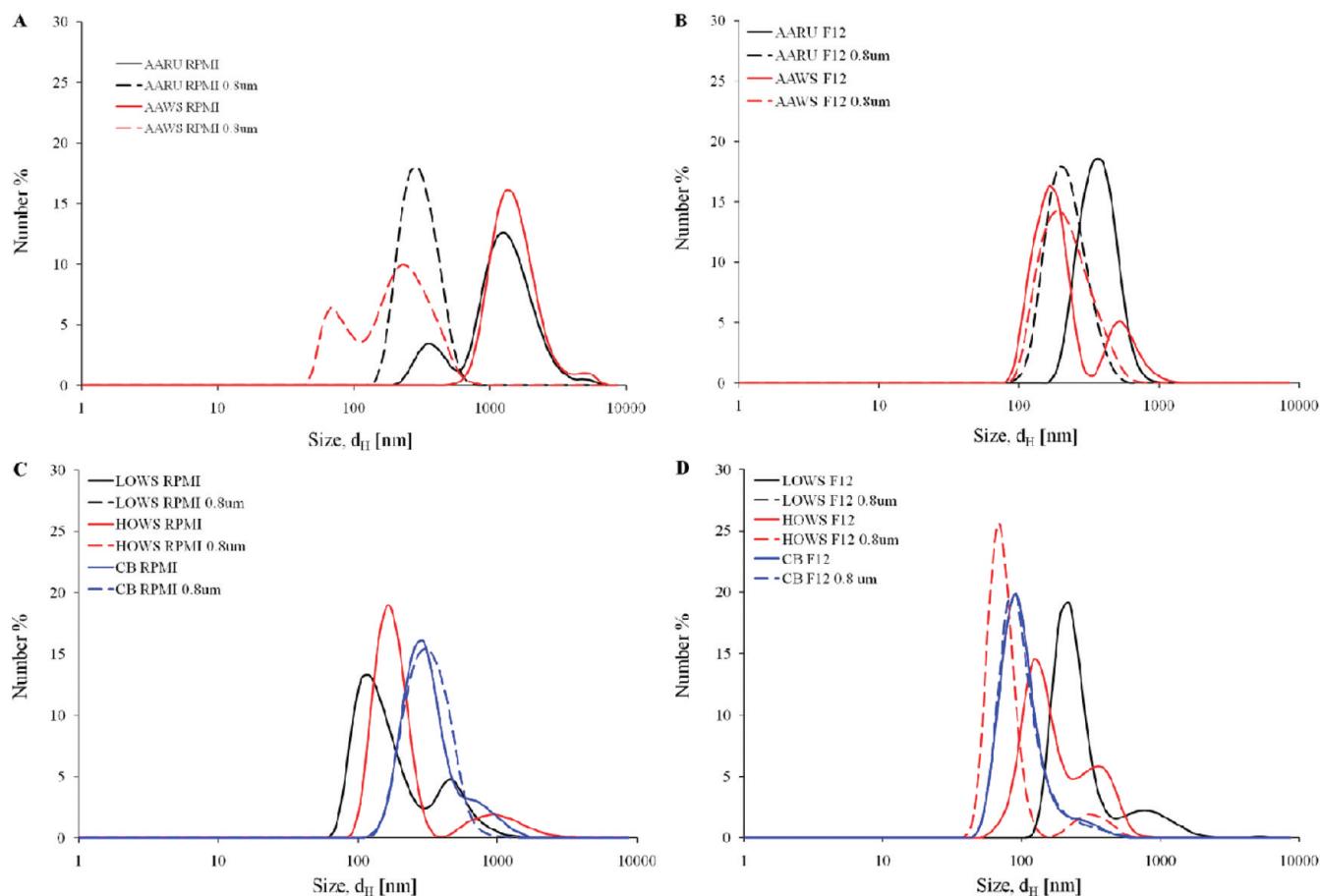


Figure 1. Hydrodynamic sized [d_H] number size distributions of particle samples suspended in RPMI and F12 cell culture medium. Size spectra are shown for unfiltered stock suspensions (500 $\mu\text{g}/\text{mL}$) and 0.8 μm filtered samples. (A) AARU and AAWS suspended in RPMI. (B) AARU and AAWS suspended in F12. (C) HOWS, LOWS, and CB suspended in RPMI. (D) HOWS and LOWS suspended in F12.

and 100 $\mu\text{g}/\text{mL}$ corresponding to 0, 1.3, 13.2, and 52.6 $\mu\text{g}/\text{cm}^2$ and incubated for 3 h. The samples were visually scored, and the primary comet assay end points in arbitrary units were transformed into lesions/ 10^6 base pairs (bp), using an investigator-specific calibration curve based on the induction of SB by ionizing radiation and assuming that the average molecular weight of a base pair is 650 Da (corresponding to approximately 6×10^9 bp or 4×10^{12} Da DNA).²⁷

The levels of 8-oxodG, 1,N⁶-etheno-2'-deoxyadenosine (edA), and 1-N²-etheno-2'-deoxyguanosine (edG) were studied only in A549 cells exposed to PM for 3 h. A total of 1.125×10^6 cells/well was seeded in a 6-well plate (9.6 cm^2 /well). The cells were treated with 5 mL of PM stock suspension diluted with cell culture medium to the following final concentrations: 0, 2.5, 25, and 100 $\mu\text{g}/\text{mL}$ corresponding to 0, 1.3, 13, and 52.1 $\mu\text{g}/\text{cm}^2$. DNA was isolated and digested as described previously.²⁸ Simultaneous determination of 8-oxodG, 1,N⁶-etheno-2'-deoxyadenosine (edA), and 1-N²-etheno-2'-deoxyguanosine (edG) was carried out by HPLC coupled to electrospray ionization tandem mass spectrometry.²⁹ External calibration with authentic standards was used. The amount of DNA was calculated from the area of the dG peak displayed in the HPLC chromatogram recorded on a UV spectrometer after the HPLC column.

The level of bulky DNA adducts were analyzed in A549 cells and THP-1 cells exposed to PM for 24 h. A total of 1.125×10^6 cells/well was seeded in a 6-well plate. The cells were treated with 5 mL of PM stock solution diluted with cell culture medium to the following final concentrations: 0, 2.5, 25, and 100 $\mu\text{g}/\text{mL}$. DNA was extracted by the phenol/chloroform/isooamyl alcohol method and digested as previously described.³⁰ Bulky DNA adducts were measured by ³²P-postlabeling

with butanol enrichment and quantification by phosphorimage analysis as previously described.³⁰ The adducts are labeled with ³²P-orthophosphate from [γ -³²P]-ATP mediated by polynucleotide kinase. For later adduct versus nucleotide quantification, a known amount of digested DNA is also labeled. The labeled mix is spotted on a polyethylenimine-cellulose thin layer chromatography plate and developed by chemical processing. The plate is then exposed on a phosphor storage screen which is scanned to locate the bulky DNA adducts. To minimize the labeling of unmodified nucleotides and to obtain more pure adduct levels and maximal adduct recovery, the DNA is extracted with butanol.

Gene Expression. The mRNA levels of TNF- α , IL-8, IL-6, LFA-1, MCP-1, HO-1, and OGG1 were measured in both THP-1 and A549 cells. A total of 1.125×10^6 cells/well were seeded in a 6-well plate. The cells were treated with 5 mL PM preparations diluted with cell culture medium to the following final concentrations: 0, 2.5, 25, and 100 $\mu\text{g}/\text{mL}$ and incubated for 3 h. The RNA was extracted using TRIzol reagent (Invitrogen A/S, Taastrup, Denmark) according to the manufacturer's protocol followed by DNase treatment (Promega Biotech AB, Denmark). Approximately 150 ng of RNA was used for cDNA synthesis in a reaction volume of 20 μL using High Capacity cDNA Reverse Transcription Kit, and the syntheses were carried out on the GeneAmpPCR system 2700 (both Applied Biosystems, Naerum, Denmark). Quantitative real-time PCR reactions were carried out in ABI PRISM 7900HT (Applied Biosystems, Naerum, Denmark), using TaqManGene Expression Assays purchased from Applied Biosystems, Naerum, Denmark. The assay IDs for the genes were as follows: MCP-1, Hs00234140_m1; IL-8, Hs00174103_m1; IL-6, Hs00174131_m1; LFA-1, Hs01035619_m1; TNF- α , Hs00174128_m1;

Table 1.^a

material	AARU	AAWS	HOWS	LOWS	CB
total amount (mg)	115	129	58	1594	
DLS size peaks (nm) in RPMI					
without 0.8 μm filter	342, 1281, 4800	1281, 4800	164, 955	122, 459	295
with 0.8 μm filter	295	68, 220	\leq	\leq	295
DLS size peaks (nm) in F12					
without 0.8 μm filter	342	164, 531	122, 342	220, 825	91, 600
with 0.8 μm filter	190	190	68, 295	\leq	91, 600
levoglucosan ($\mu\text{g}/\text{mg}$)	2.82	43.9	39.9	56.2	NA
mannosan ($\mu\text{g}/\text{mg}$)	<0.2	5.15	0.28	1.96	NA
total PAH (ng/mg)	499	281	3267	1252	0.075 ^b
soluble elements (ng/mg)					NA
As	26.9	19.3	\leq	\leq	
Se	11.6	4.0	0.9	\leq	
Ni	147.5	45.0	14.7	10.3	
Cr	27.3	25.0	21.4	21.3	
Cu	1087	323	65.6	34.4	
Fe	2427	1882	1323	1773	
Zn	2523	1773	1030	750	
Mn	113	40.6	\leq	\leq	
Pb	297	156.6	27.7	68.8	
Cd	5.8	2.0	29.4	\leq	
V	192.4	39.6	\leq	\leq	
Mo	11.5	7.3	3.8	7.7	
total	6870	3718	2517	2665	

^a \leq : Not detected or concentration below the detection limit. NA: Not analyzed. ^b The PAH concentration of Printex 90 was measured previously by Jacobsen et al.²³

HO-1, Hs00157965_m1; OGG1, Hs01114116_g1. We used 18S rRNA as the reference gene (Eukaryotic 18S rRNA Endogenous Control, 4352930E, Applied Biosystems, Naerum, Denmark). The PCR reactions were performed as described previously.³¹ The level of gene expression is reported as the ratio between the mRNA level of the target gene and the 18S rRNA reference gene using the comparative $2^{-\Delta\text{Ct}}$ method.

Statistics. The LDH data were analyzed by the nonparametric Kruskal–Wallis test, with a posthoc Tukey-type multiple comparison test. The effects on ROS generation, the comet assay, and gene expression were assessed by ANOVA with the concentration nested in the type of PM expressed as categorical. The validity of the nested ANOVA analysis was accepted on the basis of normal distribution of the residuals. Statistically significant effects were accepted at the 5% level in the overall nested ANOVA and in the posthoc least significant difference (LSD) tests. The effect of PM exposure on chromatographically determined DNA lesions was analyzed by a general linear model with particle type as categorical and dose as continuous variables. The statistical analysis was performed in Statistica 5.5 (StatSoft, Inc., Tulsa, USA).

■ RESULTS

Size Distribution and TEM of PM Samples. The particle suspensions analyzed by DLS exhibited bi- or polymodal size distributions (Figure 1). Table 1 outlines a summary of the size distribution generally showing that HOWS and LOWS had smaller average size than AARU and AAWS. Filtration of the suspensions revealed the presence of peaks in the nanosize range of AAWS, HOWS, and CB. Representative pictures of the TEM analysis are shown in Figure 2. The analysis indicated that the HOWS sample was dominated by ultrafine to fine aggregated carbonaceous soot particles with primary sizes of 40–70 nm as

well as spheres of alkali-metals (Na and K) of up to a few hundred nanometers in size and sodium chloride, both of which may occur as individual particles or in chain aggregates (Figure 2A and B). In consistence, DLS analysis showed a peak of 68 nm after filtration of HOWS in F12 medium (Table 1). The LOWS sample consisted of highly viscous tar and could not be used for conventional electron microscopic analysis. The AAWS sample was dominated by carbonaceous soot, spherical particles of alkali-metals as a major constituent, different clay minerals, and quartz (Figure 2, middle). Mineralogically, the AARU sample (Figure 2, bottom) appeared similar to the AAWS sample. Combined, the results from DLS and TEM analysis of stock suspensions of the PM samples indicate that AAWS contains smaller particles than the AARU, whereas PM directly from the wood stoves, especially HOWS, contain even smaller particles. In all samples, the finest particles were mainly soot-related.

Chemical Analysis of PM Constituents. There were similar amounts of levoglucosan in the AAWS, LOWS, and HOWS samples, whereas the amount was much lower in the AARU sample (Table 1). The concentration of the less abundant compound mannosan was highest in AAWS samples followed by the LOWS and HOWS samples, whereas it was below the limit of detection in the AARU sample.

The total concentration of the measured PAH was noticeably higher in the HOWS than in the LOWS sample, whereas the levels were lower in the AARU and particularly the AAWS samples. The PAH profile, presented as a histogram in Figure 3, showed more similarities of AAWS and AARU with HOWS than with LOWS.

The samples contained endotoxin in the following order: AARU (effect in the gel clot LAL assay at 2.5 $\mu\text{g}/\text{mL}$), AAWS,

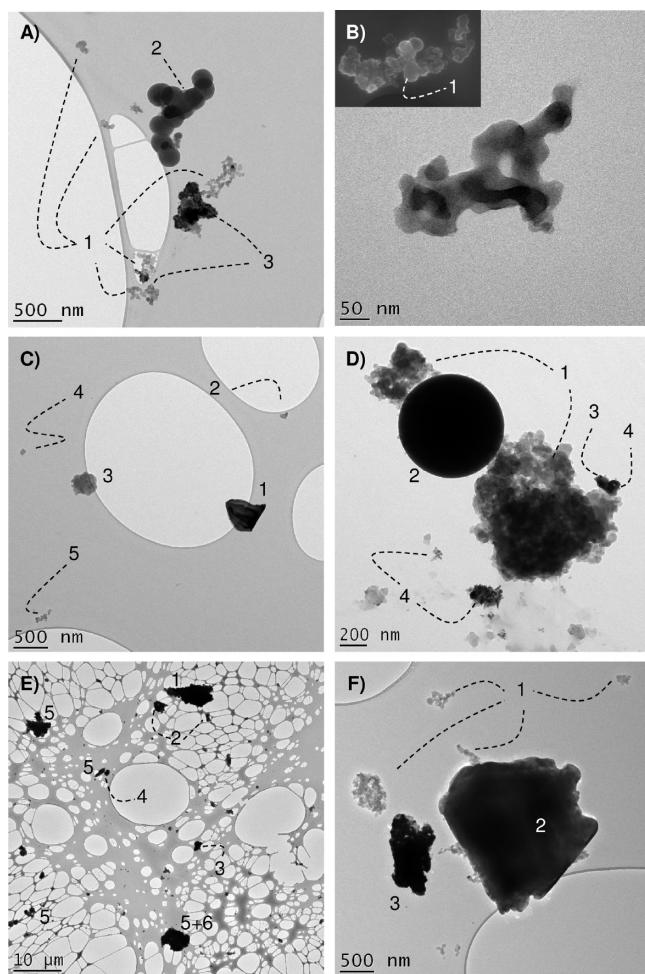


Figure 2. Transmission and scanning electron microscopy images of particle samples. (A) HAWS. 1, carbonaceous soot associated with trace concentrations of Si and NaCl; 2, NaCl chain aggregate; and 3, Ag-rich particle aggregate from the ESP sampler associated with particles of Na, Cl, S, Si and Zn. (B) High-magnification TEM-image of HAWS showing a carbonaceous soot particle with irregular primary soot spheres. The insert shows a STEM-image of another soot particle associated with K- and Ca-rich spheres (marked 1). (C) AAWS. 1, angular quartz particle; 2, K-aluminum-silicate; 3, particle rich in Si, Zn, F, and Cl; 4, Si-rich particle; and 5, carbonaceous soot with Si. (D) TEM-image showing different soot particles in AAWS. 1, carbonaceous soot associated with minor concentrations of Si; 2, fly-ash sphere mainly consisting of Si, Al, Fe, and alkali metals; 3, Ti-Al-oxide; 4, particles consisting of Ag and S. (E) Low-magnification TEM-image showing an example of several different fine to coarse particles in AARU. 1, complex aggregate consisting of carbonaceous soot, Al-silicate, and Ca-rich material; 2, carbonaceous soot associated with minor Si; 3, Ca-Zn-rich particle; 4, particle rich in Ca and Zn and minor concentrations of Fe and Pb; 5, Ag-rich particles associated with Cl and/or Si; and 6, silica-rich particle (possibly quartz) associated with Ag. (F) TEM-image showing fine mineral and soot particles in AARU. 1, carbonaceous soot with Si; 2, alkali-Al-silicate (clay mineral); 3, particle consisting of Ag and S.

HOWS, CB (25 µg/mL), and LOWS (100 µg/mL). The endotoxin concentration was determined, using the geometric mean dilution multiplied by the assay sensitivity, to be 0.6 EU/mL for LOWS, 2.4 EU/mL for AAWS and HOWS, 4.8 EU/mL for CB, and 19 EU/mL for AARU.

Table 1 shows the concentration of the 12 water-soluble elements (As, Se, Ni, Cr, Cu, Fe, Zn, Mn, Pb, Cd, V, and Mo)

chosen among the transition metals and major constituents of 11 inorganic hazardous air pollution substances defined by the US Environmental Protection Agency (<http://www.epa.gov/ttn/atw/orig189.html>). The total and all individual concentrations of water-soluble elements, except for cadmium, followed the same pattern, being most abundant by mass in the AARU sample followed by the AAWS sample, whereas the water-soluble elemental concentration (especially of Cu, Pb, V, Ni, Mn, and As) was lower or not detectable in the HOWS and LOWS samples. Furthermore, no sample showed more than 140 ng/mg water-soluble Ag.

Cytotoxicity of PM. The cytotoxicity induced by HOWS, LOWS, AARU, and AAWS in A549 and THP-1 cells was measured as LDH release after 24-h exposure (data not shown). An increase in the number of dead or membrane-damaged cells increases the LDH activity in the cell culture supernatant. For both cell lines, there was no statistically significant increase in cytotoxicity at any dose from 2.5 to 100 µg/mL ($p > 0.05$; Kruskall-Wallis test). All particles resulted in LDH release less than 6% of the maximum release, $n = 3$. In A549 cells, the LDH release was up to 16% at 100 µg/mL CB ($p = 0.061$; Kruskal-Wallis test). The WST1 assay showed no effects on the viability of A549 cells after 3 h at LOWS or CB concentrations of 2.5, 25, or 100 µg/mL.

Radical and ROS Generation. The AARU and HOWS samples produced large amounts of ROS in terms of hydroxyl radicals and superoxide anions as indicated by DMPO spin trapping, whereas the AAWS sample produced less and the LOWS sample no ROS at all detected by ESR (Figure 4A). A representative ESR spectrum of AARU is shown in Figure 4B. A large, presently unknown radical signal (g -value 2.018–2.021) was seen by ESR only in the HOWS sample without DMPO (Figure 4C and E) and still visible with DMPO, even though the ESR signal was not spin-trapped (Figure 4D and F).

Figure 5 shows the ability of the particle samples to generate ROS assessed by the DCFH-DA assay in an acellular environment (Figure 5A) as well as in both THP-1 (Figure 5B) and A549 cells (Figure 5C). The acellular experiment (Figure 5A) shows that all types of PM results in significantly increased ROS production from 25 µg/mL (AAWS, AARU, and HOWS) or from 50 µg/mL for LOWS ($p < 0.05$; posthoc LSD test). The CB positive control, had a concentration dependent ROS generation ($p < 0.05$; posthoc LSD test). The nested ANOVA analysis demonstrates that ROS production from CB and LOWS are significantly different from that produced by all other types of PM and that the ROS formation capacity of AAWS, AARU, and HOWS are comparable ($p < 0.05$, nested ANOVA). In THP-1 cells (Figure 5B), exposure to AAWS, AARU, and LOWS significantly increased DCFH-detected ROS levels only at the highest concentration of 50 µg/mL ($p < 0.05$; posthoc LSD test), whereas HOWS did not induce ROS. CB significantly induced ROS in both cell types, but contrary to the acellular experiment, the concentration-response was bell-shaped instead of linear. In the A549 cells (Figure 5C), the nested ANOVA analysis revealed that CB, LOWS, and HOWS increased ROS levels significantly from 6.25 µg/mL and were significantly different AAWS and AARU, which were similar with 50 µg/mL required to increase ROS production ($p < 0.05$; nested ANOVA and posthoc LSD tests).

DNA Damage. Figure 6 depicts the concentration-response relationships of SB and FPG sites in A549 (Figure 6A and B) and THP-1 cells A549 (Figure 6C and D). The overall nested ANOVA test showed statistically significant differences between the different PM suspensions.

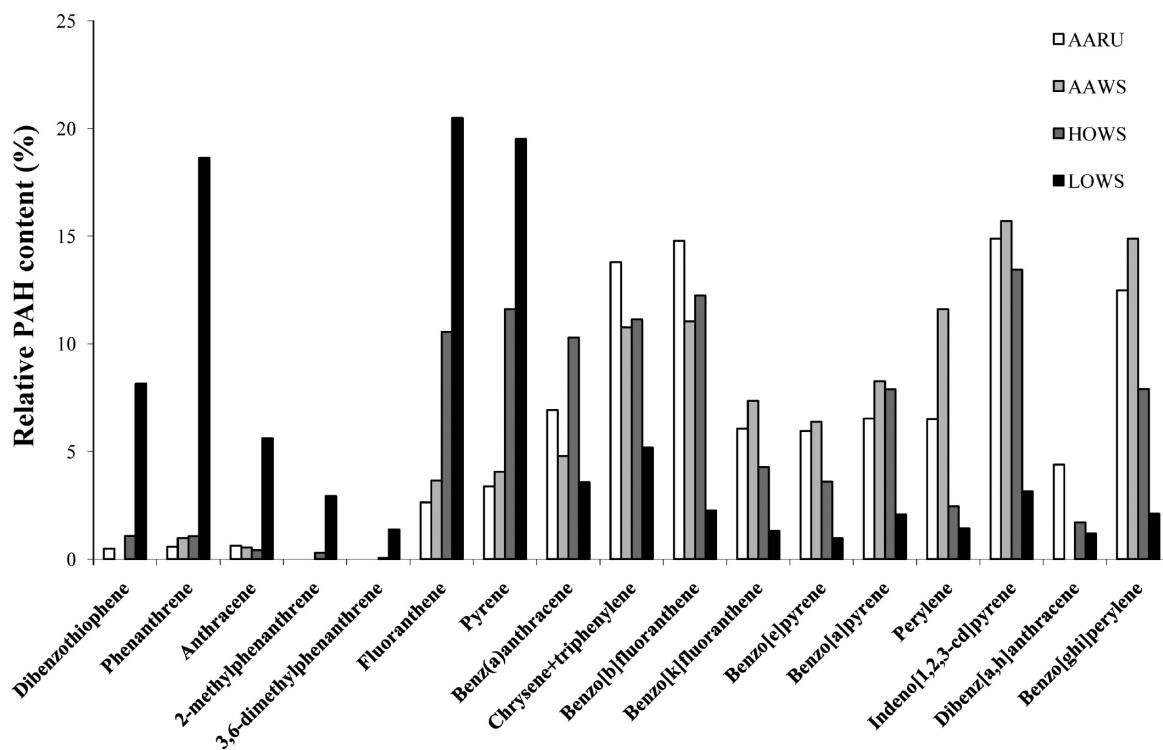


Figure 3. PAH profiles of the PM samples.

In A549 cells, the LOWS and HOWS sample generated significantly higher levels of SB than the AAWS and AARU samples ($p < 0.05$; nested ANOVA). Compared to the control, there were increased levels of SB after exposure to AARU (2.5 $\mu\text{g}/\text{mL}$), AAWS (100 $\mu\text{g}/\text{mL}$), HOWS (25 $\mu\text{g}/\text{mL}$), and LOWS (25 $\mu\text{g}/\text{mL}$) ($p < 0.05$; posthoc LSD test).

In THP-1 cells, the HOWS sample generated higher levels of SB than the other samples of PM ($p < 0.05$; nested ANOVA), whereas LOWS generated a damage level similar to that of AAWS. AARU was the least potent material to induce SB ($p < 0.05$; nested ANOVA). AAWS and HOWS increased the level of SB at concentrations above 25 $\mu\text{g}/\text{mL}$ ($p < 0.05$; posthoc LSD test), whereas AARU and LOWS increased SB only at 100 $\mu\text{g}/\text{mL}$ ($p < 0.05$; posthoc LSD test).

In A549 cells exposed to 25 and 100 $\mu\text{g}/\text{mL}$ of LOWS and HOWS, the comet assay reached a saturation stage at which the level of SB was so high that FPG sites could not be reliably measured. These concentrations were consequently excluded from Figure 6B and omitted from the statistical analysis. The level of FPG sites in A549 cells was significantly increased compared to that of the control after exposure to AAWS and HOWS at concentrations above 25 $\mu\text{g}/\text{mL}$ ($p < 0.05$; posthoc LSD test), whereas there was no significantly increased level of FPG sites after exposure to AARU ($p > 0.05$; posthoc LSD test).

For the THP-1 cells, the PM samples generated comparable levels of FPG sites, except for AARU which elicited lower levels than the others. The level of FPG sites increased for all types of PM at concentrations above 25 $\mu\text{g}/\text{mL}$ ($p < 0.05$; posthoc LSD test), except for AARU that only showed a significantly increased level of FPG sites at the highest concentration ($p < 0.05$; posthoc LSD test).

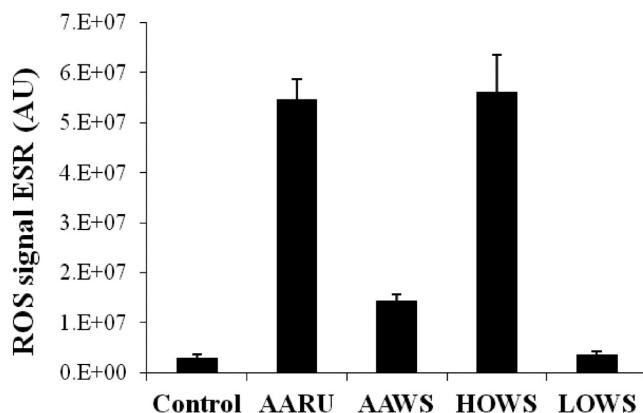
The levels of 8-oxodG, δdG , and δdA in DNA from A549 cells are shown in Figure 7. There were no statistically significant differences between the etheno-adduct levels in A549 cell exposure

to any PM preparations and the controls. The level of 8-oxoG was significantly dose-dependently increased in A549 cells by all four types of PM combined in the general linear statistical model, whereas no difference between the samples could be detected.

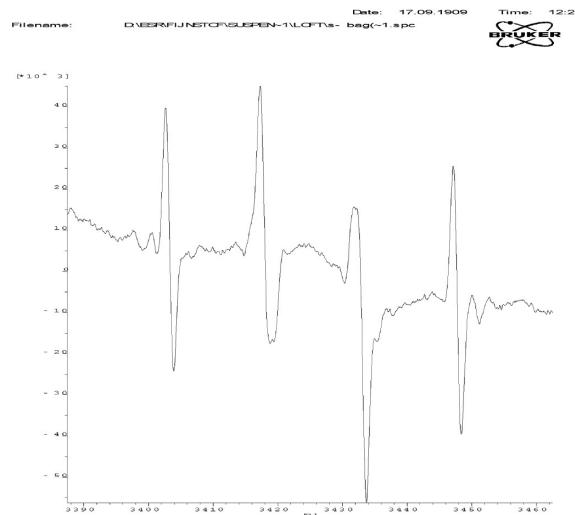
The level of bulky DNA adducts in A549 and THP-1 cells exposed to the PM preparations for 24 h is shown in Figure 8. There were no significant effects of exposure to any PM preparation on the level of bulky adducts in any of the cell types.

Gene Expression. The mRNA expression of a battery of genes after exposure to the PM preparations for 3 h is shown in Figures 9 (A549 cells) and 10 (THP-1 cells). We were not able to detect the basal expression of *LFA-1* and *TNF- α* in A549 cells and *IL-6* in THP-1 cells. Nested ANOVA of the data for the A549 cells showed no significant differences between the particles for any of the measured genes. The only significant upregulation detected was the *MCP-1* mRNA after exposure to 2.5 $\mu\text{g}/\text{mL}$ of LOWS ($p < 0.05$; posthoc LSD test). In the THP-1 cells, nested ANOVA indicated significant differences between the PM preparations for *TNF- α* and *HO-1* mRNA, whereas *IL-8* mRNA that visually seems to differ among the particles types showed inhomogeneity of variance and consequently the nested ANOVA analysis could not be performed for that gene. For the induction of *TNF- α* mRNA in THP-1 cells, AAWS significantly differed from AARU ($p < 0.05$, nested ANOVA). The statistical analysis of *HO-1* mRNA showed that AAWS and LOWS were different from AARU, whereas HOWS was different from the LOWS sample ($p < 0.05$, nested ANOVA). Compared to the control, the *MCP-1* mRNA was upregulated at the concentration of 2.5 $\mu\text{g}/\text{mL}$ of LOWS, as well as at the concentration of 25 $\mu\text{g}/\text{mL}$ of AAWS ($p < 0.05$; posthoc LSD test). The same concentrations and particles as well as 25 $\mu\text{g}/\text{mL}$ of LOWS resulted in significant upregulation of *TNF- α* . *IL-8* and *HO-1* mRNA expression was also increased in THP-1 cells by all particles at the highest concentrations. *IL-8* mRNA was significantly increased when the

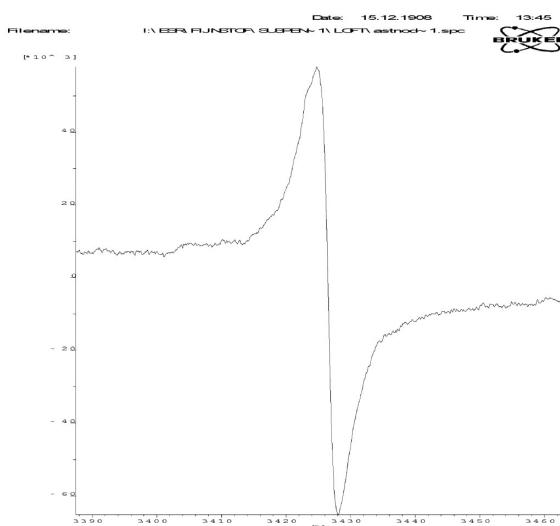
A



B



C



D

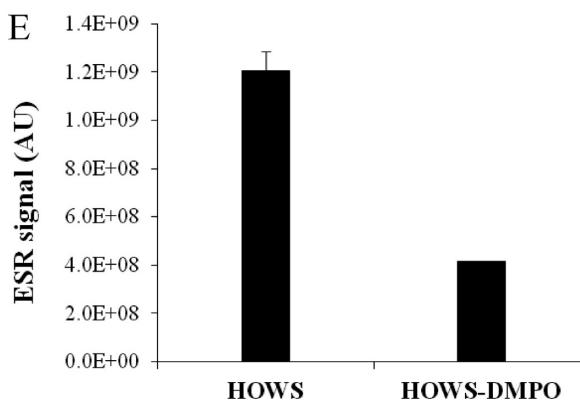
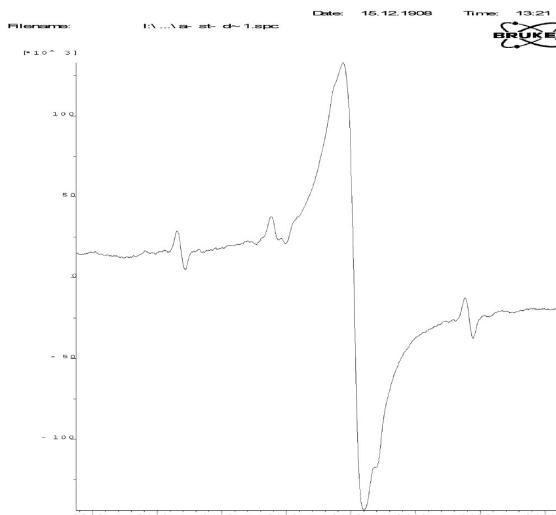


Figure 4. ROS production measured as spin-trapped hydroxyl radicals and superoxide anions in AARU, AAWS, HOWS, and LOWS (A). Representative ESR spectra of the AARU sample (B). An unknown radical was present in HOWS without (C and E) and with DMPO (D and E).

cells were exposed to AAWS at all concentrations, to AARU at 25 and 100 μ g/mL, and to HOWS at 100 μ g/mL ($p < 0.05$;

posthoc LSD test). HO-1 mRNA was significantly upregulated at concentrations 25 and 100 μ g/mL after exposure to AAWS,

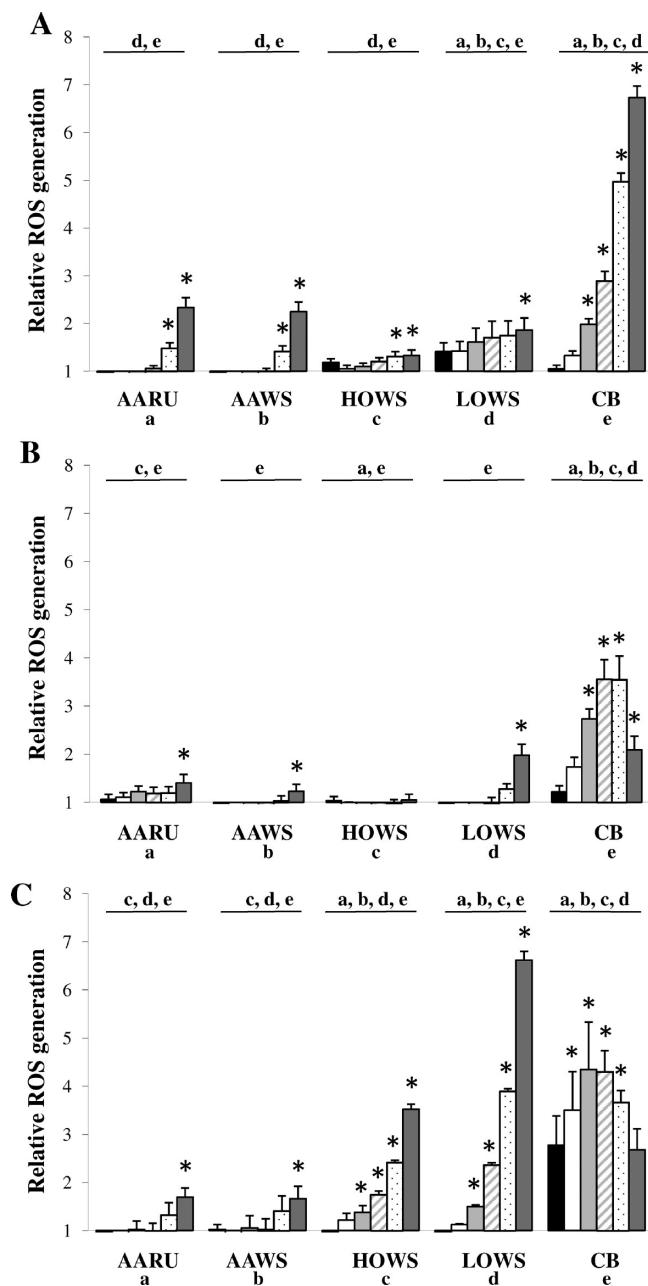


Figure 5. The relative ROS generation in acellular condition (A), in THP-1 cell cultures (B), and in A549 cell cultures (C) exposed for 3 h to PM. The data are normalized to 1. Each bar represents the mean \pm SE ($n = 3$) of cultures exposed to 1.56 (black), 3.13 (white), 6.25 (light gray), 12.5 (hatched), 25 (dotted), and 50 (dark gray) $\mu\text{g}/\text{mL}$ PM. The letters above each type of PM indicate statistically significant differences ($p < 0.05$, nested ANOVA) between that and other PM preparations. * statistically significant compared to the control ($p < 0.05$; posthoc LSD test).

AARU, and HOWS, whereas LOWS only resulted in significant upregulation at the concentration of 100 $\mu\text{g}/\text{mL}$ ($p < 0.05$; posthoc LSD test for AARU, Kruskall-Wallis test for the other samples). Combined, these results show that mRNA expression of inflammatory genes (*MCP-1*, *IL-8*, and *TNF- α*) and mRNA expression of the stress response gene *HO-1* in THP-1 cells are upregulated substantially to or above the levels expressed in A549 cells, which do not show consistent changes in the

regulation of these genes 3 h after exposure to the PM preparations.

DISCUSSION

In this study, we compared multiple toxicological end points in two relevant cell lines between PM collected from ambient air in an area with high (AAWS) and low (AARU) influence from wood stove emission as well as directly from the combustion of birch wood in a stove with high (HOWS) and low (LOWS) oxygen supplies. In general, the wood stove particles were smaller, contained more PAH and less soluble metals, and produced more free radicals, DNA damage, as well as inflammatory and oxidative stress response gene expression than did the AAWS and in particular the AARU sample as summarized in Figure 11. The results suggest that WSPM per unit mass is more toxic than rural background ambient air particulates per unit mass in terms of the increased levels of DCFH-measured ROS production, SB, FPG sites, and HO-1 expression, whereas the inflammatory potential may show less difference. In addition, we show for the first time that PM material collected from ambient air in high wood smoke emissions generate ROS and oxidatively damaged DNA in a dose-dependent manner that is similar in potency to that of pure WSPM. The strong contribution of wood smoke to AAWS was documented by the high levels of levoglucosan (1,6-anhydro- β -D-glucopyranose) and the less abundant stereoisomer mannosan, which are derived from cellulose and significant components of WSPM.³² Levoglucosan levels in PM collected from ambient air in residential areas in Denmark are substantially elevated in the evening and nights when wood stoves are being used.¹⁹ The ambient air particulates AAWS and especially AARU contained much higher concentrations of water-soluble elements, in particular copper, lead, vanadium, nickel, manganese, and arsenic than did HOWS and LOWS. These metals are tracers for the long-range transport of PM from coal and fuel oil combustion, vehicle emissions, and brake wear as well as suspended soil dust and rock material. Therefore, the AAWS sample can be considered as a mixture of both WSPM and AARU material. It is somewhat in keeping with the results showing that WSPM generated effects that were higher than those of AARU, whereas it was difficult to distinguish between the level of effects of AAWS and the other WSPM samples. More repetitions of each analysis or sampling at different times in the season might have produced clearer distinctions between the effects of the samples because of increased statistical power.

ROS were generated by all PM samples in one or more of the applied test systems and generally with a linear concentration dependence. The higher ROS production in A549 cells could be due to phagocytic activity and/or the higher activity of biotransformation as well as larger actual exposure because of sedimentation of the particles to the adherent cells at the bottom of the wells. However, this difference between the cell lines was not seen as clearly in terms of DNA damage detected by the comet assay and was actually opposite for gene expression. WSPM has previously been reported to induce the production of H_2O_2 in RAW 264.7, whereas no ROS production was observed in A549 cells by the DCFH-DA assay in contrast to our findings.^{12,33} We used CB as a positive control, resulting in increased ROS levels in both cell lines and in the acellular environment. It has been shown that CB produces ROS in alveolar macrophages in a concentration-dependent manner.^{22,23} In our study, cellular exposure to CB resulted in bell-shaped concentration-response curves, in contrast to the linear response for the acellular assay. We used ESR as

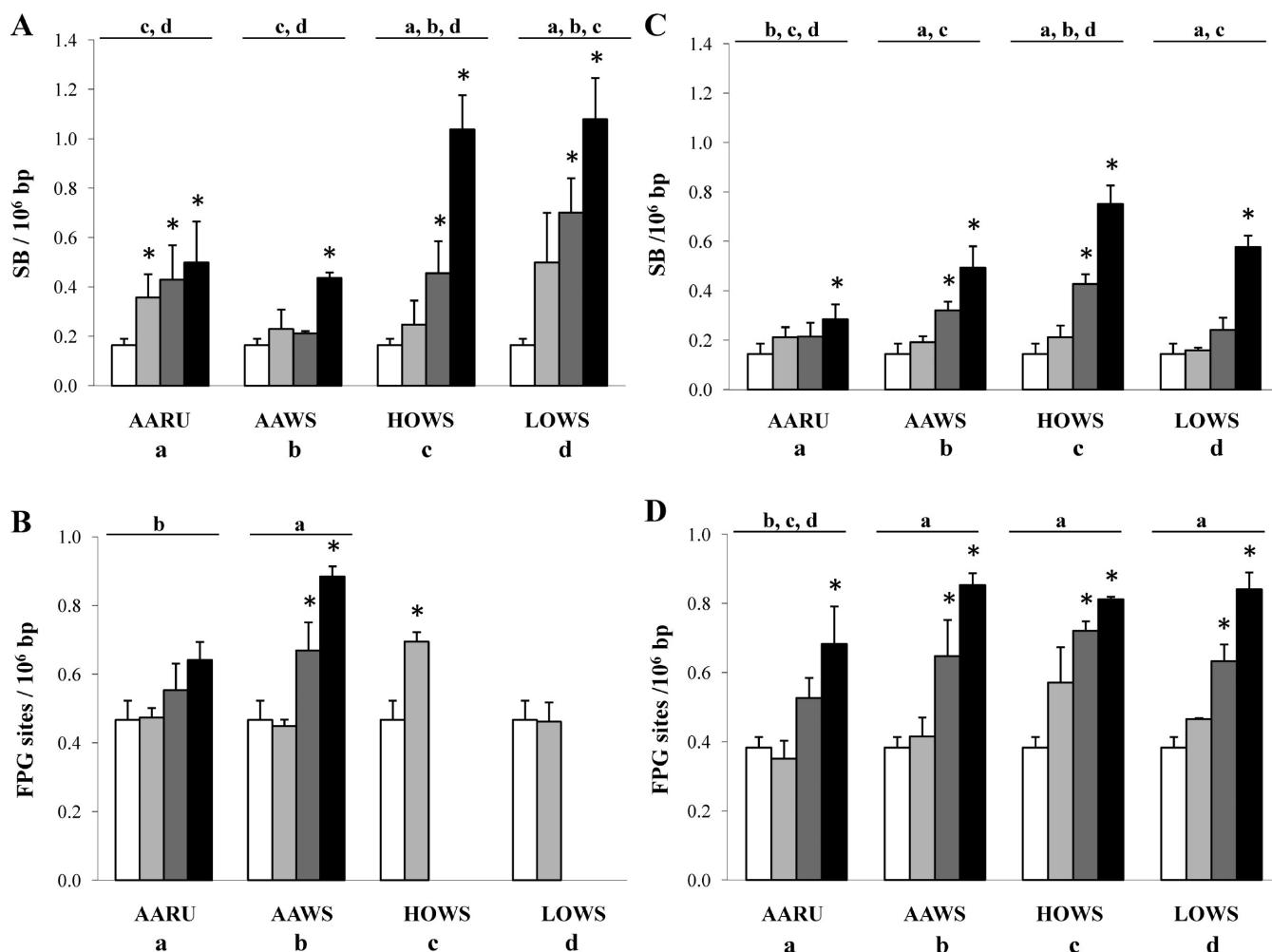


Figure 6. SB (A) and FPG sites (B) in A549 cell cultures and SB (C) and FPG sites (D) in THP-1 cell cultures exposed for 3 h to PM. Each bar represents the mean \pm SE ($n = 6$) of cultures exposed to 0 (light gray), 2.5 (white), 25 (dark gray), and 100 (black) $\mu\text{g}/\text{mL}$ PM. The letters above each type of PM indicate statistically significant differences ($p < 0.05$, nested ANOVA) between that and other PM preparations. *, statistically significant compared to the control ($p < 0.05$; posthoc LSD test).

a second method for the detection of acellular ROS production. The ESR method used here measures hydroxyl and superoxide anion radicals, whereas the DCFH-DA assay determines a wide range of unspecific ROS.³⁴ It is likely that the soluble transition metals contributed substantially to the ROS production detected by ESR in AARU and AAWS, whereas the radicals generated by HOWS are more likely to be related to organic compounds. Previous ESR investigations have identified WSPM as a source of quinoid radicals^{12,35} which can produce ROS through redox cycling.^{36,37} By comparing the ESR spectra and values given in those studies, we conclude that we are more likely to have detected some kind of carbon-centered than a semiquinone radical, although from our experience we know that most carbon-centered radicals also react with DMPO, and this was not the case in this experiment. Hydroxyl and carbon-centered radicals have been observed in WSPM in the presence of H_2O_2 ^{11,12,38} and without.³⁵ In this study, we did not add H_2O_2 to catalyze the Fenton reaction; instead, we used ascorbate, which acts both in the process of iron recycling and redox cycling of quinone-like structures.²⁵ It has been shown that PAH include or can be converted to quinone species either by cytochrome P450 peroxidase activity or by the aldo-keto reductase pathway followed by redox cycling.^{39,40} These pathways

are likely to be responsible for the intracellular ROS production from LOWS in particular due to the abundance of oxygenated organic species formed during smoldering combustion,¹⁶ whereas the soluble metals could have contributed to the intracellular ROS production from AARU and AAWS. In addition, the WSPM followed by AAWS formed the smallest particles in suspension in comparison with AARU, thus providing a larger surface area for ROS formation.

The comet assay has been extensively used in cell culture studies investigating the genotoxic effects of PM.^{6,8} The SB detected by the alkaline comet assay is an unspecific measure of genotoxicity, whereas FPG sites represent oxidized purine bases in the DNA, such as the mutagenic 8-oxoGua.⁴¹ In this study, induction of SB and FPG sites followed a concentration-dependent pattern in both cell lines. The WSPM samples elicited higher levels of SB than the ambient air samples per unit mass in both cell lines, whereas AAWS with substantial contribution of WSPM caused higher SB levels than AARU in THP-1 cells. Similarly, we observed higher levels of FPG sites in cells exposed to the WSPM samples or AAWS than in those exposed to AARU. The comet assay reached a saturation stage in the A549 cells exposed to the WSPM samples, at which the level of SB was so high that FPG sites

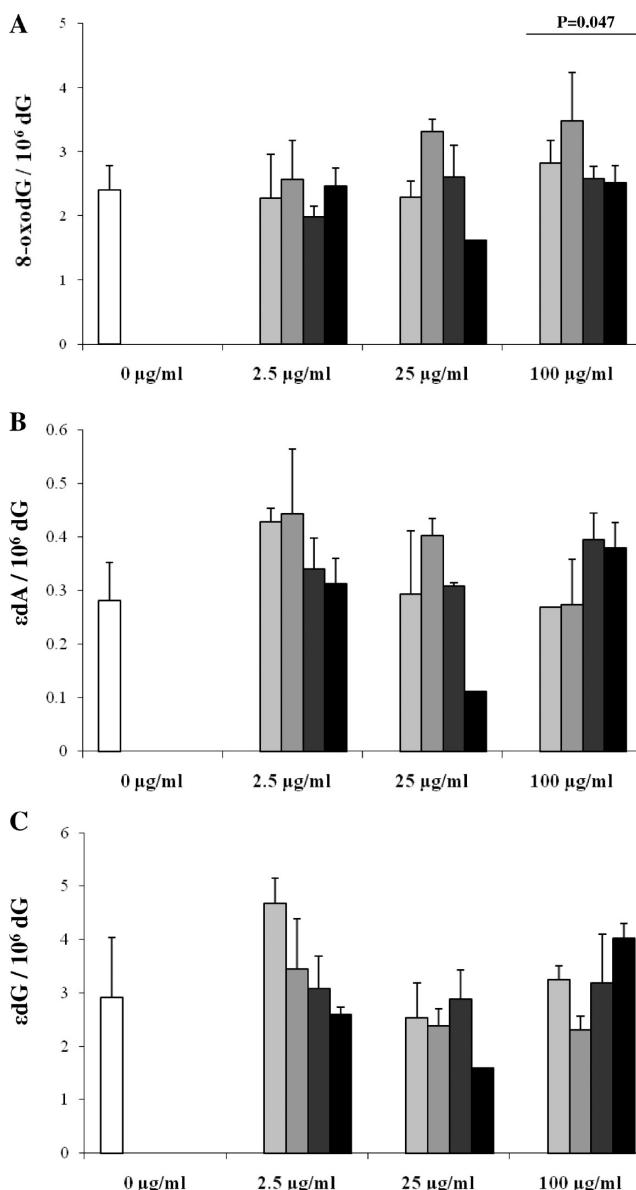


Figure 7. DNA lesions in terms of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), etheno-2'-deoxyguanosine (edG), and etheno-2'-deoxyadenosine (edA) measured by high performance liquid chromatography with tandem mass spectrometry in DNA from A549 after 3 h of exposure. The bars represent the mean \pm SE ($n = 3$) of the control (white), AARU (light gray), AAWS (gray), HOWS (dark gray), and LOWS (black). There was a significant concentration-dependent increase in the level of 8-oxodG in a general linear model analysis including all particle data.

could not be measured reliably. The substantially increased DNA damage was not associated with the increased cytotoxicity measured by LDH release. A few studies have previously shown increased generation of SB^{11,13,15,33} and FPG sensitive sites¹⁵ by exposure to WSPM in cell cultures. The DNA damage measured by the comet assay was consistent with the data on ROS production measured by the DCFH-DA with high levels in both cell lines exposed to LOWS and the A549 cell line exposed HOWS, which, however, also showed high radical formation in the ESR assay. The organic content could be very important in the potency of induction of DNA damage. Indeed, fractions of WSPM including

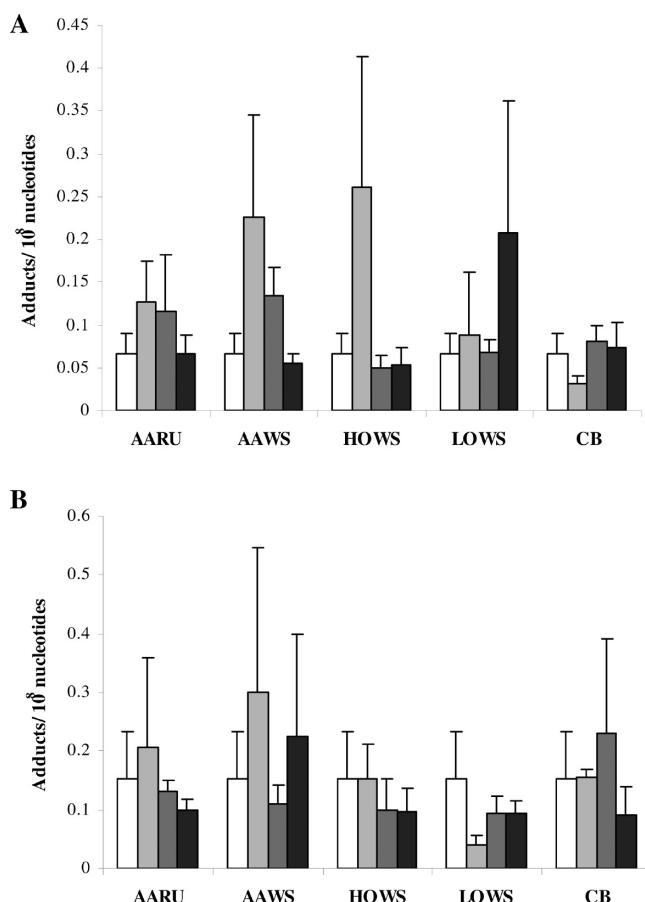


Figure 8. Bulky DNA adducts measured by ^{32}P -postlabeling in A549 (A) cells and THP-1 (B) cells after 24 h of exposure to 0 (light gray), 2.5 (white), 25 (dark gray), and 100 (black) $\mu\text{g/mL}$ PM. Each bar represents the mean \pm SE ($n = 3$). There were no significant differences between the PM preparations or effect compared to the control.

oxy-PAH and quinones contributed substantially to oxidative stress assessed as glutathione depletion in a macrophage cell line.⁴² Similarly, we showed previously that organic extracts of WSPM generated more SB than both native and washed PM.¹⁵ Additionally, it has been shown that PAH are capable of generating SB and guanine oxidation in A549 cells through ROS generation by the aldo-keto reductase pathway.⁴⁰ The measurement of oxidative damage to DNA is widely used as a biomarker in mononuclear blood cells from human populations exposed to air pollution,⁴³ and such responses in the monocyte THP-1 cell line is therefore relevant for comparison, although increased oxidative damage to DNA effects was not seen in humans exposed to wood smoke.⁴⁴

HPLC-MS/MS provides high sensitivity and specificity to the measurement of DNA lesions.⁴⁵ In accordance with the increased level of FPG sites in the comet assay, 8-oxodG was found to increase dose-dependently in A549 cells, although the data did not allow one to distinguish between the effects of the origin of the particles. We found unchanged expression of the OGG1 gene indicating that the protein responsible for the repair of 8-oxodG was not increased and thereby perturbing the results. The formation of 8-oxodG by ambient PM has mainly been investigated in calf thymus DNA or dG by HPLC-ECD or dot-blot, whereas induction of 8-oxodG in cell cultures mainly has been investigated indirectly by measuring the FPG sites in the comet assay.⁶ WSPM exposure has been shown to generate

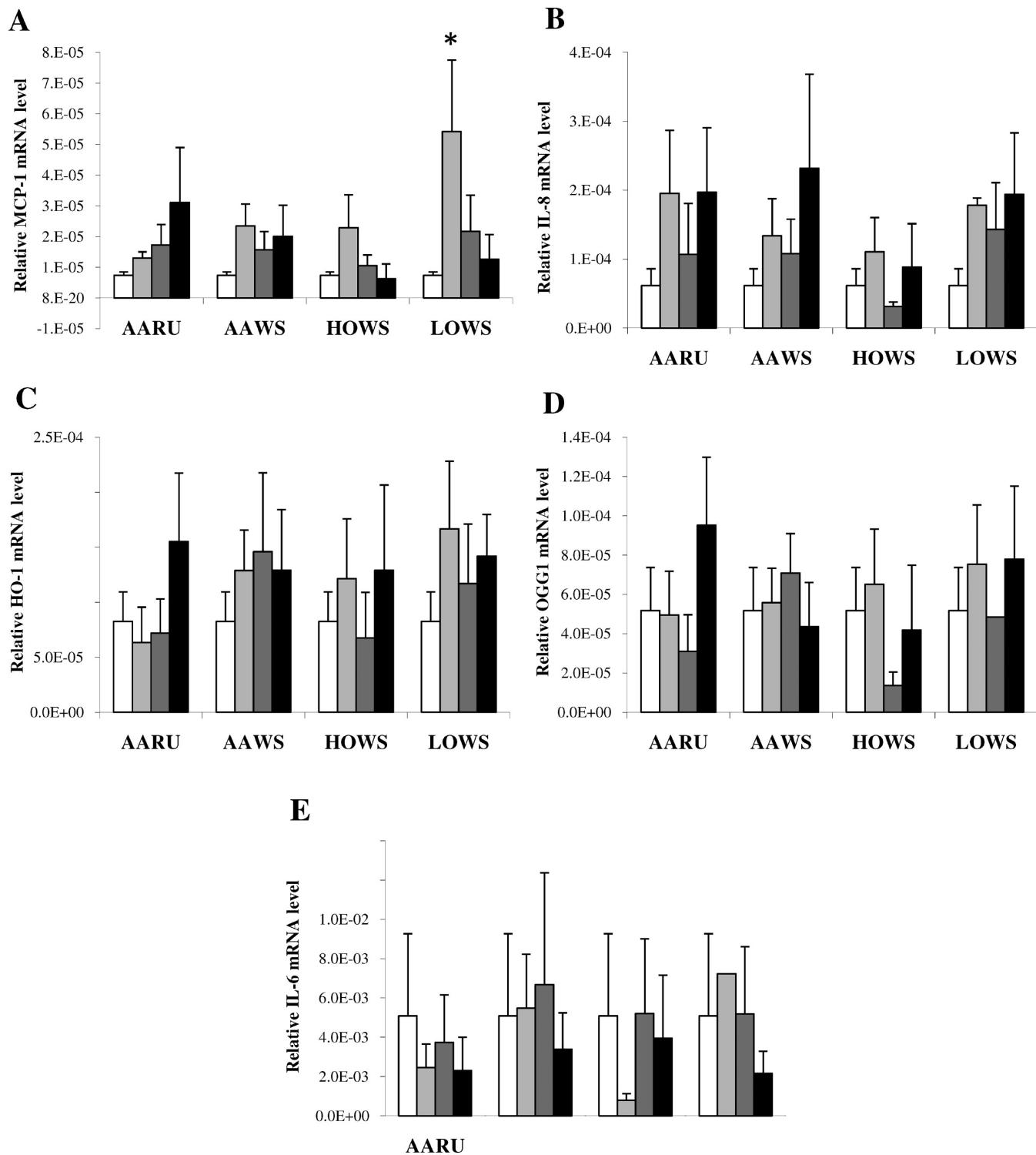


Figure 9. Gene expression in A549 cells after 3 h of exposure to 0 (light gray), 2.5 (white), 25 (dark gray), and 100 (black) $\mu\text{g}/\text{mL}$ PM. Each bar represents the mean \pm SE ($n = 3$). * statistically significant compared to the control ($p < 0.05$; posthoc LSD test).

8-oxodG from dG in solution³⁸ and FPG sites in cell cultures,¹⁵ but to our knowledge, the formation of 8-oxodG has never been investigated in cultured cells exposed to WSPM. We also measured the generation of etheno adducts because it previously has been shown that WSPM generated lipid peroxidation in RAW 264.7 cells.^{11,12} The edA and edG etheno adducts can be formed by reactions of DNA with the lipid

peroxidation-derived carbonyl compounds such as 4-hydroxy-2-nonenal.⁴⁶ To our knowledge, the formation of etheno adducts has never before been investigated in relation to general PM or WSPM exposure. Although we did not detect increased adduct levels in exposed A549 cells, it might be of interest to study etheno adducts in animals or humans exposed to PM, where inflammation is evident. Indeed, such adducts have been found in

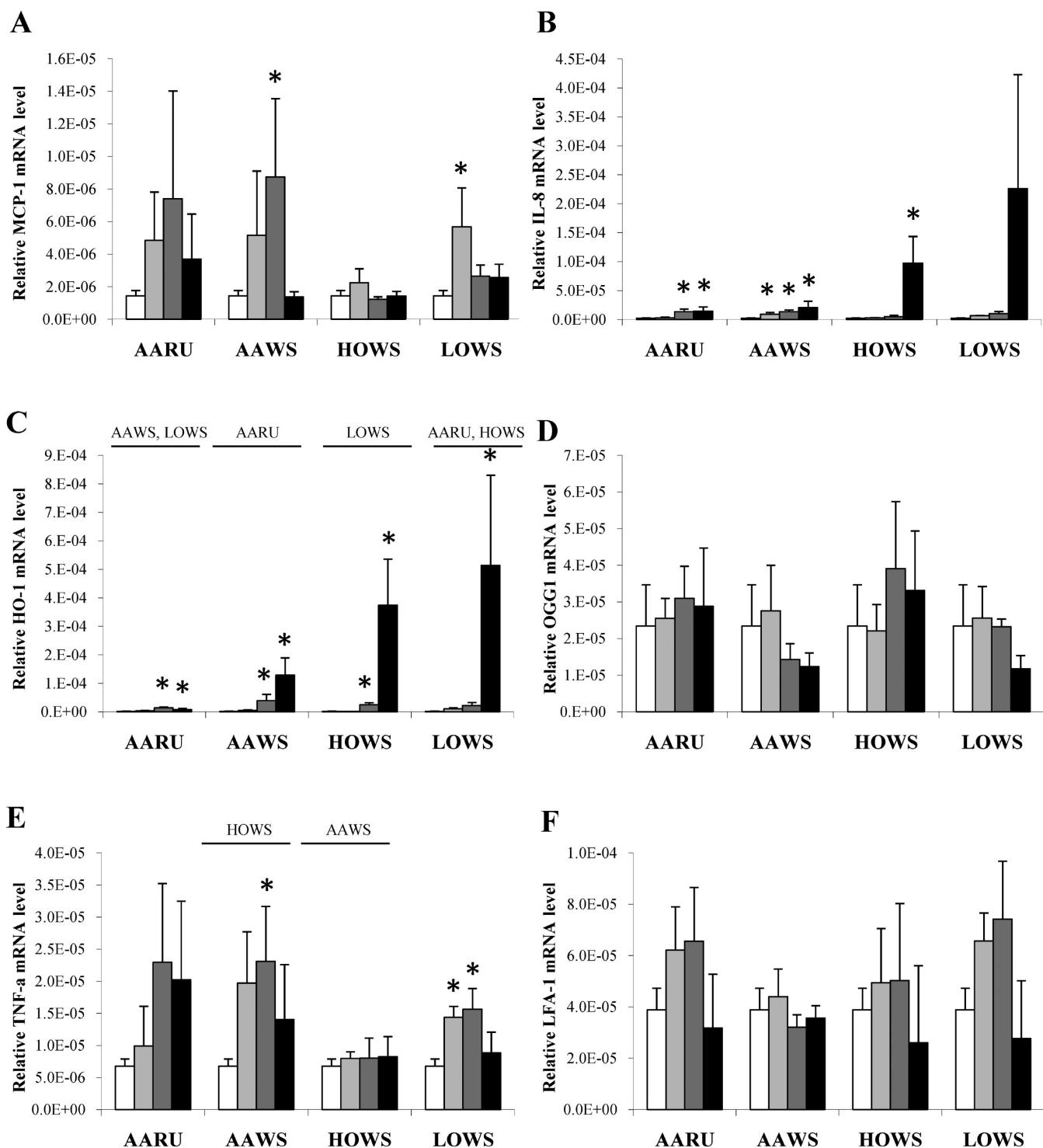


Figure 10. Gene expression in THP-1 cells after 3 h of exposure to 0 (light gray), 2.5 (white), 25 (dark gray), and 100 (black) $\mu\text{g}/\text{mL}$ PM. Each bar represents the mean \pm SE ($n = 3$). *, statistically significant compared to the control ($p < 0.05$; posthoc LSD test).

animal models of other types of inflammation and in inflammatory conditions in humans.^{47,48}

It has been estimated that residential wood combustion contributes more than 90% to the ambient level of PAH in Denmark.⁴⁹ In addition, high ambient levels of PAH have been found in residential areas with many wood stoves in Finland and Denmark.^{19,50} However, in our AAWS material the total level of PAH was slightly lower than that in AARU. The profile

of individual PAH in AARU and AAWS was similar to that of HOWS, whereas the profile was quite different in LOWS. Nevertheless, the PAH profile for LOWS with phenanthrene, anthracene, fluoranthene, and pyrene contributing more than 60% of the total mass is similar to what has been measured in PM from birch wood combustion.^{14,51} We only analyzed a limited set of PAH and the PAH concentrations in the collected PMs cannot be understood as accurate quantitative values. Sharma et al. have

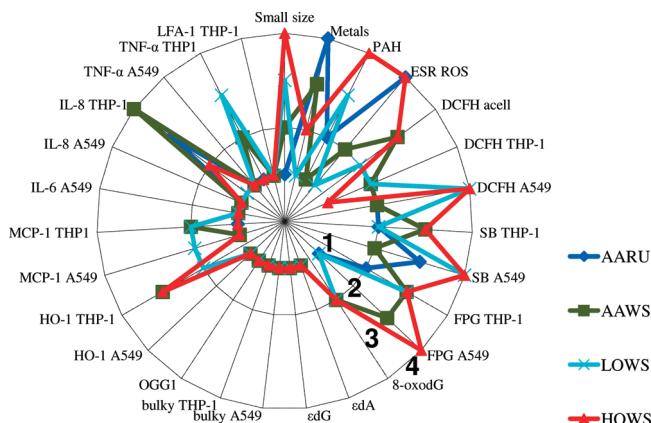


Figure 11. Summary of characteristics and effects in A549 and THP-1 cells. For small size, concentration of soluble metals, polycyclic aromatic hydrocarbons (PAH), and acellular formation of reactive oxygen species (ROS) determined by ESR and DCFH, the values 1–4 (from the center of the circle of the radar plot) indicate the rank order of the particles. For all other markers, the value 1 indicates no statistically significant difference from incubation without particles (control), whereas 2–4 indicate significant increase from the control at the high (2), medium (3), or low (4) exposure concentration.

shown that severe loss of, e.g., benzo[*a*]pyrene may occur within 24 h.¹⁷ Additionally, further PAH loss and degradation during lyophilization can also be expected. We, however, still believe that variations observed in the PAH profiles are real. Despite the relatively high levels of PAH especially in HOWS, we did not observe increased levels of bulky DNA adducts, in contrast to other studies using the extractable organic matter from air pollution PM in cell culture studies.^{52,53} To our knowledge, only one study has investigated the level of bulky DNA adducts in A549 cells exposed to native PM from ambient air, and that study showed only very low levels, which were not reliably quantifiable, even though significantly increased gene expression and activity of CYP1A1 were found.⁵⁴ PAH may be metabolically activated by P450 to reactive species, such as the diol-epoxides, that are able to form bulky adducts in the DNA,⁵⁵ and it is possible that our exposure time was too short to induce sufficient expression, especially in the THP-1 cells which express very limited CYP activity. It is also possible that intact PM exerts toxic effects through ROS that reduces the adduct formation, although this was not reflected in increased LDH release. When comparing extracts of industrial and urban samples with high and low PAH but comparable benzo[*a*]pyrene concentrations, the former induced mainly adducts, whereas the latter generated SB and no adducts.⁵⁶ Industrial and urban air samples were also compared in a study where the same sampler as that in the present study was used to collect PM samples.⁵⁷ In that study, there was no relationship between the PAH levels at the two industrial sites, urban street air and urban background air and the induction of DNA SB. PAH may also bind tightly to PM with limited bioavailability,⁵⁸ although the CB with the highest PAH concentration used in that study was able to induce quantifiable levels of bulky DNA adducts despite 2.5- and 4-fold lower levels than the WSPM samples in this study.

Our data showed statistically significant upregulation of expression of proinflammatory genes in terms of *IL-8*, *MCP-1*, and *TNF-α* as well as the oxidative stress response gene *HO-1* in THP-1 cells exposed to the particle preparations. We have found

earlier that 3 h is the optimum time point for such gene expression,⁵⁹ and we chose not to measure the cytokine proteins because the levels are affected by the presence of WSPM.⁶⁰ AAWS, HOWS, and in particular AARU contained endotoxin in levels sufficient to activate monocytes. However, AARU showed the lowest inflammatory response, and LOWS had no detectable endotoxin. In reasonable accordance with the main order of ROS production, AARU showed the lowest response in *HO-1* upregulation, while the expression of surface antigen *LFA-1* was not affected by PM. *MCP-1*, *TNF-α*, and *IL-8* were upregulated by the AAWS and LOWS samples, although *IL-8* failed to reach statistical significance for LOWS. The AARU and HOWS samples only upregulated *IL-8*. In contrast, the A549 cells showed only significant upregulation of *MCP-1* at one concentration of LOWS, and this could have been due to chance, although the same response was seen in the THP-1 cells. The limited response in A549 could be due to the fact that these cells showed levels of expression of those genes relative to 18S that were similar to the levels of exposed THP-1 cells without exposure, even though the RT-PCR method does not allow direct comparison. It is also possible that A549 cells require longer duration of exposure for full response. Previous results have shown that inflammatory potential, measured as *TNF-α* and *IL-8*, did not correlate well with genotoxicity in cell culture experiments, which might be because PAH influence the release of cytokines.^{13,14} However, in our study, the increased mRNA levels were reasonably associated with the increased level of DNA damage measured by the comet assay, where the WSPM samples showed the strongest response.

CONCLUSIONS

In conclusion, we found that PM material collected from ambient air in a small Danish village can have extensive contribution of particulates from the combustion of biomass. This material generated ROS and DNA damage in a dose-dependent manner that was stronger with respect to DNA damage than did PM collected from ambient air in a rural area without wood stove emissions. PM collected directly from wood smoke emission, in particular particles generated by combustion with high oxygen supply, was smaller in particle size, contained more PAH and less water-soluble metals, and produced more free radicals, DNA damage, as well as inflammatory and oxidative stress response gene expression in cell culture than did the rural PM material collected from ambient air without wood stoves. We found reasonable consistency between the ability of the PM preparation to induce ROS, DNA oxidation damage, and expression of inflammation and oxidative stress response genes, whereas the level of etheno and bulky DNA adducts was not increased. Our results suggest that WSPM are more toxic than rural background ambient air particulates per unit mass as measured by oxidative stress-associated damage, whereas ambient air particulates containing wood smoke-generated PM generate effects that were in between those of pure WSPM and rural background particulates.

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ABBREVIATIONS

8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; AARU, ambient air particles collected in a rural area; AAWS, ambient air particles collected in a wood stove rich area; CB, carbon black; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DLS, dynamic light scattering; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; edA, 1,N⁶-etheno-2'-deoxyadenosine; edG, 1-N²-etheno-2'-deoxyguanosine; FPG, formamidopyrimidine DNA glycosylase; HO-1, heme oxygenase-1; HOWS, high oxygen combustion wood smoke particles; IL-6, interleukin-6; IL-8, interleukin-8; LDH, lactate dehydrogenase; LFA-1, lymphocyte function-associated antigen 1; LOWS, low oxygen combustion wood smoke particles; MCP-1, monocyte chemoattractant protein-1; OGG1, oxoguanine glycosylase 1; PM, particulate matter; PAH, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; SB, strand breaks; TNF- α , tumor necrosis factor-alpha; WSPM, wood smoke particulate matter.

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