In vitro toxicological characterization of particulate emissions from residential biomass heating systems based on old and new technologies

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A B S T R A C T

Residential wood combustion causes major effects on the air quality on a global scale. The ambient particulate levels are known to be responsible for severe adverse health effects that include e.g. cardio-respiratory illnesses and cancer related effects, even mortality. It is known that biomass combustion derived emissions are affected by combustion technology, fuel being used and user-related practices. There are also indications that the health related toxicological effects are influenced by these parameters. This study we evaluated toxicological effects of particulate emissions (PM1) from seven different residential wood combusting furnaces. Two appliances i.e. log wood boiler and stove represented old batch combustion technology, whereas stove and tiled stove were designated as new batch combustion as three modern automated boilers were a log wood boiler, a woodchip boiler and a pellet boiler. The PM1 samples from the furnaces were collected in an experimental setup with a Dekati/C210 gravimetric impactor on PTFE filters with the samples being weighed and extracted from the substrates and prior to toxicological analyses. The toxicological analyses were conducted after a 24-hour exposure of the mouse RAW 264.7 macrophage cell line to four doses of emission particle samples and analysis of levels of the proinflammatory cytokine TNFα, chemokine MIP-2, cytotoxicity with three different methods (MTT, PI, cell cycle analysis) and genotoxicity with the comet assay. In the correlation analysis all the toxicological results were compared with the chemical composition of the samples. All the samples induced dose-dependent increases in the studied parameters. Combustion technology greatly affected the emissions and the concomitant toxicological responses. The modern automated boilers were usually the least potent inducers of most of the parameters while emissions from the old technology log wood boiler were the most potent. In correlation analysis, the PAH and other organic composition and inorganic ash composition affected the toxicological responses differently. In conclusion, combustion technology largely affects the particulate emissions and their toxic potential this being reflected in substantially larger responses in devices with incomplete combustion. These differences become emphasized when the large emission factors from old technology appliances are taken into account.

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1. Introduction

Wood combustion in residential homes is one of the most important sources of fine particulate emissions. The associated particulate concentrations can be at the same level, even larger as those measured near busy streets (Glæsious et al., 2006). It has been shown that combustion technology, fuel being burned and user practices affect both the emissions (Fine et al., 2002; Jordan and Seen, 2005; Tissari et al., 2008; Brunner et al., 2008; Brunner and Obernberger, 2009) and their toxicological responses (Jalava et al., 2010a). Strategically important future areas of health research on ambient air and emission particles are promoting the development and the use of clean combustion as well as emission control...
technologies in residential biomass heating systems. This is of special interest since the European Union is likely to greatly increase its consumption of biomass energy in the forthcoming years. However any increase in use of biomass energy has to be achieved without increasing the harmful effects on either human health or the environment.

In epidemiological studies, small scale wood combustion has been associated with various adverse health effects (Naether et al., 2007), especially cardio-respiratory illnesses (Andersen et al., 2007; Sarnat et al., 2008). Moreover, wood combustion emissions have been linked to asthma and other respiratory symptoms (Boman et al., 2003; Allen et al., 2008; Chio, 2008). Since severe adverse health effects are associated with ambient air PM2.5 and combustion emissions, in many countries there is growing pressure to enact more strict regulation of these emissions.

There are many toxicological mechanisms proposed to be involved in PM-induced adverse health effects e.g. cytotoxicity, inflammation associated injury (Pope and Dockery, 2006; Brook et al., 2010) oxidative stress (Tao et al., 2003) and genotoxicity (Binkova et al., 2003; Farmer et al., 2003; Knaapen et al., 2004). These mechanisms can be evoked in alveolar macrophages in response to exposure to particulate matter (Becker et al., 2005; Jalava et al., 2007; Jalava et al., 2010a). Alveolar macrophages are the primary defense cell type against inhaled particles in the lung periphery.

The physicochemical characteristics of the particulate samples have been shown to influence the biological outcomes both in vivo in the mouse lung (Happo et al., 2008) and in vitro in cell experiments (Hetland et al., 2004; Jalava et al., 2009). The combustion technologies in appliances as well as how they are operated change the physicochemical properties of the emission particles (Tissari et al., 2008; Brunner and Obernberger, 2009) and their toxic properties (Kochbach Bolling et al., 2009). We have previously shown that the compositions of particles emitted from the incomplete and more complete combustion activate different toxicological responses in vitro (Jalava et al., 2010a).

The main focus of the present study was to investigate the emissions from several old and new technology heating appliances that are commonly used in central European countries and their relation to toxicological potential in mouse cell line. The inflammatory, cytotoxic and genotoxic responses induced by the particles emitted from small scale wood combustion were investigated. Therefore, the results were compared with the physico-chemical properties of the emitted particles.

2. Materials and methods

2.1. The studied furnaces

The small-scale furnaces included in this study were as follows: pellet boiler (new technology), wood chip boiler (new technology), log wood boiler (new technology), log wood boiler (old technology), stove (new technology), stove (old technology) and tiled stove (new technology). The pellet boiler (PLB) and the woodchip boiler (WCB) were equipped with automatic ignition systems, staged combustion, automated boiler cleaning systems as well as automated de-ashing systems. Their nominal boiler capacities were 21 kW and 30 kW, respectively. The pellet boiler contained an overfed burner, a water cooled combustion chamber as well as combustion control based on the measurement of the furnace temperature. The woodchip boiler consisted of an underfeed stoker combustion system and a water cooled combustion chamber. To achieve combustion control, the flue gas temperature control and λ-control were applied. The modern logwood boiler (LWB OT) was based on down-draught combustion technology and had a nominal boiler capacity of 30 kW. The device was equipped with an automated boiler cleaning system and with an automated combustion control based on a λ-control. The old logwood boiler (LWB OT) (nominal boiler capacity: 15 kW) was a typical under-fire boiler. In this system, combustion control is based on a thermo-mechanic combustion air control by a primary air flap. In contrast to the pellet and the woodchip boiler, both logwood boilers were manually fed and also the ash removal had to be performed manually. The modern stove (ST NT) (nominal heat output: 6 kW) consisted of a primary and a secondary combustion zone. The air supply was arranged via primary and window purge air during ignition and only by window purge air during main combustion and burnout phases. The air supply control had to be manually adjusted. The old technology stove (ST OT) (nominal heat output: 6.5 kW) consisted of one burning chamber. Combustion air was divided into primary air through the grate, window purge air as well as secondary air drawn in through nozzles from the back of the combustion chamber. The air distribution was adjusted manually by a damper. The tiled stove (TL ST) (nominal heat output: 4.2 kW) was designed according to the current (2009) guidelines of the Austrian tiled stove association. The air supply entered through a vertical grate positioned in the stove door. While the investigated boiler systems were equipped with flue gas fans, the stoves represent typical natural draught systems. The emission background information of the furnaces is presented in Table 1.

For the pellet and the woodchip boiler, typical whole day load cycles have been evaluated from field measurement data in order to define a representative operation cycle. The final applied operation cycle had a duration of 10 h and included in addition to stable full and partial load operation also a considerable number of startup, load change and shut down procedures. All the batch combustion appliances were operated on the normal whole day operation cycles including all burnning phases. The emission background information is presented in Table 1. More detailed information about the combustion cycles, furnaces and the related emissions can be found in Kelz et al. (2010) and Brunner and Obernberger (2009).

2.2. Sample collection for toxicological analyses

Samples for toxicological analyses were collected in facilities of Bioenergy 2020+ /Technical University of Graz by using a previously validated novel particulate sampling system for use in toxicological and chemical analysis (Lamberg et al., 2011). PM1 was collected from diluted flue gas from each furnace on polytetrafluoroethylene (PTFE) filters (Millipore Corp., Billerica, MA, USA) with Dekati® Gravimetric Impactor (DGI, Dekati Ltd, Tampere, Finland). Blank control substrates were collected from all sampling campaigns and treated similarly as the other substrates. The DGI system consists of a heated cyclone (cut diameter: 10 µm), a porous tube diluter (PRD), the 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

<table>
<thead>
<tr>
<th>Appliance</th>
<th>PM1, mg MJ⁻¹</th>
<th>CO, mg MJ⁻¹</th>
<th>OGC, mg MJ⁻¹</th>
<th>Load, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log wood boiler, old technology</td>
<td>106</td>
<td>12,600</td>
<td>1140</td>
<td>82</td>
</tr>
<tr>
<td>Log wood boiler, new technology</td>
<td>18</td>
<td>780</td>
<td>62</td>
<td>98</td>
</tr>
<tr>
<td>Stove, old technology</td>
<td>74</td>
<td>2350</td>
<td>220</td>
<td>All phases</td>
</tr>
<tr>
<td>Stove, new technology</td>
<td>46</td>
<td>1040</td>
<td>96</td>
<td>All phases</td>
</tr>
<tr>
<td>Tiled stove, new technology</td>
<td>28</td>
<td>1010</td>
<td>46</td>
<td>All phases</td>
</tr>
<tr>
<td>Woodchip boiler, new technology</td>
<td>14</td>
<td>180</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>Pellet boiler, new technology</td>
<td>6</td>
<td>47</td>
<td>3</td>
<td>68</td>
</tr>
</tbody>
</table>
diluted flue gas. The temperature of the diluted flue gas was measured with Pt100 temperature sensors. The DGI itself consists of 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). The stages below 1 μm (PM$_1$) were taken into the toxicological and chemical analyses since the emissions occur in that size range. The purpose of the study was to compare the PM$_1$ samples from the different combustion appliances. The PM$_1$ concentrations agreed in the experiments with TSP and those measured with BLPI. This indicates that all the emissions were in the same PM$_1$ size range.

### 2.3. Chemical analyses

Chemical characterization of the fuel was arranged by wet chemical analyses. The moisture content of the fuel was determined by drying at 105 °C, the ash content was analyzed according to the standard procedure prEN/TS 14,775, the concentrations of C, H and N were determined with elemental analyzer, Si, Ca, Mg, Mn, K, Na, Zn, S were detected after pressurized multi-step digestion with HNO$_3$/HF/H$_3$BO$_3$ by ICP-OES and Cl after bomb combustion in oxygen and absorption in NaOH was detected by IC.

Determination of the chemical composition of both BLPI and DGI samples was achieved in Graz by pressurized multi-step digestion of the samples (HNO$_3$/HF/H$_3$BO$_3$) before the element detection with ICP-OES or ICP-MS. Determination of the contents of different carbon compounds including organic carbon (OC), elemental carbon (EC) and inorganic carbon (IC) in aerosol samples was conducted with a carbon/hydrogen analyzer (LECO RC-612). The sample was inserted into a quartz tube that was heated to predetermined temperatures. 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 entering 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 designated as EC, carbon detected after switching to oxidizing conditions was EC.

A total of 30 polycyclic aromatic hydrocarbons were analyzed using a gas chromatograph and a mass selective detector (6890N/5973 INERT MSD, Agilent Technologies Santa Clara, CA) after using a gas chromatograph and a mass selective detector (6890N GC, Heraeus Biofuge Fresco) and the remaining half of them were shipped to Kuopio in dry ice. Prior to the re-weighing of the filter sets were shipped to Graz, Austria for the sample collection.

After the sample collections, the filter sets were packed in laboratory grade aluminum foil and double zip lock plastic bags and shipped to Kuopio in dry ice. Prior to the re-weighing of the filters, the samples were allowed to acclimatize in the weighing room overnight. Three stages of each DGI set were weighed to form a PM$_1$ sample.

The particulate samples were prepared for the cell experiments using previously validated procedures (Jalava et al., 2005, 2006). The PM$_1$ samples were extracted with methanol from the filters. The filters were cut into pieces and placed into a 50 ml glass tube that was filled with methanol. The tubes were placed in an ultrasonic water bath, extracted for 30 min, and thereafter the procedure was repeated. The PM$_1$ samples were pooled in a flask and the excess methanol was evaporated in a rotary evaporator (Heidolph laborota 4000) that was attached to a controlled vacuum pump 150 mbar (Vacuubrand) and chiller (Lauda WK500). Finally, aliquots of the concentrated suspension, calculated on a mass basis, were dried in glass tubes under nitrogen (99.5% flow and stored at −20 °C.

The extraction efficiencies calculated from randomly selected samples were 99.7% of the mass for the new technology stove, 98.6% for the old technology log wood boiler, 98.5% for the old technology stove, 96.1% for the tiled stove, 95.3% for the woodchip boiler, 93.1% for the new technology log wood boiler, and finally 90% for the pellet boiler derived particulate samples.

### 2.5. Study design

Mouse RAW264.7 macrophages (ATCC, Rockville, MD, USA) were cultured at +37 °C and in 5% CO$_2$ in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U ml$^{-1}$ penicillin—streptomycin (Gibco, Paisley, UK). The cell suspension of 500 000 cells ml$^{-1}$ was dispensed into 6-well plates (2 ml/well, Corning Inc., New York, USA), and these cell cultures were allowed to stabilize for 24 h. Fresh cell culture medium was changed on the wells for one hour prior to the exposure to the emission particles. The particulate samples were suspended into a solution of 0.3% dimethylsulfoxide (DMSO) and pyrogen free water (W1503, Sigma—Aldrich Corp., St. Louis, MO, USA) at a concentration of 5 mg ml$^{-1}$, and treated in an ultrasonic water-bath (FinnSonic m03, FinnSonic Ltd., Lahti Finland) for 30 min. Mouse RAW264.7 macrophages were exposed for 24 h to four doses (15, 50, 150 and 300 μg ml$^{-1}$) of the emission particles from each of the studied furnaces. All the exposures were made in duplicate in three independent experiments. The 24 h exposure duration was chosen on basis of results from the time-dependency study with various different particulate samples (Jalava et al., 2005).

After the 24 h exposure, the macrophages were suspended into culture medium by scraping the well bottoms with a cell lifter. Cell viability was immediately measured from the cell suspension using the MTT test. The rest of the cell suspension was centrifuged (5 min, 6082 g, +4 °C, Heraeus Biofuge Fresco) and the remaining supernatant was stored at −80 °C for the analysis of several inflammatory mediators including the proinflammatory cytokines Tumor Necrosis Factor α (TNFα) and Interleukin 6 (IL-6) as well as the chemokine, macrophage inflammatory protein 2 (MIP-2). The cell pellets were suspended into phosphate buffered saline (PBS) and half of them were fixed with ethanol (70% v/v) for subsequent DNA content analysis with the other half being utilized in propidium iodide (PI)-staining of the fresh cells. The cells from the other duplicate well were used in the Comet assay.

### 2.6. Investigations into cell death

#### 2.6.1. MTT-assay

The viability of the macrophages was detected with the MTT-test on 96-well plates, and calculated as a percentage by comparing absorbances from cell suspensions exposed to particulate samples with those from corresponding control cells. Cell viability assessed by the MTT-test is based on the presence of functioning mitochondrial and endoplasmic reticulum in the cell suspension. In the test, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is transformed to formazan, which is spectrophotometrically detected. The test was also run with particles only and with blank samples to ensure methodological reliability.
2.6.2. PI-exclusion method

The total amount of PI-positive cells with a lowered cell membrane potential was detected by flow cytometry. Briefly, the cell suspension was centrifuged (5 min at 370 × g) to separate the cells from the culture medium and washed once with 1 ml of phosphate-buffered saline. Then, the cells were resuspended into 0.5 ml of PBS and PI (f.c. 1 g ml\(^{-1}\), Sigma—Aldrich Corp.) was added. The samples were incubated for 15 min at room temperature in the dark before flow cytometric analysis (CyAn ADP, Beckman Coulter, USA). A total of 10,000 cells per sample were analyzed with Summit software version 4.3.

2.6.3. Cell cycle analysis

Cellular DNA content and thus the cell cycle stage of non-apoptotic cells were analyzed by PI staining of permeabilized cells. The cells containing fragmented DNA were identified as apoptotic cells (SubG1) (Nicotelli et al., 1991; Darzynkiewicz et al., 1992). Briefly, the cell-ethanol-suspension was centrifuged (10 min, 400 × g) and thereafter the cells were suspended into PBS. The suspension was treated for 1 h with 0.15 mg ml\(^{-1}\) of monoclonal capture antibodies. Non-specific binding was blocked with blocking buffer. The cytokines present in the measured sample or standard were bound by antibodies on the microplate wells and non-bound material was removed by washing with washing buffer. Biotinylated monoclonal detecting antibodies for each cytokine were added and after incubation, the plates were washed three times with washing buffer. Horseradish peroxidase (HRP)-conjugated streptavidin was added to bind to the biotinylated side of the cytokine sandwich. After incubation, non-bound HRP conjugate was removed by washing and TMB substrate solution was added. During the incubation, TMB forms a blue color that is converted yellow when the stop solution is added. This yellow color was read at wavelength 450 nm (CyAn ADP, Beckman Coulter). Possible interference of the method by the particles was checked and found to be negligible.

2.7. Inflammatory parameters

Levels of the proinflammatory cytokine TNF\(_{a}\) and chemokine MIP-2 were analyzed immunochemically from cell culture medium using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) slightly modified from the manufacturer’s instructions. Shortly, 96-well plates (MaxiSorp\(^{TM}\), Nunc A/S) were coated with monoclonal capture antibodies. Non-specific binding was blocked with blocking buffer. The cytokines present in the measured sample or standard were bound by antibodies on the microplate wells and non-bound material was removed by washing with washing buffer. Biotinylated monoclonal detecting antibodies for each cytokine were added and after incubation, the plates were washed three times with washing buffer. Horseradish peroxidase (HRP)-conjugated streptavidin was added to bind to the biotinylated side of the cytokine sandwich. After incubation, non-bound HRP conjugate was removed by washing and TMB substrate solution was added. During the incubation, TMB forms a blue color that is converted yellow when the stop solution is added. This yellow color was read at wavelength 450 nm using the microplate reader. The concentrations of cytokines in samples were determined by interpolation from the standard curve.

2.8. Genotoxicity

The single cell gel (SCG)/Comet assay was used to detect DNA damage (e.g., DNA single-strand breaks, alkali-labile sites, DNA—DNA/DNA—protein cross-linking and single-strand breaks associated with incomplete excision repair sites) (Tice et al., 2000). The alkaline version of the Comet assay was performed according to the procedure originally described by Singh et al. (1988) as modified by Jalava et al. (2010b). The comet analysis was conducted in ethidium bromide-stained slides (100 cells per concentration) using the Comet assay IV (Perceptive Instruments Ltd., UK) image analysis system. The comet response parameter used in the statistical analysis was Olive tail moment (OTM) \([(\text{tail mean} – \text{head mean}) \times \text{tail}\% \text{DNA} / 100]\).

2.9. Statistical analysis

Levine’s test for equality of variances was used for all the samples before analyzing the data with the analysis of variance (ANOVA). The results from exposures to actual particulate samples were tested against corresponding blanks as well as with regard to particle dose. Paired comparisons between the different particulate doses and combustion condition were made by Dunnett’s test. ANOVA and Tukey’s test were used in the comparison of the differences between the combustion technologies. The cell-cycle and comet results were tested with the non-parametric Kruskall–Wallis test. The correlation analyses between the toxicological responses and chemical compositions were made by Spearman’s rank correlations. All the differences were regarded as statistically significant at \(p < 0.05\). The data were analyzed using the PASW-statistics version 17.0 (SPSS Inc. Chicago, IL, USA).

3. Results

3.1. Chemical composition of the samples

The chemical composition of the samples is presented in Table 2. Inorganic ash components were enriched in the emission samples from modern technology furnaces when calculated for same emission sample mass. There were substantial differences especially in the Zn concentrations, that were over 100-fold between old technology log wood boiler and pellet boiler derived samples. In addition, concentrations of other metals were higher in the modern technology samples. In contrast, PAH concentrations were substantially higher in the emissions from old technology appliances. The difference in PAH concentration between old technology stove and wood chip boiler representing the extreme ends of the PAH concentrations was almost 300-fold. Furthermore, when the emissions from old technology log wood boiler and stove were compared to the new technology log wood boiler and stove, they produced 10 and 16 fold PAH concentration, respectively.

3.2. Inflammation

The responses to blank sample and unexposed control did not significantly differ from each other in their inflammatory responses. Therefore unexposed controls were used in statistical comparison of the dose responses. All the samples evoked a dose dependent increase in the TNF\(_{a}\) responses; although the response seen with highest dose was sometimes reduced due to the extensive cell toxicity. Overall, the TNF\(_{a}\) responses were low, being little more than twice the control levels (Fig. 1). Most commonly the two highest doses were found to increase TNF\(_{a}\) production statistically significantly. Particulate samples from the log wood boiler, the tiled stove and the woodchip boiler representing new technology were somewhat less potent inducers of TNF\(_{a}\) production than the rest of the emission samples. The only statistically significant difference compared to the other samples was seen with the old technology log wood boiler. Due to extensive cytotoxicity of old technology logwood boiler sample, the TNF\(_{a}\) response was clearly lower with the highest dose used.

The MIP-2 responses were more intense than those of TNF\(_{a}\). (Fig. 2) Particles from old technology logwood boiler were statistically significantly more potent in their ability to increase MIP-2 production, when compared to the other samples. Extensive cytotoxicity affected also the MIP-2 response at the largest dose. All samples, except to that from the pellet boiler, caused a dose-dependent, statistically significantly elevation of MIP-2 responses in the macrophages. The PM samples from the pellet- and the woodchip boiler were statistically significantly less potent inducers.
of MIP-2 production in macrophages than either of the old technology furnaces studied.

### 3.3. Cytotoxicity

All the samples caused a dose dependent, statistically significant decline in cell viability/mitochondrial function when assessed with the MTT-test (Fig. 3). The new technology stove provided the least and the old technology log wood boiler the most cytotoxic of the samples. However, the new technology stove sample caused significant cytotoxicity already at the lowest dose. The new technology stove and the logwood boiler emitted less cytotoxic emission particles than the corresponding old technology furnaces but the level of statistical significance was reached only in the comparison between the old and new technology logwood boilers. Extensive cytotoxicity was also seen when the macrophages were exposed to samples from the tiled stove, the woodchip and the pellet boilers which all represented new combustion technology. The viabilities of the unexposed control and blank sample did not differ significantly from each other.

With the PI-exclusion method, the magnitude of the responses varied considerably between the samples (Fig. 4) and responses by the emission particles of the new and old technology displayed very large differences between each other. Most of the samples caused statistically significant, dose dependently increasing responses. The PI exclusion method assesses the permeability of the cell membranes and thus, the fate of the cells. The particulate mass derived from the old technology logwood boiler evoked the largest response among the samples, at the largest dose causing almost all the cells to be PI-positive. The old technology stove derived sample was the second most potent among the samples. All the other samples had rather modest response levels. The new technology logwood boiler derived sample caused cytotoxicity in this method. The unexposed control and the response by blank sample did not differ from each other.

### 3.4. Genotoxicity

All the samples exhibited a dose dependent, statistically significant increase in genotoxicity as indicated by OTM value
f) WCB (woodchip boiler) and g) PLB (pellet boiler).

OT (old technology stove); d) ST NT (new technology stove); e) TL ST (tiled stove); f) WCB (woodchip boiler) and g) PLB (pellet boiler).

Tukey and Dunnett

emission particle samples.

of the combustion decreases the genotoxic responses caused by the

macrophage cell line. Overall, it seemed that the completeness

the level of their own in being able to cause a genotoxic response in

Old technology stove samples appeared to be the most potent

inducers of apoptotic responses (SubG1 phase of the cell cycle

analysis) (Fig. 6). Tiled stove, woodchip- and pellet boiler induced

significant apoptosis at least at some of the doses. The same samples

causd also a reduction in the cell numbers in the normal resting

phase (G1). Old technology logwood boiler samples caused accu-
mulation of the cells to the G2/M phase of the cell cycle. This may be

(Fig. 5). However, the response levels from the new technology

log wood boiler and the pellet boiler derived samples could be

considered as low or very low. Both old and new technology stoves

evoked increased genotoxicity, old technology samples being more

potent. With the old technology logwood boiler samples were at

the level of their own in being able to cause a genotoxic response in

the macrophage cell line. Overall, it seemed that the completeness

of the combustion decreases the genotoxic responses caused by the

emission particle samples.

3.5. Cell cycle

Old technology stove samples appeared to be the most potent

inducers of apoptotic responses (SubG1 phase of the cell cycle

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Fig. 1. Tumor necrosis factor alpha (TNFα) responses (pg ml⁻¹) after 24 h exposure to

PM₁₀ emission samples from different combustion appliances. Bars present control and

four doses (15, 50, 150 and 300 μg ml⁻¹) and whiskers the standard error of mean (SEM).
Asterisks indicate statistical significance compared to control (p < 0.05) ANOVA and Dunnett’s test. Letters indicate statistically larger response (p < 0.05) ANOVA, Tukey’s test compared to other appliances. a) LWB OT (old technology log wood boiler); b) LWB NT (new technology log wood boiler); c) ST OT (old technology stove); d) ST NT (new technology stove); e) TL ST (tiled stove); f) WCB (woodchip boiler) and g) PLB (pellet boiler).

Fig. 2. Macrophage inflammatory protein 2 (MIP-2) responses (pg ml⁻¹) after 24 h

exposure to PM₁₀ emission samples from different combustion appliances. Bars represent control and four doses (15, 50, 150 and 300 μg ml⁻¹) and whiskers the standard error of mean (SEM). Asterisks indicate a statistical significance compared to control (p < 0.05) ANOVA and Dunnett’s test. Letters indicate statistically larger response (p < 0.05) ANOVA, Tukey’s test compared to other appliances. a) LWB OT (old technology log wood boiler); b) LWB NT (new technology log wood boiler); c) ST OT (old technology stove); d) ST NT (new technology stove); e) TL ST (tiled stove); f) WCB (woodchip boiler) and g) PLB (pellet boiler).

Fig. 3. Cell viability assessed with MTT test after 24 h exposure to PM₁₀ emission samples from different combustion appliances. Bars present control and four doses (15, 50, 150 and 300 μg ml⁻¹) and whiskers the standard error of mean (SEM). Asterisks indicate statistical significance compared to control (p < 0.05) ANOVA and Dunnett’s test. Letters indicate statistically larger response (p < 0.05) ANOVA, Tukey’s test compared to other appliances. a) LWB OT (old technology log wood boiler); b) LWB NT (new technology log wood boiler); c) ST OT (old technology stove); d) ST NT (new technology stove); e) TL ST (tiled stove); f) WCB (woodchip boiler) and g) PLB (pellet boiler).

Fig. 4. Cell membrane permeability (cytotoxicity) assessed with PI exclusion test after

24 h exposure to PM₁₀ emission samples from different combustion appliances. Bars represent control and four doses (15, 50, 150 and 300 μg ml⁻¹) and whiskers the standard error of mean (SEM). Asterisks indicate statistical significance compared to control (p < 0.05) ANOVA and Dunnett’s test. Letters indicate statistically larger response (p < 0.05) ANOVA, Tukey’s test compared to other appliances. a) LWB OT (old technology log wood boiler); b) LWB NT (new technology log wood boiler); c) ST OT (old technology stove); d) ST NT (new technology stove); e) TL ST (tiled stove); f) WCB (woodchip boiler) and g) PLB (pellet boiler).
due to cell cycle arrest that occurs when cells are trying to repair damaged DNA. The tiled stove sample affected the cell cycle even though the samples did not appear to be very potent inducers of other responses in macrophages. Blank levels were similar to those of unexposed controls.

3.6. Effects of chemical composition

The correlation coefficients between the detected toxicological parameters and chemical composition of the particulate samples from the different furnaces are shown in Tables 3 and 4. Most of the inorganic constituents displayed negative correlations with the detected toxicological responses. In contrast, PAH compounds were consistently associated with the increased inflammatory responses and moreover, they were linked to the disrupted cell cycle in the macrophages as indicated via apoptosis and the decreasing proportion of cells in their normal resting phase. Overall, these findings indicate that in this experiment with seven different furnaces, those devices achieving more complete combustion were the least potent in each of the toxicological parameters. In contrast, emission particles from the old technology furnaces which represented more incomplete combustion in this experimental setup were the most potent inducers of toxicological responses. These differences were responsible for the negative correlation for the inorganic ash compositions and the positive correlations for PAH and other organic compositions.

3.7. Toxicological effects weighed with emission factor

When the emitted particulate mass calculated for the same produced energy (mg MJ⁻¹) was taken into account in the evaluation of the toxicological potency between the samples, large differences were revealed when compared to those derived from mass based calculations (Table 5). From the appliances that were used in the present study, the old technology logwood boiler emitted the largest particulate mass (106 mg MJ⁻¹) and the toxicological potency in most parameters was also large. Emissions from the old technology logwood boiler were estimated as being up to 570 times more potent than the emissions from the pellet boiler, in terms of the genotoxic response. With emission factor weighed results, this appliance type exceeded the relative harmfulness of all the other devices in terms of all parameters. In addition the old technology stove was emitting substantial amount (74 mg MJ⁻¹) of particulate mass, and the genotoxic and inflammatory parameters were increased in macrophages. The new technology stove had a similar toxicological profile yet with a slightly lower level when compared to the corresponding old technology stove, but it did emit significantly less particulate mass per MJ. These furnaces were followed by decreasing emissions from the tiled stove, the new technology logwood boiler and the woodchip boiler. Finally, the pellet boiler had the smallest emissions, which also caused the smallest toxicological responses in the cells. This emphasizes the low toxicological potential of this sample in mouse macrophages.

4. Discussion

We studied the particulate emissions and their toxicological responses with seven different small scale heating appliances representing the types of modern and old biomass combustion furnaces that are in widespread use in households all over Central and Northern Europe. The role of the combustion technology on the ambient air quality has been previously observed in the woodstove change out programme in Libby MT (Bergauff et al., 2009). The toxic potential of the emissions seemed to be determined by the combustion quality as seen previously by Jalava et al. (2010a) and Danielsen et al. (2011). Thus, the effect of combustion technology may be important in determining the toxicological potency of the particulate samples. However, it must be remembered that these results only represent differences between the furnaces used in the present study. The high emitting old technology furnaces may have multifold impacts on the local air quality and consequently in the potential adverse health effects in the population when compared to the low emission new technology appliances as revealed in this extensive in vitro evaluation of toxicological effects of emissions from different combustion appliances. New biomass heating systems will be used in both replacing the old biomass based heating systems but
also the heating systems based on oil combustion and electricity. Therefore combustion technologies as good as possible should be preferred to avoid the excess particulate exposures of the people.

4.1. Emissions from the furnaces

The furnaces examined in the present study had rather different emission profiles. This can be seen from the emission background information in Table 1 where there were as much as 17-fold differences in \( PM_1 \) emissions between the best (pellet boiler) and the worst (old technology logwood boiler) appliances. Moreover, the differences between the furnace types for the emissions of gaseous organic compounds were almost 400-fold and for the PAH the differences between the furnaces examined in the present study had rather different emission profiles. This is most probably applicable combustion quality monitoring with the previous results about poor and ordinary batch state-of-the-art heating systems, even more importantly in batch combustion also wet fuels almost completely. Thus, also in the new, small scale systems. Instead, larger scale systems are able to combust also wet fuels almost completely. Thus, also in the new, state-of-the-art heating systems, even more importantly in batch operated, the combustion quality is largely dependent on the fuels and user related practices.

4.2. Toxicological responses induced by the particulate samples

Emissions from residential wood combustion are responsible for local air pollution and greater exposure and adverse health effects of the people living in their vicinity (Schreuder et al., 2006; Boman et al., 2003). The present in vitro data showed clearly that the emission particles from the old technology logwood boiler were the most potent in their ability to cause cytotoxic responses in macrophages. They also disturbed the normal cell cycle and markedly increased genotoxicity. Instead, the emissions of new technology logwood boiler with air staging, and automated lambda control were significantly less harmful in this respect. The responses by the woodchip- and pellet boiler derived samples triggered consistently the lowest toxicological responses, except for cytotoxicity. Increased acute cytotoxicity has also been seen previously with samples representing good combustion quality (Jalava et al., 2010a; Tapanainen et al., 2011). This may be due to inorganic components of ash damaging the cells and causing cytotoxicity. Combined with the fact that these ash-rich samples have also greater metal ratio compared to the organic compounds, those may well be, at least partially, responsible for the cellular effects. However, this hypothesis does not fully account for the low inflammatory potential of these samples. The samples from other new technology furnaces also induced only modest or low responses in the toxicological analyses. Thus, the lower toxicological response levels were mostly associated with the appliances that had smaller emission rates. The finding is in line with our previous studies (Jalava et al., 2010a; Tapanainen et al., 2011).

4.3. Cytotoxicity induced by the particulate samples

There were large differences between the cytotoxic potentials of emissions from the studied furnaces. As detected with MTT-test, the old technology logwood boiler had the largest response levels with the two largest doses. Moreover, samples from the old technology logwood boiler derived samples seemed to have a certain cytotoxic potential, but not as high as the emissions from the studied furnaces. As detected with MTT-test, the old technology logwood boiler had the largest response levels with the two largest doses. Moreover, samples from the old technology logwood boiler derived samples seemed to have a certain cytotoxic potential, but not as high as the emissions from the studied furnaces.
threshold dose level that caused a major decline in the viability of the cells. Such large differences were not observed between the other appliances and in fact, acute cytotoxicity was initiated by smaller concentrations of the samples from the wood chip boiler and the pellet boiler. Instead, in the PI-exclusion method, the two smaller concentrations of the samples from the wood chip boiler and the pellet boiler induced consistently more cell death than all of the new technology appliances. With respect to programmed cell death, the old technology stove and the tiled stove were the most potent samples. Wood combustion particles have also been shown previously to induce cell death (Kocbach et al., 2008a) but in that experiment the samples did not significantly increase apoptosis. These discrepancies between the different parameters of cytotoxicity show that emission particulate samples are able to induce different types of cellular damage. It is most likely the cytotoxic responses of pellet boiler and wood chip boiler derived samples were associated with the inorganic ash composition of the samples. Moreover, the activated cellular pathway leading to cytotoxicity associated with these furnaces is probably different from that of the old technology logwood boiler. This interpretation is also supported by the present finding that particulate samples from old technology appliances evoked generally larger genotoxic responses than those emerging from new technology devices. Similar differences have been observed in comparison of good and poor batch combustion in the same heater (Jalava et al., 2010a) and in comparison of conventional batch combustion with continuous combustion in pellet boiler (Tapanainen et al., 2011). Overall, the present results suggest that the combustion conditions and type of combustion (batch, continuous combustion, air staging, and automation) as well as the combustion appliance type influence the relative harmfulness of the particulate emissions. This has implications in understanding the mechanisms behind the adverse health effects. In this respect, the automated operation new technology furnaces seem to have the least harmful emissions among the studied appliances in the present study.

4.4. Inflammatory responses

In the present study, the inflammatory responses were often low at the most only moderate. There is supporting epidemiological evidence in line with this finding. It has been observed that combustion generated PM fraction of ambient air is responsible for impaired lung functions whereas other components evoke an inflammatory response (Allen et al., 2008). This may explain the latter discrepancies of the associations between chemical compounds and evoked responses in the correlation analysis. With respect to the chemokine MIP-2 response, the samples representing old combustion technology evoked larger responses than those from the modern appliances. This is supported by our previous studies on normal and poor batch combustion (Jalava et al., 2010a) and also by the study of Danielsen et al. (2011). MIP-2 has a specific role in initiating inflammation by recruiting other immunological cells to the site of inflammation. It is possible that cytotoxicity either increases the levels of this chemokine or alternatively the proteins are produced before the cytotoxicity is able to disturb the protein synthesis. In the present study, the differences in composition of the emissions indicate different roles of ash and soot compositions in the toxicological responses. It has also been seen previously that the small (1 μm) size range of the particles does not cause extensive inflammatory responses in the macrophages (Jalava et al., 2007) and that cytotoxicity may decrease the cytokine levels. Moreover, the increased solubility of the particles has decreased the responses in macrophages (Jalava et al., 2008). In the present study, secondary mechanisms may have either up- or down-regulated the inflammatory responses which will be further discussed in the methodological considerations section. Overall, these low inflammatory responses are in line with previous studies with wood combustion particles (Kocbach et al., 2008a,b; Karlsson et al., 2006).

4.5. Genotoxicity of the particulate samples

The wood combustion derived particulate samples have previously been shown to cause DNA damage in toxicological settings (e.g. Karlsson et al., 2006; Danielsen et al., 2009, 2011) which together with increased cell proliferation may lead to lung cancer (Knaapen et al., 2004). In the present study, both old technology furnaces and new the technology stove evoked marked genotoxicity in the SCG/Comet assay. For other samples, the genotoxic responses were small, even though they were all statistically significantly increased. The genotoxic response seemed to be largely dependent on the combustion quality. The compounds most likely responsible for these responses are PAHs that are known to cause genetic damage by forming adducts with DNA (WHO-IPCS, 1998; Knaapen et al., 2004). PAH compounds cause also oxidative DNA damage in cell lines (Leonard et al., 2000; Karlsson et al., 2006; Danielsen et al., 2009). Some of the DNA damage observed in the present study may be related to the extensive cytotoxicity seen with the highest sample doses.

4.6. Effects of chemical composition on toxicological responses

We have recently shown that the chemical composition of urban air fine particles influences their toxicological responses in a highly complex manner (Jalava et al., 2007, 2009; Hoppo et al., 2008, 2010b). This is most probably the case also with combustion derived particles, but the mechanisms involved may be different because of their strong cytotoxic activity. Particle-induced DNA-damage and increased cell proliferation might lead to the development of lung cancer in humans (Knaapen et al., 2004). In the present study, particulate samples induced significant DNA-damage in mouse macrophages. These cells are not specifically the target cell type for genotoxic effects. However, the genotoxic effects in macrophages and epithelial cells have correlated well in our analyses (unpublished results). The particulate samples from old technology heaters in this...
setting can be considered significantly more harmful due to their large genotoxic potential. These findings are in good agreement with various in vitro studies demonstrating the genotoxicity of particles from incomplete biomass combustion (Leonard et al., 2000; Karlsson et al., 2006; Danielsen et al., 2008, 2009, 2011). The genotoxicity of the particles can also occur via different mechanisms than direct strand breaks. Also PAH-DNA adducts can be formed during the repair mechanisms (Tarantini et al., 2009). PAH compounds were positively associated with the inflammatory activity in macrophages in this study. However, it cannot be concluded that they would be the causative components instead they may simply be surrogates of the combustion situation. In the previous urban air studies, PAH concentrations have consistently decreased inflammation (Jalava et al., 2009; Happo et al., 2008) most probably due to immunomodulating effects of these compounds (Li et al., 2009). However, there are also opposite findings in cases of small differences in the composition between the samples (Steerenberg et al., 2006; Happo et al., 2010a,b). The PAHs present may also be converted to even more potent chemicals in the atmosphere due to photochemical transformation (Squadrito et al., 2001). These components affect the redox-cycling processes and produce radicals that evoke oxidative stress. The emissions from the old technology furnaces caused apoptosis in the macrophages which can be viewed as evidence for the failure of some intracellular repair mechanisms, and the response is closely linked to genotoxicity. A similar effect was also seen in our earlier study with a wood-smoke rich PM2.5 sample from Prague (Jalava et al., 2009; Happo et al., 2008).

Elemental composition was mostly associated with either minor proinflammatory responses or even negligible effects. Instead, chemokine MIP-2 production was associated with the relatively small elemental composition, compared to the organic compounds which displayed negative correlations with most of the detected elements. Previously, it has been observed that the metal composition increases the inflammatory potency of urban air particulate samples (Merolla and Richards, 2005; Frampton et al., 1999). This was however not the case in the present study with small scale wood combustion particles. It is possible that the higher combustion temperatures in the more complete combustion processes cause more vaporization of the metals. Moreover, the elemental composition had a very limited effect on the cytotoxicity detected with MTT test. According to the correlation analyses, it seemed that these correlations were almost completely driven by the organic compositions from the soot rich incomplete combustion. Consequently, the relatively large metal concentrations were indirectly associated to lower toxicological potency. This result again suggests that the extreme ends of the combustion quality drive the correlations and does not necessary mean the metal concentrations to be insignificant. Indeed, there were indications that the ash rich samples from good combustion induced cytotoxicity even at lower doses than those of poor combustion.

In general, combustion particles have a tendency toward lower inflammatory potential than urban air fine particles, which may be attributable to their selected and more uniform chemical composition. (Jalava et al., 2010a,b; Happo et al., 2008). Moreover, in our previous study with RAW264.7 macrophages, we found that the insoluble components are the most potent and that organic or water soluble extract of urban air particles have only minor effects on inflammatory parameters (Jalava et al., 2008). In urban air, the overall mixture varies extensively in terms of its inorganic and organic compositions more than any difference in the chemical composition in particles originating from a single source.

4.7. Relative harmfulness of the emission particles

The toxicological experiments in this study were conducted with the same mass doses regardless of the emitted particulate mass from the furnaces. Therefore, the detected cytotoxic and inflammatory responses of the emission particulate samples were weighed with the emission factor (mg MJ⁻¹). This was done in order to gain an insight into the particulate exposures in real-life situations. It has been observed that wood combustion emissions may increase the exposure of the people living in areas with a high density of wood combustion (Boman et al., 2003; Schreuder et al., 2006; Allen et al., 2008) The energy unit-weighed toxicity results emphasized the detrimental impact of old technology furnaces in the toxicological responses and their possibly larger role on the local air quality. This kind of effect on the local air quality has been seen when large numbers of stoves have been changed to newer and better combustion quality appliances (Bergauff et al., 2009). The improvement of air quality was not seen in outdoor, but also in indoor air where the PM concentrations were reduced substantially (Ward et al., 2008). Overall, the relative harmfulness of the particles is largely dependent both on the completeness of the combustion and on the emitted particulate mass.

4.8. Methodological considerations

The present results revealed major differences, between the particulate samples derived from different combustion appliances, in their toxicological responses in macrophages. These cells are the first in line of defense in the respiratory tract against inhaled substances. However, the adverse health effects in humans are evoked in various cell types and also in other organs than lungs after particulate exposure. Moreover, even though there were many different types of furnaces included in this study the results represent only these and should not be generalized to comparison of new and old technology. Therefore, no final conclusion of the results can be drawn on the basis of these cell experiments only.

One important issue to be considered is the sample treatment after the collection. The samples were collected using high quality standards and all potential contamination sources were excluded as far as possible. The weighings and sample preparations were well controlled as well. However, the sample extractions from the filter potentially change the particulate properties and at least it may be possible that the particles do not occur in the original size. The small difference in extraction efficiencies between the samples may have caused slightly uneven recovery of different compounds that may affect the toxic properties of the samples. Furthermore, when the methanol-particulate-suspension is evaporated from the sample tubes, the particles become attached very strongly to the walls of the tubes. Therefore, a small, non-toxic amount of DMSO is needed in the suspension to dissolve it in an aqueous solution. The dose has been tested as being non-toxic, but it cannot be certain that it may slightly alter the particle properties and their reactivity/transportation inside the cells. Moreover, DMSO may interfere with reactive oxygen species related mechanisms of toxicity.

There was extensive cytotoxicity caused by some of the samples. The low levels of inflammatory mediators could be due to cell death. Moreover, the generally small differences in the inflammatory parameters tend to decrease the reliability of any correlation analyses. When the associations of different response parameters were calculated in the correlation analysis, it was observed that the cytotoxicity as assessed by the MTT test did not affect the production of inflammatory mediators. However, when the cell membrane permeability increased, also the levels of inflammatory mediators also increased. This may be due to the release of intracellular cytokines to the culture medium directly through the cell membrane. This effect is somewhat different to the active transport of the cytokines outside the cells and this kind of rapid release of cytokines can also cause a major effect in the lungs. In general, cell death could decrease the numbers of cells so extensively that it would reduce the
production of inflammatory mediators but this was obviously not the case in this data. One possible explanation for low inflammatory responses would have been the binding of cytokines to carbonaceous material (Kochbach et al., 2008c). However, in the present study we used FBS and BSA to avoid the non-specific binding of cytokines. Moreover, the cytokine responses were largest with the samples of largest carbon content. These together indicate the absence of this kind of binding in the present study. It simply seems that the composition of the combustion derived particulate samples do not cause extensive inflammation which is different from the situation for urban air samples with their high content of soil derived particles.

5. Conclusions

The present data revealed clear differences both in the emissions and their toxicological effects. It was found, based on the results from the furnaces used in the present study that modern technology furnaces emitted substantially less particulate mass and this mass was less harmful to cells than the corresponding particles emitted from old technology heaters. One open question how the atmospheric transformation and other components of the complex ambient mixture affect the potency of the different emissions in the atmosphere, remains open. This will need to be evaluated in further studies. Some insight can be obtained from Allen et al. (2008) who noted that the combustion generated aerosol was responsible for impaired lung functions in children. The present data also demonstrated clearly that toxicological methods can be applied in the development of new combustion technologies when planning strategies for attaining less harmful emissions from domestic biomass heating appliances and thus, cleaner air. However, the final conclusions for the health effects caused by the appliance types cannot, be drawn solely on the basis of the present macrophage results. In this assessment, the aerosols from modern residential biomass combustion systems were considerably less harmful than those emerging from comparable old systems both due to the reasons of considerably lower aerosol emissions as well as due to the lower toxicological potential of emissions from a good combustion. Therefore, low emission appliances should be preferred when replacing old biomass heating systems and especially when replacing the heating systems based on oil or electricity.

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