Science of the Total Environment 443 (2013) 256–266

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Pulmonary inflammation and tissue damage in the mouse lung after exposure to PM samples from biomass heating appliances of old and modern technologies

Mikko S. Happo [a,*], Oskari Uski [a,b], Pasi I. Jalava [a], Joachim Kelz [c], Thomas Brunner [c,d,e], Pasi Hakulinen [b], Jorma Mäki-Paakkanen [b], Veli-Matti Kosma [f], Jorma Jokiniemi [a,g], Ingwald Obernberger [c,d,e], Maija-Riitta Hirvonen [a,b]

[a] Department of Environmental Science, University of Eastern Finland, Kuopio, FI-70211, Finland
[b] Department of Environmental Health, National Institute for Health and Welfare, Kuopio, FI-70211, Finland
[c] BIOENERGY2020 + GmbH, Graz, A-8010, Austria
[d] Institut for Process and Particle Engineering, Graz University of Technology, Graz, A-8010, Austria
[e] BIOS BIOENERGIESYSTEME GmbH, Graz, A-8010, Austria
[f] Institute of Clinical Medicine, Pathology and Forensic Medicine, University of Eastern Finland and Kuopio University Hospital, FI-70211, Finland
[g] VTT Technical Research Centre of Finland, FI-02044, VTT, Finland

HIGHLIGHTS

- The highest particle concentrations were emitted from the old technology appliances.
- Almost all of the acute responses in mouse lungs caused by PM_1 were at the low level.
- Pellet- and woodchip boiler emissions induced moderate inflammation in mouse lungs.
- Inflammatory activity was associated with ash related components of the emissions.

ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 29 August 2012
Accepted 2 November 2012
Available online 28 November 2012

Keywords:
Small-scale wood combustion
Particulate matter
Cytotoxicity
Genotoxicity
Inflammation
Chemical composition

ABSTRACT

Current levels of ambient air fine particulate matter ($PM_{2.5}$) are associated with mortality and morbidity in urban populations worldwide. In residential areas wood combustion is one of the main sources of $PM_{2.5}$ emissions, especially during wintertime. However, the adverse health effects of particulate emissions from the modern heating appliances and fuels are poorly known. In this study, health related toxicological properties of PM_1 emissions from five modern and two old technology appliances were examined. The PM_1 samples were collected by using a Dekati® Gravimetric Impactor (DGI). The collected samples were weighed and extracted with methanol for chemical and toxicological analyses. Healthy C57BL/6J mice were intratracheally exposed to a single dose of 1, 3, 10 or 15 mg/kg of the particulate samples for 4, 18 or 24 h. Thereafter, the lungs were lavaged and bronchoalveolar lavage fluid (BALF) was assayed for indicators of inflammation, cytotoxicity and genotoxicity. Lungs of 24 h exposed mice were collected for inspection of pulmonary tissue damage. There were substantial differences in the combustion qualities of old and modern technology appliances. Modern technology appliances had the lowest PM_1 (mg/MJ) emissions, but they induced the highest inflammatory, cytotoxic and genotoxic activities. In contrast, old technology appliances had clearly the highest PM_1 (mg/MJ) emissions, but their effect in the mouse lungs were the lowest. Increased inflammatory activity was associated with ash related components of the emissions, whereas high PAH concentrations were correlating with the smallest detected responses, possibly due to their immunosuppressive effect.

1. Introduction

The current levels of ambient air fine particulate matter ($PM_{2.5}$; particle diameter (D_p)<2.5 μm) are associated with mortality and morbidity in urban areas, especially in susceptible population groups (WHO, 2003, 2005). In residential areas, wood combustion is one of the main sources of $PM_{2.5}$ emissions, especially during wintertime. The influence of residential wood combustion on local air quality has been found to be comparable to the contribution of local traffic on busy streets (Glasius et al., 2006). On the other hand, climate change is linked to the CO_2 emissions from traffic and fossil fuel based energy production that are also substantial contributors to total $PM_{2.5}$ concentrations in European countries. Therefore, the EU has decreed to increase the share of renewable energy to 20% of the total energy

* Corresponding author at: University of Eastern Finland, Department of Environmental Science, P.O. Box 1627, FI-70211, Kuopio, Finland. Tel.: +358 40 482 2759.
E-mail address: mikko.happo@uef.fi (M.S. Happo).

0048-9697/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scitotenv.2012.11.004

consumption by 2020, which may also increase the small-scale biomass combustion in local heating systems. Thus, there is an urgent need for extensive development of new technologies for heating appliances and fuel types. In order to minimize the health risks and to develop safe small-scale biomass heating technologies, manufacturers need to know the harmful toxicological characteristics of the particulate emissions of their appliances and their relationship to physicochemical characteristics. However, the toxicological properties of particulate emissions from the modern heating appliances and fuels are poorly understood and the available toxicological data is highly limited.

The size-distribution of particulate emissions from combustion processes consists mostly of particles smaller than 1 μm of aerodynamic diameter. These particles are usually deposited in the tracheobronchial and alveolar regions of the respiratory tract (Schwarze et al., 2006). The ultrafine particles can even penetrate through the lung tissue and reach the capillary blood vessel and circulating cells and subsequently other target organs, such as liver and brain (Oberdörster et al., 2004). Particulate emissions from combustion derived sources, like local oil combustion and vehicle engines, are associated with increased inflammatory activity (Happo et al., 2008; Jalava et al., 2009; Steerenberg et al., 2006). It has been proposed that particulate induced inflammation is the main contributor to disease exacerbations in both respiratory and cardiovascular subjects (Pope and Dockery, 2006). In contrast to oil combustion and traffic related sources, products from incomplete small-scale wood and coal combustion are associated with low or moderate inflammatory activity (Happo et al., 2008; Jalava et al., 2009; Steerenberg et al., 2006; Seagrave et al., 2006). In addition, hardwood smoke has been shown to cause only mild toxic effects in the rats and mice in subchronic experiments (Reed et al., 2006). However, incomplete combustion emissions have significantly high concentrations of polycyclic hydrocarbons (PAHs) that are associated with immunosuppressive effects such as decreases in the cytokine production (Kong et al., 1994). It has been recently shown that technology and operational practice in wood combustion, such as air supply, fuel type and load, affect the chemical characteristics of particulate emissions (Brunner and Obernberger, 2009; Tissari et al., 2008) and subsequently modify the induced toxicological responses in immunological cells in vitro (Jalava et al., 2010, 2012). At present, there is a need to confirm the results from those in vitro studies with animal model since the acute pulmonary effects of wood combustion emissions in the experimental animals are poorly understood.

In this study, the overall aim was to examine the acute phase responses and evaluate the toxicological properties of small-scale biomass heating appliances particulate emission by using an animal model. Several markers of inflammation, cytotoxicity and genotoxicity as well as histopathological changes were investigated in lung tissue which had been exposed to PM_1 samples obtained from different heating appliances representing both old and modern technologies. In addition, the relationships between PM chemical composition and toxicological characteristics were evaluated. The following small-scale combustion appliances were evaluated: logwood boiler (old technology), logwood boiler (modern technology), stove (old technology), stove (modern technology), tiled stove (modern technology), woodchip boiler (modern technology), and pellet boiler (modern technology). All of these devices are commonly used in Central European countries.

2. Methods

2.1. The heating appliances

The modern technology logwood boiler (Log NT) was based on downdraught combustion technology. It was equipped with an automated boiler cleaning system and with an automated combustion control based on a λ-control. The old technology logwood boiler (Log OT) was a typical under-fire boiler. In this system, the combustion regulation was based on a thermo-mechanic combustion air control by a primary air flap. Both logwood boilers were manually fed and the ash removal was also done manually.

The modern technology stove (Stove NT) consisted of a primary and a secondary combustion zone. The air supply came via primary and window purge air during ignition, but only through the window purge air during main combustion and burnout phases. The air supply control was manually adjusted. The old technology stove (Stove OT) consisted of only one burning chamber. Combustion air was divided into primary air through the grate, window purge air as well as secondary air injected through nozzles from the back of the combustion chamber. The air distribution was adjusted manually by a damper. The tiled stove (Tiled ST) was designed according to the current guidelines of the Austrian Tiled Stove Association. The air supply came through a vertical grate positioned in the stove door. While the investigated boiler systems were equipped with flue gas fans, the stoves represented typical natural draught systems.

The modern technology pellet boiler as well as the woodchip boiler were equipped with automatic ignition systems, staged combustion, automated boiler cleaning systems as well as automated de-ashing systems. The pellet boiler was equipped with an overfed burner, a water cooled combustion chamber as well as a combustion control which was based on the measurement of the furnace temperature. The woodchip boiler consisted of an underfeed stoker combustion system and a water cooled combustion chamber. Flue gas temperature control and λ-control were utilized to achieve combustion control.

The furnaces and detailed information about the combustion cycles and the related emissions have been reported by Kelz et al. (2010), Brunner and Obernberger (2009), and by Jalava et al. (2012). The test runs performed were based on simulated whole day operation cycles and, thus, takes also transient operation phases into account. Before the test runs, typical whole day load cycles for furnaces were evaluated from field measurement data in order to define a representative operation cycle. Wood pellets according to ÖNORM M 7135, wood chips according to ÖNORM M 7133 as well as logwood according to ÖNORM M 7132 and ÖNORM CEN/TS 14961 were used as fuels (Austrian Standards Institute). The fuels applied during the test runs are representative for the respective fuel category (Kelz et al., 2010).

2.2. Sample collection for toxicological analyses

Particulate samples for toxicological analyses were collected by using previously validated methods for toxicological and chemical analyses (Lamberg et al., 2011). The filters (Fluoropore PTFE filters, Millipore Corp., Billerica, MA, USA) were washed twice with methanol and weighed before and after sampling, as well as after methanol extraction with an analytical balance (Mettler Toledo XP105DR, Mettler-Toledo Inc., Columbus, OH) equipped with a built-in electrostatic charge remover. The effects of surrounding temperature, pressure and humidity were corrected using control substrates and appropriate conditioning times (24 h, $+21.9 \pm 0.9$ °C, relative humidity 37 ± 14%, barometric pressure 1000.7 ± 11 hPa). All of the weighings were performed in the same laboratory located in Kuopio, Finland. Sets of five consecutive stages were prepared for the sample collections and shipped to Graz, Austria.

All the test runs for sample collections were performed at a test stand at BIOENERGY 2020+ GmbH, Austria. PM_1 was collected from diluted flue gas of each heating appliance on PTFE-filters (Millipore) with Dekati® Gravimetric Impactor (DGI, Dekati Ltd., Tampere, Finland). Blank control substrates were treated equally with other substrates. The used sample collection system consists of a heated cyclone (cut diameter: 10 μm), a porous tube diluter (PRD), a gravimetric impactor and a pump (Ruusunen et al., 2011). Mass flow controllers were used to control the flow rates of the pre-cleaned particle free dilution air and the diluted flue gas. The temperature of the diluted flue gas was measured with Pt100

temperature sensors. The DGI itself had four impaction stages with cut diameters of 2.5, 1, 0.5 and 0.2 μm as well as a backup filter (<0.2 μm). After the sample collection, the filter sets were transported from Austria to Finland in dry ice, where the PM samples were prepared for chemical and toxicological analyses. Prior to the re-weighing of the filters, the samples were allowed to acclimatize 24 h. Three stages of each DGI set were weighed to form a PM_1 sample.

The PM_1 samples were extracted from filters by using methanol extraction method, which is described in detail by Jalava et al. (2005). The filters were cut into a few pieces and placed into a 50 ml glass tube that was filled with methanol. Thereafter, the samples were treated in a water bath sonicator (FinnSonic m20, FinnSonic Oy, Lahti, Finland) for 2×30 min at +20 °C. The PM_1 samples of each appliance were pooled and the excess methanol was evaporated in a rotary evaporator (Heidolph laborota 4000) that was attached to a controlled vacuum pump (Vacuubrand) and chiller (Lauda WK500). Subsequently, the concentrated suspension was divided into 10 ml glass tubes as defined amounts of particulate mass and dried under nitrogen (99.5%) flow and stored at −20 °C. A similar procedure was used in the preparation of the corresponding blanks. The extraction efficiency was calculated by comparing masses of filters before and after methanol extraction to the weight measured before sample collection. The average extraction efficiency of the samples was 95.9% (range: 90.0–99.7%).

In the animal exposures, the dry particulate samples and blanks were thawed and stabilized to room conditions for 30 min. Thereafter, 32 μl of dimethyl sulfoxide (DMSO) (Uvasol®, Merck KGaA, Darmstadt, Germany) was added to 10 mg of particulate mass or to the corresponding blank sample and the sample was suspended by mixing with a glass rod. Then, 968 μl of pathogen-free water was added and the sample was sonicated for 30 min in a water-bath sonicator. The suspension was diluted in pathogen-free water to obtain final concentrations of 0.5, 1.5, 5 and 7.5 mg/ml to be used in the animal exposures. The blank sample was diluted in an equal volume of pathogen-free water to ensure that the vehicle of particulate suspension and possible impurities in methanol extraction were not the sources of the toxic activity.

2.3. Chemical analyses

Chemical characterization of the fuel was applied by wet chemical analyses. The moisture content of the fuel was determined by drying it at 105 °C. The ash content of the fuel was analyzed according to the standard procedure prCEN/TS 14775 (determination of the loss of ignition at +550 °C). The determination of the chemical composition (Si, Ca, Mg, Mn, K, Na, Zn, S) of DGI samples was done by pressurized multi-step digestion of the samples with $HNO_3/HF/H_3BO_3$ by Paar Multiwave 3000 (Anton Paar GmbH, Graz, Austria) before the element detection with ICP-OES or ICP-MS. The Cl concentration was measured by bomb combustion in oxygen and absorption in NaOH by using ion chromatography (ICS 90 Dionex).

The contents of different carbon compounds including organic carbon (OC), elemental carbon (EC) and inorganic carbon (IC) in aerosol samples were determined with a carbon/hydrogen analyzer (LECO RC-612). The sample was inserted into a quartz tube that was heated to defined temperatures. In this study, we applied different temperature ramps from ambient temperature up to +950 °C. Carbon containing compounds released from the sample were oxidized to CO_2, which was selectively detected by infrared cells. By choosing appropriate temperatures and carrier gases in the quartz tube, total carbon (TC) as well as the fractions OC, EC and IC could be distinguished. Carbon released in a temperature window from 200 to 600 °C under an inert atmosphere was assigned as OC, carbon released between 600 and 900 °C was considered as IC, carbon detected after switching to oxidizing conditions was defined as EC. A total of 30 polycyclic aromatic hydrocarbons were analyzed by using a gas chromatograph and a mass selective detector (6890N GC-5973 INERT MSD, Agilent Technologies) after extraction of the particulate samples with dichloromethane (Lamberg et al., 2011).

2.4. Animals

Pathogen-free male C57BL/6J mice 8 to 9 week-old (weight 22.3 ± 1.6 g, SE 0.09 g) were used in the studies (n = 6 mice/group). The animals were obtained from the Laboratory Animal Center of the University of Eastern Finland. They were transferred from the barrier unit to a conventional animal room two weeks before the experiments. After a one-week acclimatization period, the animals were transferred into metal cages and they were housed singly on aspen wood chips and had access to water and maintenance diet ad libitum. The animals were kept on a 12-hour light cycle at room temperature (22 ± 1 °C and humidity 55 ± 15%).

2.5. Experimental design

A well established mouse-model was used in animal experiments. National Animal Experiment Board (Eläinkoelautakunta ELLA) approved all present experiments and they were carried out in accordance with EU Directive, 2010/63/EU for animal experiments.

At the first part of the study, dose–response screenings of two particulate samples at two different time-points were examined. The logwood boiler (old technology) sample was selected on the basis of in vitro results (Jalava et al., 2012) with the same samples, which revealed that it possessed the highest inflammatory and cytotoxic activity of the tested heating appliance emission particles. The woodchip boiler sample was selected as a comparative point of view between old and new technologies, and because of the relatively low toxic responses observed in vitro. The following control groups were used 1) untreated mice, 2) mice intratracheally exposed with pathogen-free water (Sigma, W1503) as a carrier control, 3) mice exposed to solutions extracted from blank filters as a negative control in statistical analyses, 4) diesel PM_1 sample (EURO II non-road engine), 5) $PM_{10–2.5}$ Athens urban air sample (GCO) (Pennanen et al., 2007) and 6) LPS (lipopolysaccharides from *Escherichia coli* (0111:B4), Sigma L2630-10MG lot. 030M41114) as a methodological control (40 μg/animal). The mice were exposed to a single dose of particulate samples via intratracheal aspiration (50 μl/animal), using 1, 3, 10 and 15 mg/kg doses. Bronchoalveolar lavage fluids (BALF) from the mice were collected at 4 h and 18 h after the exposure.

In the second part of the study, all of the heating appliance PM samples were tested in a comparative way. Mice were exposed to 10 mg/kg sample and BALF was collected 4 h and 18 h after the treatment. The optimal time point of response recording was used in the statistical analyses to reveal associations between inflammatory responses and chemical constituents of PM samples.

In the third part of the study, the lungs of mice were dissected 24 h after the treatment with emission particles at a dose of 10 mg/kg; the tissue was then subjected to histopathological examination.

2.6. Intratracheal aspiration

The mice were anesthetized with vaporized 4.5% sevoflurane (Abbott, IL, USA) and placed in a 66° upward bent position with incisors placed on a thin wire. The administration of particles was performed under visual control with the tongue pulled out with forceps to prevent the mouse from swallowing. The sample was delivered onto the vocal folds with a Finn pipette tip. The nostrils were covered forcing the mouse to inspire the particle suspension.

2.7. Bronchoalveolar lavage

The mice were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), and thereafter, they were exsanguinated by cardiac puncture. The lungs were cannulated with polyethylene tubing and lungs were lavaged with two portions of sterile saline (30 ml/kg), three times each. These two portions of BALF were combined and kept on ice. LDH and total protein concentrations were analyzed from fresh supernatant, while the remaining portion was frozen and stored (−80 °C) for subsequent cytokine analyses.

2.8. Cell count and cell differential

Cells were separated from the BALF by centrifugation (500 *g*, 10 min) and the supernatant was removed for separate analyses. The separated cell pellet was resuspended into 220 μl of sterile saline for cell counting. Total cell number and share of dead cells was microscopically counted from each pellet by using a Bürker chamber and trypan blue exclusion method.

The remaining cell suspension was used for differential counting of cells by cytospin (210 μl, 500 rpm, 8 min; Megafuge, Heraus Instruments, Germany). Slides were fixed with May-Grünwald–Giemsa dye. For cell differential, at least 300 cells (63×) were counted from each stained cytospin slide by using a light microscope (Zeiss Axio Observer Z1). The mean percentages for macrophages, neutrophils, lymphocytes and other types of white blood cells were calculated.

2.9. Biochemical and immunochemical analyses

Lactate dehydrogenase (LDH) and protein concentration were analyzed from fresh supernatants. LDH was analyzed by using a cytotoxicity detection kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Total protein was analyzed by using a DC Protein Assay (Bio-Rad, Hercules, California, USA). Concentrations of LDH and total proteins were spectrophotometrically measured from 96-well plates at wavelengths of 492 nm and 690 nm, respectively (PerkinElmer Victor^3).

Interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) concentrations were analyzed from BALF supernatants of the animals used in the dose response screening. Cytokine analyses were made with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cytokine concentrations were spectrophotometrically measured from 96-well plates at a wavelength of 450 nm (PerkinElmer Victor^3).

2.10. Genotoxicity analysis

The single cell gel (SCG)/Comet assay was used to detect DNA damage in the cells present in the bronchoalveolar lavage fluid (BALF). The alkaline version of the Comet assay was performed according to the procedure originally described by Singh et al. (1988). The slightly modified Comet analysis (Jalava et al., 2010) was done in ethidium bromide-stained slides (100 cells from each animal) using the Comet assay IV (Perceptive Instruments Ltd., UK) image analysis system. The comet response parameter used in the statistical analysis was the Olive Tail Moment (OTM) [(tail mean − head mean) × tail%DNA / 100].

2.11. Histopathological analyses

The lungs of the animals that were not lavaged in BALF collections were used for histopathological examination. Lungs were removed and filled with 10% phosphate buffered formalin, which was also used in the preservation of the tissue samples. Thereafter, the lungs were trimmed and embedded in paraffin and cut at 5 μm sections. After cutting, the tissues were stained with hematoxylin and eosin for the subsequent examination. Sections of both left and right lungs were examined under a light microscope. Lesions were semi-quantitatively scored as follows: 0 = absent, 1 = minimal, 2 = slight, 3 = moderate and 4 = marked. The same scoring system was applied to the particulate matter accumulation in the lumen of bronchi and/or peribronchial area and/or in alveoli. All tissue samples were analyzed and scored by the same, experienced pathologist.

2.12. Statistical methods

All the measured values were first analyzed with Levene's test for equality of variances. Statistical differences in the measured BALF parameters between the particulate sample-treated animals and blank sample-treated control animals were determined with the analysis of variance (ANOVA) and Dunnett's test. In cases where Levene's test gave values <.05, the Kruskal–Wallis test was used. The differences in data were regarded as statistically significant at $p<.05$. Differences between the heating appliances were tested by Tukey's HSD or Dunnett's C test. The extent of histopathological lesions in the mouse lungs was tested for statistical significance by using two-tailed Mann–Whitney test ($p<.05$). Results of the Comet assay were analyzed using Student's *t*-test ($p<.05$). All the measured values were analyzed with Spearman's rank correlation (two-tailed) to examine out the linear relationships between the variables. Correlation coefficients (ρ) between the different variables were regarded as statistically significant at $p<.05$ level. The optimal time points for toxicity markers (4 h for comet assay and 18 h for cell differentials and total protein) were used in the statistical analyses. All the data were analyzed using the SPSS statistics version 17.0 (SPSS, Inc., Chicago, IL) or IBM SPSS statistics 19.0 (IBM®, New York, NY).

3. Results

3.1. Emissions from the tested boilers and stoves

The emission data revealed that the combustion quality was better in modern automated biomass boilers (pellet, woodchip and logwood boilers), than in modern natural draught stoves and tiled stoves. The worst combustion quality was seen in the old technology stoves and logwood boilers. As an example, the average CO emissions over the whole operation cycle increased from the values of pellet boiler (0.047 g/MJ) over the modern logwood stove and the tiled stove (around 1 g/MJ) and the old technology logwood stove (2.4 g/MJ) up to the old technology logwood boiler, which showed exceptionally high emissions of more than 12.6 g/MJ (see Table 1). A clear correlation between the average CO and organic gaseous compound (OGC) emissions could be detected ($\rho = 1.000$). Moreover, also the average PM_1 emissions ranging from around 6 mg/MJ for the pellet boiler to about 106 mg/MJ for the old technology logwood boiler correlated well with the OGC ($\rho = 1.000$) and CO emissions ($\rho = 1.000$). As shown in the chemical analyses of PM_1 samples taken during the test runs (Kelz et al., 2010), the concentrations of organic carbon and soot in the emission aerosol increase with decreasing burnout quality and consequently, the higher PM_1 emissions less efficient combustion situations can be attributed to the formation of carbonaceous aerosols. Moreover, it was seen that particle bound PAH emissions also correlate with the OGC ($\rho = 0.893$) and CO ($\rho = 0.893$) emissions and consequently with the burnout quality. The chemical composition of the particulate samples emitted by the heating devices has been presented in Jalava et al. (2012).

3.2. Dose dependences of particulate responses

The total cell numbers in BALF at 4 h and 18 h after exposure to PM_1 from logwood boiler (OT) and woodchip boiler were not significantly increased at any of the doses used, when compared to the

Table 1
Nominal heat outputs, mean values for gaseous and PM_1 emissions and detected PAH concentrations for the different investigated biomass combustion systems. OGC = organic gaseous compounds.

Combustion system	Nominal heat output (kW)	O_2 vol.%	CO mg/MJ	OGC mg/MJ	PM_1 mg/MJ	Sum of PAHs ng mg^{-1}
Logwood boiler OT	15	11	**12,632**	**1143**	**106**	13,776
Logwood boiler NT	**30**	**9**	793	62	18	1796
Stove OT	7	11	2355	223	74	**35,810**
Stove NT	6	12	1035	96	46	2496
Tiled stove	**4**	**15**	1007	69	28	989
Woodchip boiler	21	12	182	5	14	**143**
Pellet boiler	**30**	13	**47**	**3**	**6**	800

Note. The largest and smallest values are in bold.

control level (data not shown). Moreover, no differences between blank control, untreated animal and carrier control groups were detected. With respect to the positive controls, GCO and LPS increased greatly the total cell number 18 h after the exposure, whereas no such effect was detected with the diesel sample.

No increases in BALF total protein concentrations were observed after exposure to 1 mg/kg or 3 mg/kg doses. Only the woodchip boiler samples increased the total protein concentration at the higher doses, 10 mg/kg and 15 mg/kg. Diesel and GCO of the positive controls showed relatively small increases in the total protein concentration at both of the time points used, whereas LPS induced the strongest, over 3-fold response 18 h after the exposure, when compared to the blank sample (data not shown). No differences were detected between the control samples. Particulate samples from both old technology logwood boiler and woodchip boiler induced inconsistent changes in LDH with all of the doses examined. However, statistically significant increases in the LDH concentration were found only from the BALF of animals exposed for 18 h to woodchip boiler derived particulate samples.

The dose-dependences of IL-6 at 4 h and 18 h after exposure to PM_1 from logwood boiler (OT) and woodchip boiler are shown in Fig. 1. The IL-6 concentrations remained at the control level with the lowest examined doses (1 and 3 mg/kg). The highest dose used (15 mg/kg) of woodchip boiler sample increased statistically significantly the IL-6 concentration in the mouse lungs at 4 h after the treatment. At 18 h after the exposure, both 10 mg/kg and 15 mg/kg doses had increased BALF IL-6 concentration significantly. In contrast, the responses to logwood boiler (OT) samples were minimal at both time points. LPS induced a major increase in IL-6 production at both used time points but GCO only at 4 h after the exposure (data not shown). The diesel particulate sample exhibited a slight increase in the IL-6 concentration at 4 h after treatment, but no increase was observed after 18 h. No consistent MIP-2 responses were detected at any of the used particulate doses (data not shown).

3.3. Comparison of emission toxicities from different heating appliances

The dose of 10 mg/kg was selected for use in the comparative part of the study, because it displayed at least some statistically significant increases in the responses in the dose dependence study.

3.3.1. BALF analyses

Overall, the responses in total cell number were relatively small and all of the samples induced their highest increases 18 h after the exposure. Nevertheless, in agreement with the dose dependence study, no statistically significant change in the total number of cells in BALF was observed with any of the particulate samples, when compared to the corresponding blank control. However, there were statistically significant differences on macrophage and lymphocyte number in BALF, caused by the PM_1 samples from different wood combustion systems (Fig. 2). Macrophages were clearly the dominating cell type in BALF with all of the samples used at 4 h after the exposure. At the 18 h time point, the number of macrophages was slightly increased in all of the samples. However, with the pellet boiler sample, the number of macrophages was statistically significantly lower than the control level. This was largely due to cell death, which was also observed microscopically by trypan blue exclusion method (data not shown). Only few neutrophils were seen at 4 h after the exposure, but their number was clearly increased from the control level at 18 h after the exposure, especially with the modern technology heater samples. However, the detected increases were not found to be statistically significant. The number of lymphocytes was low at both of the time points. Nevertheless, stove (OT) sample

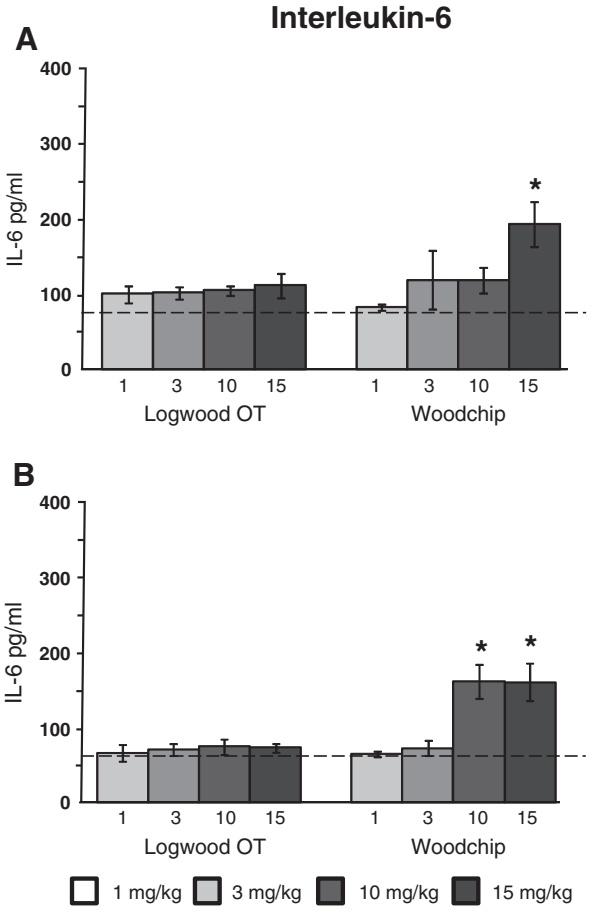

Fig. 1. Interleukin-6 concentration in BALF at 4 h (A) and 18 h (B) after intratracheal instillation of a single dose (1, 3, 10 or 15 mg/kg) of particles (PM_1) from heating appliances or an instillation of a corresponding blank sample in healthy C57BL/6J mice. Each bar shows mean ± SE (n = 4–6). An asterisk indicates a statistically significant difference from the blank control (Dunnett's C test, $p<0.05$). Dash line represents the control level.

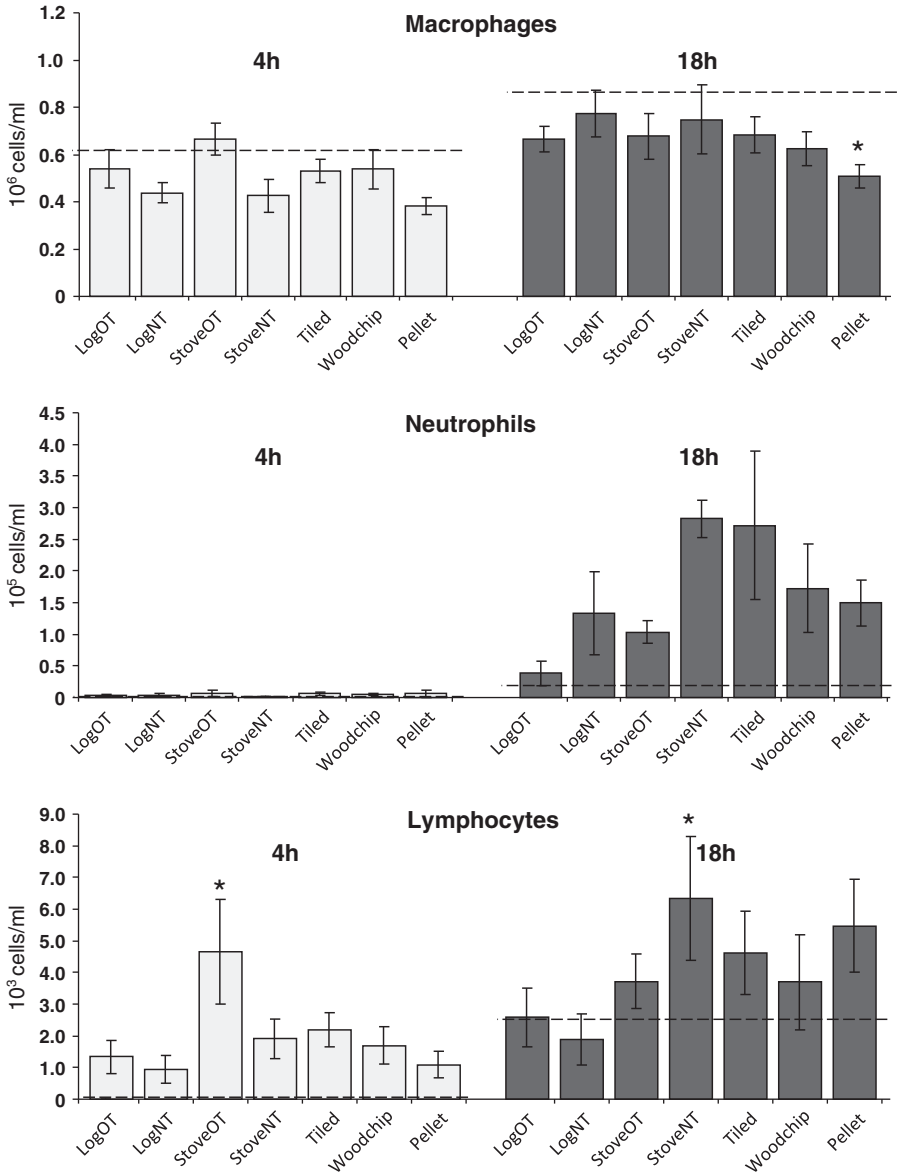

Fig. 2. Number of macrophages, neutrophils and lymphocytes in BALF from healthy C57BL/6J mice (n = 5–6) 4 (A) and 18 h (B) after intratracheal instillation exposure to a single dose of particulate samples (10 mg/kg). Each bar shows mean ± SE (n = 5–6). An asterisk indicates a statistically significant difference from the blank control (Dunnett's C test, $p < 0.05$). Dash line represents the control level.

was found to increase lymphocyte number in BALF statistically significantly at 4 h and stove (NT) sample at 18 h after the exposure. Of the other cell types, only individual eosinophils were detected from BALF.

Total protein concentrations in BALF 18 h after exposure to PM_1 samples from different heating appliances are shown in Fig. 3A. There were no statistically significant differences between samples and control level 4 h after the exposure. In contrast, a statistically significantly higher total protein concentration was measured with pellet boiler sample 18 h after the exposure, when compared to the blank or any of the other particulate samples. No consistent differences in LDH concentrations between the responses evoked by PM_1 samples or the corresponding blank sample were found.

Fig. 3B shows DNA damage as expressed via the olive tail moment (OTM) in the BALF cells of mice exposed to PM_1 samples obtained from the heating appliances at 4 h after exposure. All of the emission particle samples induced their highest responses 4 h after the exposure. Woodchip boiler ($p = 0.063$) and pellet boiler ($p = 0.004$) revealed an over 2-fold increase in DNA damage from the control level, whereas other samples had a lesser effect. However, no signs of genotoxic activity were measured at the latest 18 h time point.

3.3.2. Histopathological analyses

Only mild pathological changes in the lungs of mice were observed 24 h after intratracheal aspiration of 10 mg/kg of PM_1 from different heating appliances (Table 2). The detected pathological changes in the mouse lungs consisted mostly of granulocytes in the lumen of bronchi, and/or peribronchially, and/or in alveoli (Fig. 4). In this study, only one single mouse that had been exposed to stove (NT) sample, exhibited moderate inflammatory changes in the lungs. In most of the cases, only minimal or slight focal inflammatory changes were found in the lung tissue. Instead, the animals exposed to stove (OT) and tiled stove samples displayed statistically significantly more changes in their lung tissues than the negative control animals.

Emission particles (10 mg/kg dose) from both of the old technology systems were found to statistically significantly increase accumulation of particulate matter into inflammatory cells in the mouse

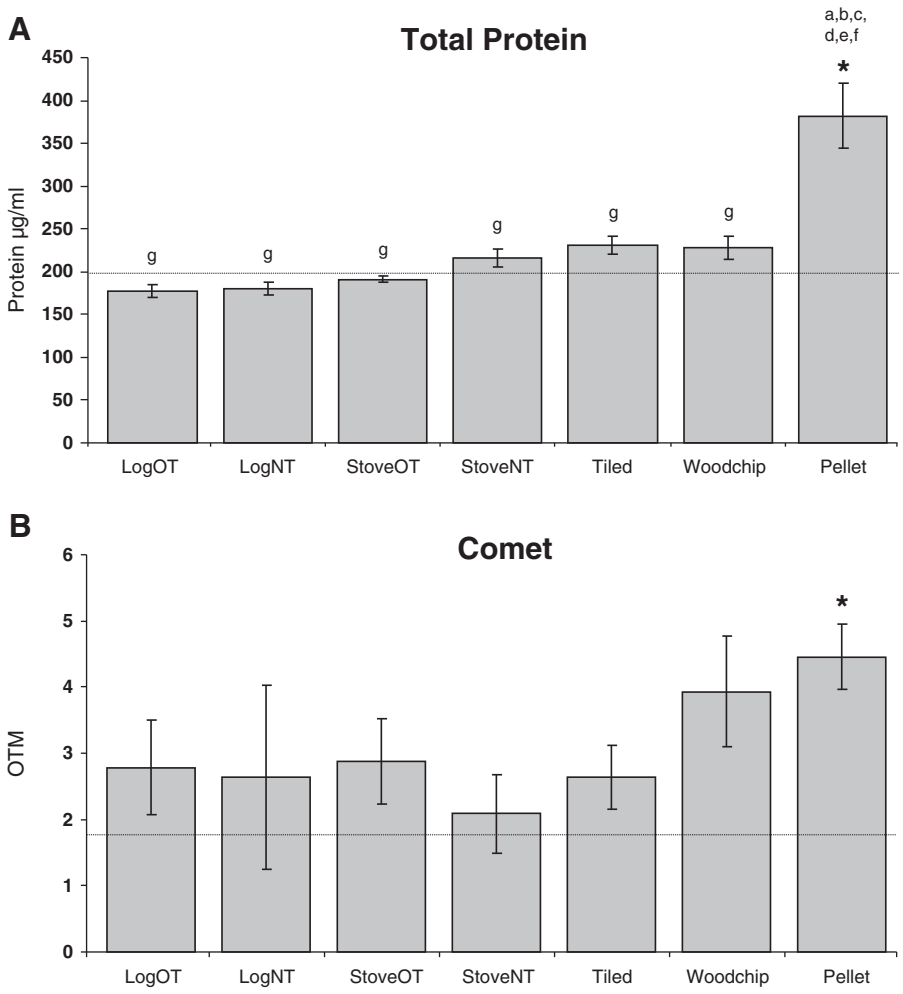

Fig. 3. Total protein concentration in BALF (A) at 18 h after intratracheal instillation of single dose (10 mg/kg) of particles (PM_1) from heating appliances or an instillation of a corresponding blank sample in healthy C57BL/6J mice. Each bar shows mean ± SE (n = 5–6). An asterisk indicates a statistically significant difference from the blank control (Dunnett's C test, $p<0.05$). Letters from a, b, c, d, e, f and g indicate statistically significant differences from the responses to other emission samples (Tukey-HSD test, $p<0.05$). Subpanel B shows the genotoxic responses (mean OTM values ± SEM) in BALF (n = 5–6, 100 OTM values were analyzed/animal) 4 h after the exposure of the same samples. An asterisk indicates a statistically significant difference to control mice ($p<0.05$, Student's *t*-test). Dash line represents the control level.

lungs. With respect to the new technology heating systems, stove NT, tiled stove and woodchip boiler had a similar outcome. Instead, emission particles from the pellet boiler and logwood boiler NT were not found to be focally accumulated in the lumen of bronchi, and/or peribronchially, and/or in alveoli. When the BALF cells were investigated with the cytospin method, the accumulation of particles into the macrophages was seen mostly with the samples originating from stove NT (Fig. 4), tiled stove and woodchip boiler at 18 h after the exposure.

3.4. Effects of chemical composition

The coefficients for correlations of the selected detected chemical constituents and sum of PAH-compounds of PM_1 with the measured

Table 2
Summary of pulmonary lesions in the mouse lungs after 24 h single dose (n = 5–6) exposure to the particle samples (10 mg/kg) from different heating appliances or corresponding blank sample. Untreated animal and a diesel sample were used as negative and positive controls.

		Untreated	Blank	Diesel	LogOT	LogNT	StoveOT	StoveNT	Tiled	Woodchip	Pellet
Inflammatory changes	None (0)	6	3	1	2	3	–	–	–	1	–
	Minimal (1)	–	3	2	3	3	**2**	4	**3**	2	4
	Slight (2)	–	–	3	–	–	**4**	1	**3**	3	1
	Moderate (3)	–	–	–	–	–	–	1	–	–	–
	Marked (4)	–	–	–	–	–	–	–	–	–	–
Particulate matter accumulation	None (0)	6	6	–	–	6	–	–	–	–	5
	Minimal (1)	–	–	–	**5**	–	–	–	–	**5**	–
	Slight (2)	–	–	**6**	–	–	**3**	–	**6**	**1**	–
	Moderate (3)	–	–	–	–	–	**3**	**6**	–	–	–
	Marked (4)	–	–	–	–	–	–	–	–	–	–

Note. Values in bold are indicators of statistically significant difference from blank sample (two-tailed Mann–Whitney *U*-test, $p<.05$).
Abbreviations: LogOT = logwood boiler old technology, LogNT = logwood boiler new technology, and StoveOT = stove old technology.
StoveNT = stove new technology, Tiled = tiled stove, Woodchip = woodchip boiler, and Pellet = pellet boiler.

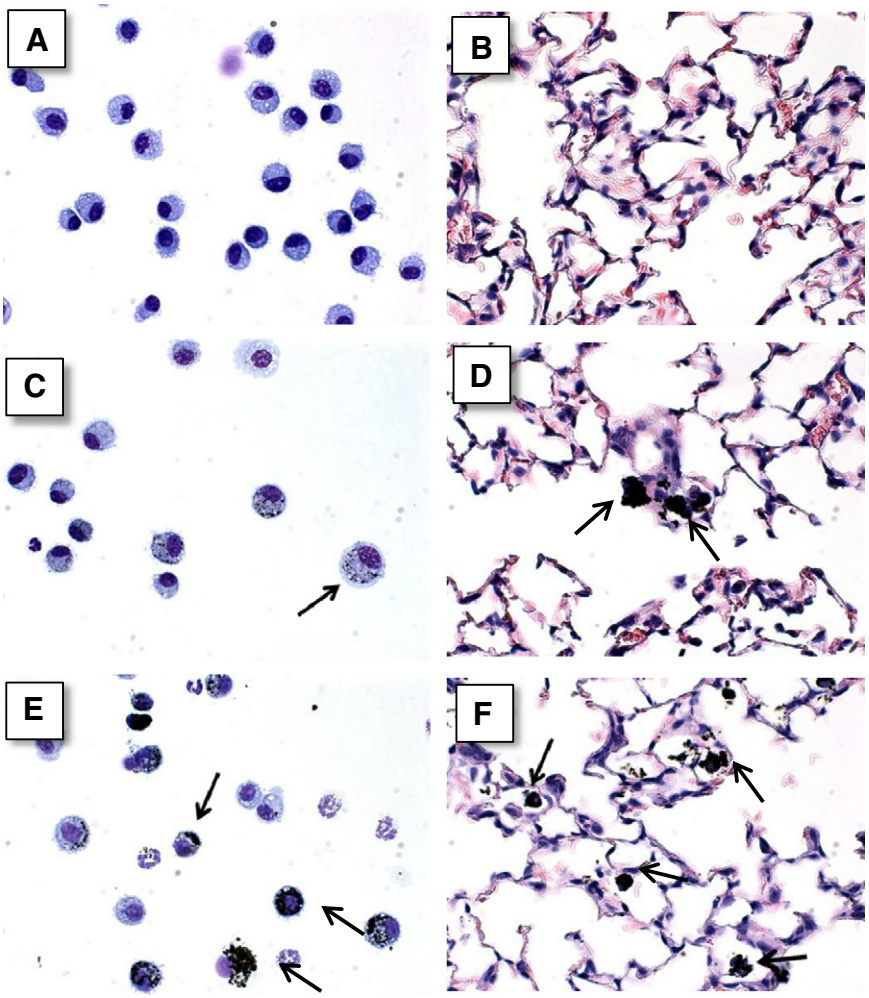

Fig. 4. Inflammatory cells in BALF of mice at 18 h and representative lung sections from mice at 24 h after a single dose of PM_1 (10 mg/kg) from different heating appliances (40×). A–B: blank sample (negative control), C–D: logwood boiler, old technology, E–F: stove, modern technology. Arrows indicate particulate matter accumulation into the inflammatory cells/alveoli.

toxicological responses are shown in Table 3. With respect to the PM_1 samples, both positive and negative statistically significant correlations between chemical constituents and detected toxicological parameters were measured. Most of the associations between the detected constituents and the total cell number or different cell types were inconsistent and had no statistically significant difference. On the other hand, the concentrations of Ca, Zn, S, Cl and Cd exhibited statistically significant positive correlations with the total protein concentration. In contrast, sum of PAHs had a high negative, but not statistically significant correlation with total protein in BALF. Overall, the genotoxic activity correlated similarly as total protein concentration with the chemical constituents. Most of the chemical constituents, especially Mn, Zn and

Table 3
The calculated correlation coefficients (rho) between the chemical composition of PM_1 emissions and the measured responses in BALF, at their most feasible time-points for recording (18 h for total cells, different cell types and total protein, and 4 h for comet assay).

	Total cells	Macrophages	Neutrophils	Lymphocytes	Total protein	Comet
OC	0.162	0.126	0.18	0.414	−0.234	**−0.757***
EC	0.259	0.334	0.074	0.408	−0.185	−0.667
Ca	0.071	−0.429	0.464	0.214	**0.786***	**0.857***
Mg	0.107	−0.321	0.393	−0.036	0.571	**0.786***
Mn	−0.111	−0.445	0.185	−0.037	0.63	**0.927****
K	0.143	−0.1443	0.321	0.071	0.607	**0.786***
Na	0.179	−0.143	0.429	0.214	0.714	0.75
Zn	0.143	−0.393	0.5	0.286	**0.857***	**0.893****
S	0.0	−0.464	0.321	0.179	**0.821***	**0.964****
Cl	0.286	−0.321	0.679	0.571	**0.964****	0.75
Cd	0.252	−0.342	0.667	0.541	**0.937****	0.739
Sum of PAHs	−0.143	0.393	−0.500	−0.107	−0.714	**−0.821***

Note. The values in this table are Spearman correlation coefficients (ρ). Boldfaced values indicate statistically significant correlations.
* $p<0.05$.
** $p<0.01$.

S, exhibited a strong positive correlation with OTM in the Comet assay. In contrast, OC and sum of PAH-compounds had negative and statistically significant correlation with genotoxicity. The six criteria PAH compounds (Directive, 2004/107/EC, 2005) correlated similarly as the sum of total PAH compounds with the measured responses.

4. Discussion

The present study detected differences in the ability of PM_1 samples originating from different wood heating appliances representing old and modern technologies in their abilities to induce inflammatory and cytotoxic responses as well as evoking tissue damage in the mouse lungs. Overall, both the old and modern technology heating appliances induced relatively small acute toxicological responses in mouse lungs. On equal mass basis, PM_1 samples from modern technology appliances were found to induce stronger acute pulmonary responses than samples from old technology devices. However, sub-chronic and chronic effects of the biomass combustion aerosols in the lungs or other target organs were not studied in this experiment.

4.1. Inflammatory responses to PM_1 emission samples

In the present study, the overall responses evoked in mouse lungs by PM_1 samples were at a relatively low level, when compared to those detected in our previous study with ambient air $PM_{10–2.5}$ and $PM_{2.5–0.2}$ samples (Happo et al., 2007). However, the particle size-ranges ($PM_{1–0.2}$ and $PM_{0.2}$) corresponding with the PM_1 in the present study were found to have much lower inflammatory activity in mouse lungs than the coarse and fine particles (Happo et al., 2010a). Moreover, wood smoke-rich particulate samples as well as inhaled wood smoke have been mostly associated with relatively low inflammatory activity in in vivo studies (Reed et al., 2006; Seagrave et al., 2005, 2006; Happo et al., 2008) as well as in in vitro studies (Jalava et al., 2009; Kocbach et al., 2008a, 2008b; Karlsson et al., 2006). This could be due to an immunosuppressive effect caused by the PAH compounds leading to lower inflammatory responses in mouse lungs.

One of the earliest responses in host defense during acute pulmonary inflammation after exposure to particulate matter is the production of cytokines and chemokines by the alveolar macrophages, respiratory epithelial cells and neutrophils. According to the findings on acute phase inflammatory responses in previous studies, the early 4 h time point was selected to assess levels of cytokines in BALF after particulate exposure. However, only slight or negligible IL-6 and MIP-2 responses were detected in the present dose-dependence study. The low cytokine and chemokine responses may also explain the relatively small number of infiltrated neutrophils in the lungs at the subsequent 18 h time point.

We observed slight increases in BALF total cell number at 18 h after the aspiration of the samples. This increase was mainly due to the increased number of neutrophils (Fig. 2), which have also been seen in many other studies on particulate matter (Adamson et al. 2004; Gavett et al., 2003; Gerlofs-Nijland et al., 2005; Schins et al., 2004). However, despite the infiltration of neutrophils into the lungs, the macrophages were still the prevailing cell type in BALF at the later time point. It is possible that the emissions from old technology boiler and stove were more rapidly transported into the lymph or bloodstream than the samples from new technology appliances, which subsequently affected the extent of cell migration into the area of inflammation. We also observed that the number of macrophages were usually lower in the BALF of animals exposed to PM_1 samples than in those exposed to the blank control samples. Similar effect has been seen in a study with $PM_{10–2.5}$ and $PM_{2.5}$ samples collected during California wildfires 2008 (Wegesser et al., 2009). One suggested explanation for this is that macrophages may have migrated in some extent into the tissue or adhered more tightly to the surfaces of airways.

4.2. Cytotoxicity and genotoxicity induced by PM_1 emission samples

In this study, the cytotoxicity of the emission samples was studied by measuring total protein and LDH concentrations in the BALF of mice. In contrast to in vitro studies with same emission samples (Jalava et al., 2012), only mild cytotoxicity was detected with both old and modern technology furnaces. The only statistically significant increase in the total protein concentration was induced by the pellet boiler sample at 18 h after the exposure. Overall, no systematic increases were detected, which reflects the relatively low acute cytotoxic potency of emission particles in the mouse lung at the doses used. The non-systematic LDH response has also been reported in other studies with mice (Dick et al., 2003; Gerlofs-Nijland et al., 2005; Seagrave et al., 2005; Happo et al., 2007). The low lung toxicity of wood smoke in subchronic exposures with rats and mice is also reported by Reed et al. (2006). They also reported that there was no accumulation of PM in mouse lungs after exposure to hardwood smoke, but with diesel emissions the situation was different. In the present study, accumulation of PM into the alveolar macrophages and alveoli was detected with most of the samples, but, similarly to Reed et al. (2006), no particles from modern logwood- and pellet boiler samples were seen in the microscopic observation. Moreover, diesel engine derived particles (positive control) were clearly accumulating into the inflammatory cells.

The highest genotoxicity in collected BALF cells was observed 4 h after the exposure to the PM_1 samples. However, only pellet boiler sample was found to increase statistically significantly DNA damage. At that time point, the BALF cells consisted mostly of macrophages (Fig. 2). Pellet boiler sample was also associated with the highest inflammatory potential of all the studied samples, as well as with low total PAH concentration. Therefore, it is possible that the measured DNA-damage was mainly secondary genotoxicity. It has been shown that particle-elicited inflammation generates reactive oxygen and nitrogen species during inflammation, which can cause to genetic damage and result in secondary genotoxicity (Schins and Knaapen, 2007).

4.3. Effect of chemical composition on toxicological responses

There was a systematic difference in the chemical composition of the emissions from old and new technology heaters. The old technology heaters clearly had the highest emissions of total particulate mass organics, soot and PAH compounds. Instead, with the low emission new technology appliances, especially pellet boiler and woodchip boiler, the following elements were enriched in the fine particle ash: Ca, Mg, Mn, K, Na, Zn, S, Cl and Cd. This finding is in good agreement with earlier studies (Werkelin et al., 2005; Sippula, 2010).

The present study observed negative correlations between observed toxicological responses and PAH compounds, confirming many previous studies. In those studies, the high concentration of PAH compounds or wood smoke-rich particulate samples has been associated with immunosuppressive effects (Jalava et al., 2008; Happo et al., 2008, 2010b), low inflammatory activity (Seagrave et al., 2006) as well as a reduction in the levels of cytokines in BALF (Kong et al., 1994). It has also been shown that a PAH-rich sample is likely to cause cell cycle arrest in G2/M phase of mouse macrophages (Jalava et al., 2007). Therefore, it is possible that the relatively low inflammatory responses induced by old technology appliances is linked to the high PAH concentrations of their PM_1 emissions.

In contrast to PAH compounds, several detected chemical constituents were found to have statistically significant positive correlations with BALF total protein concentration as well as with genotoxicity (Table 3). On the other hand, no systematic association was found between the constituents and the total cell number, different cell types

or LDH concentration. With respect to the detected constituents, Zn is known to have an important role in determining the pulmonary cell reactivity to inhaled particles (Adamson et al., 2000). It is also noteworthy that the highest Zn concentration was detected from the pellet boiler sample (Kochbach Bølling et al., 2009), which also caused the most extensive responses in several measured parameters in the present study. Added to this, concentrated metal content of the modern technology emission particles may be inducing at least some of the observed responses. This point of view is supported in many studies with urban air particles (Dye et al., 2001; Hutchison et al., 2005). However, there are large differences in the water-soluble metal abilities to induce pulmonary inflammation (Rice et al., 2001).

Of the other measured constituents, Ca was also found to be associated with increased inflammation and cytotoxicity. This was also the result of our previous studies with urban air particles (Happo et al., 2008, 2010a, 2010b; Jalava et al., 2008). However, in those studies, chloride, sulfate, sodium, magnesium and cadmium were not linked to toxicological responses in $PM_{2.5–0.2}$ and $PM_{1–0.2}$ size ranges, which were now associated with measured responses.

4.4. Methodological considerations

In this study, a controlled intratracheal aspiration technique was used to achieve the delivery of emission particle samples to the lower airways of mice. With this technique, a particulate sample needs to be suspended into water before administration to the animals. However, particulate samples with a high mass concentration of organic material are not readily suspended into water only by sonication. Therefore one needs to use DMSO during the suspension of particulate samples. DMSO is widely used in in vitro and in vivo studies, because of its ability to dissolve hydrophobic substances. It can also easily penetrate through biological membranes and cellular barriers, and is, therefore, used as a vehicle to carry medicine through skin (Colucci et al., 2008). Therefore it is possible that the transportation of particles through the epithelium in respiratory tract and in surfaces of alveoli has been enhanced. In addition, known inflammatory and anti-inflammatory effects of DMSO (Colucci et al., 2008) were excluded in pilot studies both in vivo and in vitro, which showed no systemic difference in the inflammatory activity between samples with or without DMSO (data not shown).

The intratracheal aspiration technique used in this study naturally differs from the inhalation exposure to emission particles. However, it has been shown that intratracheal instillation and inhalation exposure have resulted in semiquantitatively similar particulate distribution patterns and inflammatory responses in rodent lungs (Driscoll et al., 2000; Costa et al., 2006). It was reported in the other study that inhalation exposure and intratracheal aspiration to carbon nanotubes leads to similar outcomes, but there is a difference in the strength of the responses (Shedova et al., 2008). It was suggested that in intratracheal aspiration particles in suspension contain micrometer-size agglomerates, which lower their reactivity when compared to dry aerosol exposure. This agglomeration effect may also explain, at least partly, the relatively low responses to PM_1 emissions that were measured in the present study.

The doses used in our present study seem relatively high when compared to doses that are likely to be delivered acutely to the lungs in concentrated ambient air particles (CAP) and inhalation studies. However, they are no higher than the instilled doses that have usually induced inflammatory responses in rodent lungs according to the cited literature (Adamson et al., 1999; Schins et al., 2004; Gerlofs-Nijland et al., 2005; Park et al., 2011). We emphasize that this kind of doses are required to reveal statistically significant differences in the acute inflammatory activity and cytotoxicity between the PM_1 samples, especially when small groups of healthy animals are used. Nevertheless, no signs of lung overloading were observed even with the highest used dose of 15 mg/kg.

In our previous studies, we have detected high correlations between in vivo and in vitro results with ambient air particles (Happo et al., 2007; Jalava et al., 2007). Instead, in the present study, there were some differences between the results of cell and animal studies. The difference in clearance mechanisms between these two experimental set-ups may be one of the main causes behind this apparent discrepancy. In the cell culture dish, macrophages are exposed to particulate sample for the duration of the experiment, whereas in mice, the duration of particle contact with inflammatory cells in the lungs will vary depending on the efficiency of the clearance mechanisms. Therefore, clear cytotoxicity was seen mostly in the in vitro study (Jalava et al., 2012), whereas in the in vivo studies its role was not as clear. However, highly cytotoxic and genotoxic samples will also influence the excretion of proinflammatory cytokines, which may also explain their relatively low levels in the in vitro study, whereas in the animal study, some significant increases of inflammatory markers were detected.

Eventually, all of the experiments in the present study were conducted on equal mass basis regardless of the emitted particulate mass from the heaters. It is noteworthy that the heaters used in this study had huge differences in the PM_1 total mass emissions between old and modern combustion technologies (Table 1), which may also have large role on the local air quality in the areas with a high density of wood combustion. This effect on local air quality has been seen, when large numbers of stoves were changed to newer and better combustion quality appliances (Bergauff et al., 2009). The change in the air quality was also measured indoors, where the PM concentrations were reduced significantly (Ward et al., 2008). In addition to this, the highest emitted PM_1 total mass together with the highest concentrations of PAH compounds may disturb the cell cycle (Jalava et al., 2007), and cause subsequent activation of immunosuppressive mechanisms in the lungs, such like decrease in cytokine production (Kong et al., 1994). The higher dose of particles may also be able to exacerbate existing chronic diseases, such as asthma and allergy. Therefore, modern technology pellet boilers as well as stoves and log-wood boiler is suggested to have clearly lower harmfulness than those of the corresponding old technology appliances, which was also concluded in the in vitro study with the same PM_1 samples than used in the present study (Jalava et al., 2012).

4.5. Conclusions

Substantial differences in the burnout qualities of old and modern technology appliances were detected, which also affected to chemical composition and toxicity of the emissions. Overall, almost all of the observed acute responses in mouse lungs caused by PM_1 emission samples were at a very low level. Modern technology appliances had the smallest PM_1 (mg/MJ) emissions, but they induced the highest inflammatory, cytotoxic and genotoxic activities. In contrast, old technology appliances had clearly the highest PM_1 (mg/MJ) emissions that had the lowest effects in the lungs of mice. Increased inflammatory activity was associated with ash related components of the emissions, whereas high PAH concentrations were correlating with the smallest detected responses, potentially due to their immunosuppressive effect.

Declaration of interest

The authors report no conflicts of interest.

Acknowledgments

This study is funded by the Austrian Kplus program of the Federal Government of Austria, the State Government of Styria and the State Government of Lower Austria and belongs to lead program of University of Eastern Finland (Sustainable Bioenergy, Climate Change and

Health). The laboratory assistance in the toxicological experiments by Heli Martikainen and Miia Koistinen is highly appreciated.

References

Adamson IYR, Prieditis H, Vincent R. Pulmonary toxicity of an atmospheric particulate sample is due to the soluble fraction. Toxicol Appl Pharmacol 1999;157:43–50.

Adamson IY, Prieditis H, Hedgecock C, Vincent R. Zinc is the toxic factor in the lung response to an atmospheric particulate sample. Toxicol Appl Pharmacol 2000;166(2):111–9.

Adamson IYR, Prieditis H, Vincent R. Soluble and insoluble air particle fractions induce differential production of tumor necrosis factor α in rat lung. Exp Lung Res 2004;30:355–368.

Bergauff MA, Ward TJ, Noonan CW, Palmer CP. The effect of a woodstove changeout on ambient levels of $PM_{2.5}$ and chemical tracers for woodsmoke in Libby, Montana. Atmos Environ 2009;43:2938–43.

Brunner T, Obernberger I. Primary measures for low-emission residential wood combustion — comparison of old with optimised modern systems. in: Proc. of the 17th European Biomass Conference, June 2009, Hamburg, Germany, ISBN 978-88-89407-57-3, ETA-Renewable Energies (Ed.), Florence, Italy, pp. 2009;1319-28.

Colucci M, Maione F, Bonito MC, Piscopo A, Di Giannuario A, Pieretti S. New insights of dimethyl sulphoxide effects (DMSO) on experimental in vivo models of nociception and inflammation. Pharmacol Res 2008;57(6):419–25.

Costa DL, Lehmann JR, Winsett D, Richards J, Ledbetter AD, Dreher KL. Comparative pulmonary toxicological assessment of oil combustion particles following inhalation or instillation exposure. Toxicol Sci 2006;91:237–46.

Dick CA, Singh P, Daniels M, Evansky P, Becker S, Gilmour MI. Murine pulmonary inflammatory responses following instillation of size-fractioned ambient particulate matter. J Toxicol Environ Health A 2003;66:2193–207.

Directive 2004/107/EC of the European Parliament and of the Council of 15 December 2004 relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air. Off J 2005;L 23. [26/01].

Directive 2010/63/EU for animal experiments. http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdörster G, Salem H, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicol Sci 2000;55:24–35.

Dye JA, Lehmann JR, McGee JK, Winsett DW, Ledbetter AD, Everitt JI, et al. Acute pulmonary toxicity of particulate matter filter extracts in rats: coherence with epidemiological studies in Utah Valley residents. Environ Health Perspect 2001;109:395–403.

Gavett SH, Haykal-Coates N, Copeland LB, Heinrich J, Gilmour MI. Metal composition of ambient $PM_{2.5}$ influences severity of allergic airways disease in mice. Environ Health Perspect 2003;111:1471–7.

Gerlofs-Nijland ME, Boere AJF, Leseman DLAC, Dormans JAMA, Sandström T, Salonen RO, et al. Effects of particulate matter on the pulmonary and vascular system: time course in spontaneously hypertensive rats. Part Fibre Toxicol 2005;2:2. [[Online] Available at: http://www.particleandfibretoxicology.com/content/2/1/2].

Glasius M, Ketze M, Wåhlin P, Jensen B, Mønster J, Berkowicz B, et al. Impact of wood combustion on particle levels in a residential area in Denmark. Atmos Environ 2006;40:7115–24.

Happo MS, Salonen RO, Hälinen AI, Jalava PI, Pennanen AS, Kosma VM, et al. Dose- and time dependency of inflammatory responses in the mouse lung to urban air coarse, fine and ultrafine particles from six European cities. Inhal Toxicol 2007;19:227–46.

Happo MS, Hirvonen M-R, Hälinen AI, Jalava PI, Pennanen AS, Sillanpää M, et al. Chemical compositions responsible for inflammation and tissue damage in the mouse lung by coarse and fine particulate samples from contrasting air pollution in Europe. Inhal Toxicol 2008;20:1-17.

Happo MS, Hirvonen M-R, Hälinen AI, Jalava PI, Pennanen AS, Sillanpää M, et al. Seasonal variation in chemical composition of size-segregated urban air particles and the inflammatory activity in the mouse lung. Inhal Toxicol 2010a;22:17–32.

Happo MS, Salonen RO, Hälinen AI, Jalava PI, Pennanen AS, Dormans JA, et al. Inflammation and tissue damage in mouse lung by single and repeated dosing of urban air coarse and fine particles collected from six European cities. Inhal Toxicol 2010b;22:402–16.

Hutchison GR, Brown DM, Hibbs LR, Heal MR, Donaldson K, Maynard RL, et al. The effect of refurbishing a UK steel plant on PM_{10} metal composition and ability to induce inflammation. Respir Res 2005;6:43.

Jalava P, Salonen RO, Hälinen AI, Sillanpää M, Sandell E, Hirvonen M-R. Effects of sample preparation on chemistry, cytotoxicity, and inflammatory responses induced by air particulate matter. Inhal Toxicol 2005;17:107–17.

Jalava PI, Salonen RO, Pennanen AS, Sillanpää M, Hälinen AI, Happo MS, et al. Heterogeneities in inflammatory and cytotoxic responses of RAW 264.7 macrophage cell line to urban air coarse, fine and ultrafine particles from six European sampling campaigns. Inhal Toxicol 2007;19:213–25.

Jalava PI, Salonen RO, Pennanen AS, Happo MS, Penttinen P, Hälinen AI, et al. Effects of solubility of urban air fine and coarse particles on cytotoxic and inflammatory responses in RAW 264.7 macrophage cell line. Toxicol Appl Pharmacol 2008;229(2):146–60.

Jalava PI, Hirvonen M-R, Sillanpää M, Pennanen AS, Happo MS, Hillamo R, et al. Associations of urban air particulate composition with inflammatory and cytotoxic responses in RAW 264.7 cell line. Inhal Toxicol 2009;21:994-1006.

Jalava PI, Salonen RO, Nuutinen K, Pennanen AS, Happo MS, Penttinen P, et al. Effect of combustion condition on cytotoxic and inflammatory activity of residential wood combustion particles. Atmos Environ 2010;44:1691–8.

Jalava PI, Happo MS, Kelz J, Brunner T, Hakulinen P, Mäki-Paakkanen J, et al. *In vitro* toxicological characterization of particulate emissions from residential biomass heating systems based on old and new technologies. Atmos Environ 2012;50:24–35.

Karlsson HL, Ljungman AG, Lindbom J, Moller L. Comparison of genotoxic and inflammatory effects of particles generated by wood combustion, a road simulator and collected from street and subway. Toxicol Lett 2006;165:203–11.

Kelz J, Brunner T, Obernberger I, Jalava P, Hirvonen M-R. Untersuchung des Gesundheitsgefährungspotentials von Feinstaubemissionen aus Biomasse-Kleinfeuerungsanlagen. Endbericht Teil 1, Bericht Nr. TR I-2-13 01, BIOENERGY 2020+ GmbH (ed.), Graz, Austria (In German). 2010.

Kocbach A, Herseth JI, Låg M, Refsnes M, Schwarze PE. Particles from wood combustion and traffic induce differential pro-inflammatory response patterns in co-cultures. Toxicol Appl Pharmacol 2008a;232:317–26.

Kocbach A, Namork E, Schwarze PE. Pro-inflammatory potential of wood smoke and traffic-derived particles in a monocytic cell line. Toxicology 2008b;247:123–32.

Kochbach Bølling A, Pagels J, Yttri KE, Barregard L, Sallsten G, Schwarze PE, et al. Health effects of residential wood smoke particles: the importance of combustion conditions and physicochemical particle properties. Part Fibre Toxicol 2009;6:29. [[Online] Available at: http://www.particleandfibretoxicology.com/content/6/1/29].

Kong L-Y, Luster MI, Dixon D, O'Grady J, Rosenthal GJ. Inhibition of lung immunity after intratracheal instillation of benzo(a)pyrene. Am J Respir Crit Care Med 1994;150:1123–9.

Lamberg H, Nuutinen K, Tissari J, Ruusunen J, Yli-Pirilä P, Sippula O, et al. Physicochemical characterization of fine particles from small-scale wood combustion. Atmos Environ 2011;45:7635–43.

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, et al. Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 2004;16:437–45.

Park E-J, Roh J, Kim Y, Park K, Kim D-S, Yu S-D. PM 2.5 collected in a residential area induced Th1-type inflammatory responses with oxidative stress in mice. Environ Res 2011;111:348–55.

Pennanen AS, Sillanpää M, Hillamo R, Quass U, John AC, Branis M, et al. Performance of a high-volume cascade impactor in six European urban environments: mass measurement and chemical characterization of size-segregated particulate samples. Sci Total Environ 2007;374:297–310.

Pope CA, Dockery DW. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manage Assoc 2006;56:709–42.

Reed MD, Campen MJ, Gigliotti AP, Harrod KS, McDonald JD, Seagrave JC, et al. Health effects of subchronic exposure to environmental levels of hardwood smoke. Inhal Toxicol 2006;18:523–39.

Rice TM, Clarke RW, Godleski JJ, Al-Mutairi E, Jiang N-F, Hauser R, et al. Differential ability of transition metals to induce pulmonary inflammation. Toxicol Appl Pharmacol 2001;177:46–53.

Ruusunen J, Tapanainen M, Sippula O, Jalava PI, Lamberg H, Nuutinen K, et al. A novel particle sampling system for physico-chemical and toxicological characterization of emissions. Anal Bioanal Chem 2011;401:3183–95.

Schins RP, Knaapen AM. Genotoxicity of poorly soluble particles. Inhal Toxicol 2007;19:189–98.

Schins RPF, Lightbody JH, Borm PJA, Shi T, Donaldson K, Stone V. Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents. Toxicol Appl Pharmacol 2004;195:1-11.

Schwarze PE, Øvrevik M, Låg M, Refsnes P, Nafstad RB, Hetland RB, et al. Particulate matter properties and health effects: consistency of epidemiological and toxicological studies. Hum Exp Toxicol 2006;25:559–79.

Seagrave JC, McDonald JD, Reed MD, Seilkop SK, Mauderly JL. Responses to subchronic inhalation of low concentrations of diesel exhaust and hardwood smoke measured in rat bronchoalveolar lavage fluid. Inhal Toxicol 2005;17:657–70.

Seagrave JC, McDonald JD, Bedrick E, Edgerton ES, Gigliotti AP, Jansen JJ, et al. Lung toxicity of ambient particulate matter from southeastern U.S. sites with different contributing sources: relationships between composition and effects. Environ Health Perspect 2006;114:1387–93.

Shedova AA, Kisin E, Murray AR, Johnson VJ, Gorelik O, Arepalli S, et al. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inlammation, fibrosis, oxidative stress and mutagenesis. Am J Physiol Lung Cell Mol Physiol 2008;295:552–65.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low-levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.

Steerenberg PA, van Amelsvoort L, Lovik M, Hetland RB, Alberg T, Halatek T, et al. Relation between sources of particulate air pollution and biological effect parameters in samples from four European cities: an exploratory study. InhalToxicol 2006;18:333–46.

Sippula O. Fine particle formation and emissions in biomass combustion. Report series in aerosol science, 108, ISBN: 978-952-5822-14-4; 2010 [[Online] Available at: http://www.atm.helsinki.fi/FAAR/].

Tissari J, Lyyränen J, Hytönen K, Sippula O, Tapper U, Frey A, et al. Fine particle and gaseous emissions from normal and smouldering wood combustion in a conventional masonry heater. Atmos Environ 2008;42:7862–73.

Ward T, Palmer C, Bergauff M, Hooper K, Noonan C. Results of a residential indoor PM2.5 sampling program before and after a woodstove changeout. Indoor Air 2008;18:408–15.

Wegesser TC, Pinkerton KE, Last JA. California Wildfires of 2008: coarse and fine particulate matter toxicity. Environ Health Perspect 2009;117:893–7.

Werkelin J, Skrifvars B, Hupa M. Ash-forming elements in four Scandinavian wood species. Part 1: summer harvest. Biomass Bioenergy 2005;29:451–66.

World Health Organization (WHO). Review of health aspects of air pollution with particulate matter, ozone and nitrogen dioxide. Report EUR/03/5042688 of working group, Bonn, Germany, 13–15 January 2003. Copenhagen, Denmark: WHO Regional Office for Europe; 2003.

World Health Organization (WHO). WHO air quality guidelines global update 2005. Report WHOLIS E87950 of Working Group Meeting, Bonn, Germany, 18–20 October 2005. Copenhagen, Denmark: WHO Regional Office for Europe; 2005. [[Online] Available at: http://www.euro.who.int/Document/E87950.pdf].