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Oskari Uski

Toxicological Effects of Fine Particles from Small-scale Biomass Combustion

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ABSTRACT

Biomass combustion in residential heating is recognized as one of the most important sources of fine particulate matter ($PM_{2.5}$: particles ≤ 2.5 μm in aerodynamic diameter) not only in the developing countries but also in the developed countries and in urban environments. Epidemiological studies have revealed that exposure to current levels of urban air inhalable particulate matter (PM_{10}: particles ≤ 10 μm in aerodynamic diameter) and $PM_{2.5}$ increases both mortality and morbidity. In addition to size, shape and mass concentration, the chemical composition of the PM is important in determining the adverse health effects. One putative factor in the outcomes induced may be traced back to the presence of inorganic transition metals in the ultrafine particles (particles ≤ 100 nm in aerodynamic diameter). Zinc is one of the main transition metals in ash emissions from biomass combustion. It is known to induce toxic effects in animal lung as well as in pulmonary cells. The aim of this thesis was to compare the toxic properties of particulate samples derived from old and modern small-scale wood combustion appliances using *in vivo* and *in vitro* models. A specific aim was to gather information on the possible role of zinc in these toxic effects.

Healthy mice were intratracheally exposed to emission particles from five new and two old technology appliances. It was found that even though the modern technology appliances had clearly the smallest PM_1 (mg/MJ) emissions, they induced the highest toxicological responses in the mouse lungs when equal doses were compared. Ash related PM components, such as transition metals (including Zn) of the emissions increased the inflammatory, cytotoxic and genotoxic responses, whereas the highest polycyclic aromatic hydrocarbon (PAH) concentrations were associated with immunosuppressive effects and thus low inflammatory responses.

In an attempt to verify the findings from the animal study, an *in vitro* mouse macrophage cell line (RAW 264.7) was used to characterize the role of zinc among the other chemical components of the emissions. The first step was to duplicate as

similar combustion conditions as utilized in previous animal experiments by using a grate combustion reactor. In the next step, synthesized nanoparticles were made containing defined amounts of potassium, sulfur and zinc. These constituents are the major components forming inorganic particles during wood combustion. Finally, the toxic role of zinc was investigated by burning pellets containing different concentrations of added zinc using similar pellet boiler as in the animal experiments. It was shown that both synthetic zinc nanoparticles as well as particles derived from combustion of zinc-enriched pellets exhibited a toxicity profile very similar to that found after exposure of macrophages to the particles from reactor-made efficient combustion. Moreover, other components (potassium and sulfur) found in efficient combustion PM emissions were not capable of evoking toxic responses.

In conclusion, zinc has an important role in the induced toxic effects of PM from efficient continuous wood combustion. In contrast, high concentrations of organic compounds (i.e. PAHs) are likely to induce immunosuppressive effects, especially from inefficient combustion conditions. If one wishes to prevent potential adverse health effects, more attention should be paid to the quality of the biomass fuel as well as by ensuring efficient combustion conditions to lower the total mass of particulate emissions.

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CAB Thesaurus: air pollutants; combustion; burning; wood; wood smoke; particles; aerosols; chemical composition; health hazards; toxicity; cytotoxicity; genotoxicity; inflammation; lungs; histopathology; reactive oxygen species; metals; transition elements; zinc; mice

TIIVISTELMÄ

Kotitalouksien biomassan polton tiedetään olevan yksi tärkeimmistä pienhiukkasten ($PM_{2.5}$: hiukkaset ≤ 2,5 μm aerodynaaminen halkaisija) lähteistä, ei vain kehitysmaissa, vaan myös kehittyneissä maissa. Epidemiologisissa tutkimuksissa on havaittu kaupunki-ilman hengitettävien hiukkasten (PM_{10}: hiukkaset ≤ 10 μm aerodynaaminen halkaisija) ja erityisesti pienhiukkasten lisäävän kuolleisuutta ja sairastuvuutta. Haittavaikutusten syntyyn vaikuttaa hiukkasten koon, muodon ja massapitoisuuden lisäksi niiden kemiallinen koostumus. Yhdeksi haittavaikutuksia aiheuttavaksi tekijäksi on ehdotettu ultrapienissä hiukkasissa (hiukkaset ≤ 100 nm aerodynaaminen halkaisija) esiintyviä siirtymämetalleja. Sinkki on yksi tärkeimmistä biomassan poltossa vapautuvien tuhkahiukkasten siirtymämetalleista. Sinkin tiedetään aiheuttavan toksisia vasteita niin koe-eläinten keuhkoissa kuin viljellyissä keuhkosoluissa. Tämän väitöskirjan tavoitteena oli tutkia modernien ja perinteisten puun polttolaitteiden hiukkaspäästöjen haittavaikutuksia, sekä erityisesti sinkin osuutta todetuissa vasteissa. Tutkimukset toteutettiin käyttäen koe-eläin- ja solumallia.

Hiukkasten toksikologisia vaikutuksia tutkittiin terveillä hiirillä, jotka altistettiin intratrakeaalisesti viidelle modernin ja kahdelle perinteisen kotitalouskokoluokan polttolaitteen hiukkasille. Modernien puunpolttolaitteiden päästöt olivat pienimmät tuotettua energiayksikköä (mg/MJ) kohti, mutta samalla aiheuttivat voimakkaimmat toksiset vasteet hiiren keuhkoissa kun hiukkasnäytteitä annosteltiin massaperusteisesti. Hiukkasissa olevat tuhkakomponentit kuten siirtymämetallit (mukaan lukien sinkki) liittyivät havaittuihin tulehduksen, solukuoleman ja perimävaurion vasteisiin. Sitä vastoin hiukkasissa olevat polysykliset aromaattiset hiilivedyt (PAH) liittyivät immuunivasteen estymiseen ja näin ollen matalaan tulehdukseen.

Eläinkokeiden tuloksia tutkittiin tarkemmin hiiren makrofagisolulinjassa jotta sinkin ja muiden kemiallisten

komponenttien roolia puunpolton päästöissä ymmärrettäisiin paremmin. Solukokeita varten valmistettiin polttoreaktorissa samankaltaisia hiukkasia kuin eläinkokeissa oli tutkittu. Seuraavaksi syntetisoitiin nanohiukkasia jotka sisälsivät ennalta määrätyn pitoisuuden kaliumia, rikkiä ja sinkkiä. Nämä ovat tärkeimpiä komponentteja, jotka muodostavat epäorgaanisia pienhiukkasia puunpoltossa. Lopuksi tutkittiin sinkin toksista roolia päästöhiukkasissa polttamalla pellettikattilassa koetta varten valmistettuja pellettejä, joihin oli lisätty tunnettuja pitoisuuksia sinkkiä. Tulokset osoittivat että synteettisillä sinkki-nanohiukkasilla sekä sinkillä rikastettujen pellettien polton hiukkasilla oli samankaltainen toksisuusprofiili kuin tehokkaan palamisen näytteellä, joka oli peräisin polttoreaktorista. Sen sijaan muut lähes täydellisessä puun palamisessa vapautuvien hiukkasten pääkomponentit (kalium ja rikki) eivät aiheuttaneet havaittavia toksisia vasteita.

Tässä väitöskirjassa saatujen tulosten perusteella voidaan sanoa, että sinkillä on merkittävä rooli jatkuvatoimisten polttolaitteiden päästöhiukkasten aiheuttamien toksisten vasteiden synnyssä. Sitä vastoin epätäydellisessä palamisessa vapautuvat orgaaniset yhdisteet aiheuttavat todennäköisesti immuunivasteen heikentymistä. Näin ollen hiukkasten terveyshaittojen vähentämiseksi on erityisen tärkeää että kiinnitetään enemmän huomiota biopolttoaineiden laatuun. Tämän lisäksi on tärkeää polttaa biopolttoaineita optimoiduissa olosuhteissa päästöjen hiukkasmassan vähentämiseksi.

Yleinen suomalainen asiasanasto: ilma - epäpuhtaudet; pienhiukkaset; aerosolit; poltto; palaminen; biopolttoaineet; puu; päästöt; savukaasut; kemiallinen koostumus; terveysvaikutukset; terveyshaitat; myrkyllisyys; tulehdus; keuhkot; happiradikaalit; metallit; sinkki; hiiret

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary disease
DCF	2′,7′- dichlorodihydrofluorescein
DGI	Dekati® Gravimetric Impactor
DMSO	Dimethyl sulfoxide
Dp	Particle diameter
EDS	Energy dispersive X-ray spectrometry
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FSP	Flame spray pyrolysis
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HPLC-MS	High-performance liquid chromatograph mass spectrometer
HSD	honest significant difference
IC	Inorganic carbon
ICP/MS	Inductively-coupled plasma mass spectrometer
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
KC	Keratinocyte-derived chemokine
LDH	Lactate dehydrogenase
LPA	Lymphocyte proliferation assays
MIP-2	Macrophage inflammatory protein 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NBT	Nitroblue tetrazolium
NO	Nitric oxide
NP	Nanoparticle
NT	New technology
OC	Organic carbon
OGC	Organic gaseous carbon

OT	Old technology
OTM	Olive tail moment
OVA	Ovalbumin
PAH	Polycyclic aromatic hydrocarbon
PBMC	Peripheral blood mononucleate cell
PBS	Phosphate-buffered saline
PI	Propidium iodide
PM_x	Particulate matter with aerodynamic diameter less than x μm
PM_{x-y}	Particulate matter with aerodynamic diameter between x and y μm
PRD	Porous tube diluter
PTFE	Fluoropore™ membrane filter
RAW 264.7	Abelson murine leukemia virus transformed mouse macrophage/monocyte cell line
RNS	Reactive nitrogen species
ROFA	Residual oil fly ash
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
SCGE	Single cell gel electrophoresis assay
SEM	Standard error of the mean
SIM	Selected ion monitoring
TC	Total carbon
TEM	Transmission electron microscopy
THC	Total hydrocarbon
TNF-α	Tumor necrosis factor α
VOC	Volatile organic compound

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals I-V.

I Happo M S, Uski O, Jalava P I, Kelz J, Brunner T, Hakulinen P, Mäki-Paakkanen J, Kosma V M, Jokiniemi J, Obernberger I and Hirvonen M R. Pulmonary inflammation and tissue damage in the mouse lung after exposure to PM samples from biomass heating appliances of old and modern technologies.
Science of the Total Environment 443: 256-266, 2013.

II Uski O, Happo M S, Jalava P I, Brunner T, Kelz J, Obernberger I, Jokiniemi J and Hirvonen M R. Acute systemic and lung inflammation in C57Bl/6J mice after intratracheal aspiration of particulate matter from small-scale biomass combustion appliances based on old and modern technologies.
Inhalation Toxicology 14: 952-965, 2012.

III Uski O, Jalava P I, Happo M S, Leskinen J, Sippula O, Tissari J, Mäki-Paakkanen J, Jokiniemi J and Hirvonen M R. Different toxic mechanisms are activated by emission PM depending on combustion efficiency.
Atmospheric Environment 89: 623-632, 2014.

IV Torvela T, Uski O, Karhunen T, Lähde A, Jalava P I, Sippula O, Tissari J, Hirvonen M R and Jokiniemi J. Reference particles for toxicological studies of wood combustion: formation, characteristics and toxicity compared to real wood combustion PM.
Chemical Research in Toxicology 27(9): 1516-1527, 2014.

V Uski O, Jalava P I, Happo M S, Torvela T, Leskinen J, Mäki-Paakkanen J, Tissari J, Sippula O, Lamberg H, Jokiniemi J and Hirvonen M R. Zinc enhances toxicological responses of PM_1 from pellet combustion *in vitro*. Submitted.

AUTHOR'S CONTRIBUTION

The publications in this dissertation are original research papers on how PM emission from wood combustion can exert biological effects.

In vivo studies (Study I and II): The author participated in conducting the toxicological animal experiments, analysis of the samples, and preparation of manuscript I. Manuscript II was mainly written by author.

In vitro studies (Studies III-V): The author participated in planning and conducting all of the toxicological experiments and carried out the toxicological analysis of the samples. The author also performed the statistical analysis presented in figures and tables. The author wrote manuscripts III and V and mainly wrote the toxicological parts of manuscript IV.

All papers were finalized in significant co-operation with the co-authors.

Contents

1 Introduction

Airborne particulate matter (PM) originates from many different sources, e.g. traffic, industry and energy production. PM is known to cause adverse health effects in humans (WHO 2004). Fine particles ($PM_{2.5}$: particles ≤ 2.5 μm in aerodynamic diameter) are regarded as one of the most harmful pollutants present in ambient air (WHO 2003; USEPA 2004; Pope & Dockery, 2006). Residential biomass combustion is a major source of those particles in both the developing and the developed countries (Boman et al., 2003; Saarikoski at al., 2008; Krecl et al., 2008; Favez et al., 2009; Ward & Lange, 2010; Zhang et al., 2010). In epidemiological studies wood smoke exposure has been linked to increased incidence of asthma and respiratory symptoms (Boman et al., 2003; Allen et al., 2008; Ghio, 2008; Mirabelli et al., 2009), cardiovascular effects and increased hospital admissions of cardiorespiratory patients (Orozco-Levi et al., 2006; Schreuder et al., 2006; Andersen et al., 2007; Sarnat et al., 2008). Moreover, epidemiological studies which have been conducted in the developing countries have detected a high incidence of lung cancer in those women who use open fire stoves indoors (Xiao et al., 2012; Hu et al., 2014). There is also increasing evidence from both *in vivo* and *in vitro* experiments as well as from controlled human studies, demonstrating that exposure to fresh biomass combustion particles is able to evoke adverse effects, including inflammation, cytotoxic effects, genotoxic effects, oxidative stress and arterial stiffness (Barregard et al., 2006; Sevastyanova et al., 2007; Jalava et al., 2012; Unosson et al., 2013).

In addition to the direct health hazards, it is well known that atmospheric aerosols can influence climate (IPCC, 2013), and thus may cause indirect health impacts (Gabriel & Endlicher, 2011; Yardley et al., 2011). Wood combustion produces soot, which warms the atmosphere by absorbing solar radiation

(Jacobson et al., 2010). Furthermore, incomplete wood combustion produces greenhouse gases such as nitrous oxides and methane (Brassarda et al., 2014). Nonetheless, the usage of fossil fuel for heat production is discouraged, since its emissions are considered harmful to climate and the exploitation of renewable energy sources is strongly encouraged as a way to reduce greenhouse gas emission (EU Directive 2009/287EC; The President's Climate Action Plan, June 2013). This policy will increase all kinds of biomass energy in the future.

The emissions from small-scale biomass combustion are not only dependent on the technology used in the heating appliance, the fuel quality and operating conditions, such as load and the air staging settings are also key factors determining the amount and the composition of particles being emitted from the heater. Thus, emissions from small-scale combustion have been demonstrated to be extremely heterogenic between appliances (Nussbaumer, 2003; Johansson et al., 2003, 2004; Sippula et al., 2007a) as well as between operation practices with the same appliance (e.g. Jordan & Seen, 2005; Leskinen et al., 2014).

Overall, there is only limited amount of data available on the toxicological mechanisms behind the reported adverse health effects of PM from small-scale wood combustion. In particular, there is a lack of studies which would have compared the physical and chemical properties of the PM (e.g. particle size and morphology, number and mass concentration, chemical composition) with their induced toxicological end points (e.g. Kelly & Fussell, 2012). This kind of multidisciplinary data are urgently needed to help in the new formulation of ambient air quality standards and when targeted PM control strategies are planned. In particular, focused restrictions on the most toxic constituents of $PM_{2.5}$ and their sources could protect the general public more efficiently than adherence to the current PM mass based standards (Mauderly et al., 2010).

This thesis studied the significance of different chemical composition influencing the toxicity of fine PM emission from new (NT) and old (OT) technology small-scale wood

combustion appliances. More specifically, the goal was to increase knowledge concerning the toxicity of emissions from NT wood combustion furnaces and to evaluate the possible causative role of zinc in the emission PM. The investigation was based on animal model and cell line experiments which involved an extensive physiochemical analysis of the particles with respect to health related toxicological endpoints. The literature review part of this thesis concentrates on the PM emissions from wood combustion and their toxic effects.

2 Literature review

Inhalable aerosols in the air form a complex mixture of solid, liquid and gaseous components, which have originated from a wide range of anthropogenic and natural sources. The particulate size distribution in ambient air and indoors usually consists of four modes: nuclei mode (particle diameter Dp < 100 nm), Aitken mode (Dp < 0.1 μm), accumulation mode (0.1 μm < Dp < 1 μm) and coarse mode (Dp > 1 μm) (Friedlander, 1971). In epidemiological studies, particles are divided into ultrafine particles (particles ≤ 100 nm in aerodynamic diameter), fine particles (particles ≤ 2.5 μm in aerodynamic diameter) and coarse thoracic particles (particles ≤ 10 μm in aerodynamic diameter) (USEPA 2004). Each of these particle size ranges has a distinct chemical composition, source and formation mechanism. The ultrafine particles originate from high temperature processes, atmospheric transformation and combustion sources e.g. diesel engine exhaust and small-scale combustion. These particles grow very rapidly due to coagulation as well as through the condensation of water on their surface. Most of the ultrafine particles gradually grow into accumulation particles. The ultrafine particles contain sulfates, elemental (EC) and organic carbon (OC) and trace metals that are mostly derived from the combustion sources. The fine particles are partly formed from ultrafine particles and originate from coal, oil, gas, diesel and biomass combustion. Thus their origin, the chemistry of the fine particles is related to ultrafine particles but they contain more sulfate, nitrate and water. Finally coarse thoracic particles arise from re-suspended industrial and road dust, suspension from disturbed soil, tire and brake pad as well as road wear debris, sea spray and biogenic material. The chemical composition of coarse particles' includes nitrates, chlorides and sulfates, oxides of crustal elements and metals. In addition, coarse particles contain pollen

and microbe fragments as well as fungal spores (USEPA 2004, 2009).

There is an impressive body of toxicological and epidemiological data describing both the short- and long-term adverse effects of atmospheric PM on human health. Concerns have been raised about fine and ultrafine particles. In epidemiological studies, these particles have been associated with an increased prevalence of stroke and cardiac outcomes (Madl & Pinkerton, 2009; Mills et al., 2009; Franchini & Mannucci, 2009, 2011). Their detrimental effects are thought to be attributable to both reactive organic species and metals (Mills et al., 2009). Moreover, there is only a limited amount of scientific data about the toxicological properties and the related chemical constituents of fine and ultrafine particles, which are released from primary combustion sources e.g. small-scale wood combustion.

Existing air quality monitoring is based on the measurement of PM_{10} and $PM_{2.5}$ size fractions (WHO 2003, 2004). However, it is known that ambient air contains several different size fractions which all exert their own adverse health effects. Those can be evaluated using toxicological studies. Ambient air samples for toxicological studies are collected using high volume cascade impactors in order to gather a sufficient PM mass and to size segregate the collected particles (Fruin et al., 2014). This approach makes it possible to studying toxic effects of different size fractions and sources (Novák et al., 2014). The same methods that are used to collect ambient air PM can be used to collect PM directly from the combustion sources. The emission particles coming from combustion sources are usually very small (tens to few hundreds nm in diameter) and their concentration in the emission gas is very high. In addition, emissions directly after combustion source are extremely hot and are under constant change. In order to overcome those problems, the emissions need to be diluted, which also stabilizes the emission and makes it possible to collect homogenous PM sample (Giechaskiel et al., 2014). In this thesis, a previously validated Dekati® Gravimetric Impactor (DGI) (Ruusunen et al.,

2011) was used to collect PM from small-scale wood combustion. In the DGI, a high flow rate of 70 l/min is used which allows a high PM collection capacity in short collection time to enable simultaneous chemical analyses and toxicological studies with a variety of *in vivo* and *in vitro* methods.

2.1 CHEMICAL CHARACTERISTICS OF WOOD COMBUSTION EMISSIONS

Wood combustion emissions affect local, regional and global environments. At the local level, residential wood combustion is responsible for the release of significant amount of fine PM (e.g. Glasius et al., 2006; Hellén et al., 2008). Many chemicals are bound to those particles e.g. polyaromatic hydrocarbons (PAHs) as well as chlorine and many metals such as potassium, calcium, zinc, manganese, copper, and lead (Marchand et al., 2004; Molnar et al., 2005). Wood smoke also contains volatile organic compounds (Mandalakis et al., 2005; Gaeggeler et al., 2008) which may have negative health effects. Residential wood combustion increases exposure to 1,3-butadiene and benzene within those houses where wood is being used for heating (Gustafson et al., 2007). The regional environment is affected by biomass combustion emissions, since these reduce the air quality. Finally, wood combustion can affect the global environment via emissions of greenhouse gases and PM, especially black carbon (Fountoukis et al., 2014). Black carbon has a large light absorption capacity (Hansen & Nazarenko, 2004) and thus it is estimated to be the second-greatest contributor to global warming after carbon dioxide (Jacobson, 2001; Ramanathan & Carmichael, 2008).

2.1.1 Effects of fuel on emissions

The chemical composition of the fuel has an important effect on the emissions appearing after biomass combustion. The volatile matter content in wood is high in contrast to many other solid fuels. Wood is composed mainly of cellulose (40–45% of dry

weight), hemicellulose (20–35%) and lignin (15–30%) (Rowell, 1984). In addition, wood fuel contains water. There are also other organic and inorganic elements and compounds which are bound to the structure of wood. The main elements are calcium, potassium, magnesium, manganese, sulphur, chlorine, phosphorus, iron, aluminum and zinc (Sippula et al., 2007b). However, the chemical composition of wood fuel varies depending on species, age, habitat as well as which part of the plant is being used as the fuel (Fogel & Cromack, 1977; Rowell, 1984; Sippula et al., 2007b). In particular, bark contains high amounts of ash forming components including zinc (Sippula et al., 2007b).

2.1.2 Effects of combustion technology and combustion conditions on emission

Combustion technology and operational practice of appliances have a major impact of physicochemical properties of the emitted particles (Figure 1.) (e.g. Tissari et al., 2008; Kocbach Bølling et al., 2009). It is known that residential wood combustion in OT furnaces is a major source of $PM_{2.5}$ emissions, PAHs and certain gaseous pollutants such as volatile organic compounds (VOCs) (e.g. Karvosenoja et al., 2008). Instead, if one can achieve efficient biomass combustion, the complex carbon compounds are reduced to CO_2 and H_2O almost completely but that still leaves incombustible volatile alkali and transition metals which were present in the fuel, leading to the formation of fine fly ash particles, while the non-volatile species typically form large ash particles (Oser et al., 2001; Boman et al., 2004; Sippula et al., 2007a,b). It is noteworthy that inefficient combustion may produce equivalent amounts of inorganic ash components as efficient combustion when the emission factor (mg/MJ fuel energy content) is taken into account (Leskinen et al., 2014).

In general, the fine particles emitted from residential wood combustion appliances may be divided roughly into two characteristic classes based on the combustion efficiency (Obaidullah et al., 2012). When there is inefficient combustion,

the particles are relatively large and contain carbon in a variety of forms. The particle emissions from efficient combustion devices are dominated by ash species such as potassium, sulfate, chloride and many transition metals (Tissari et al., 2008; Wiinikka et al., 2013). Those particles are usually small and their number is far greater than the particles emerging from inefficient combustion (Tissari et al., 2008; Kocbach Bølling et al., 2009). It should be emphasized that in real combustion situations, the particle classes co-exist and interact (Torvela et al., 2014).

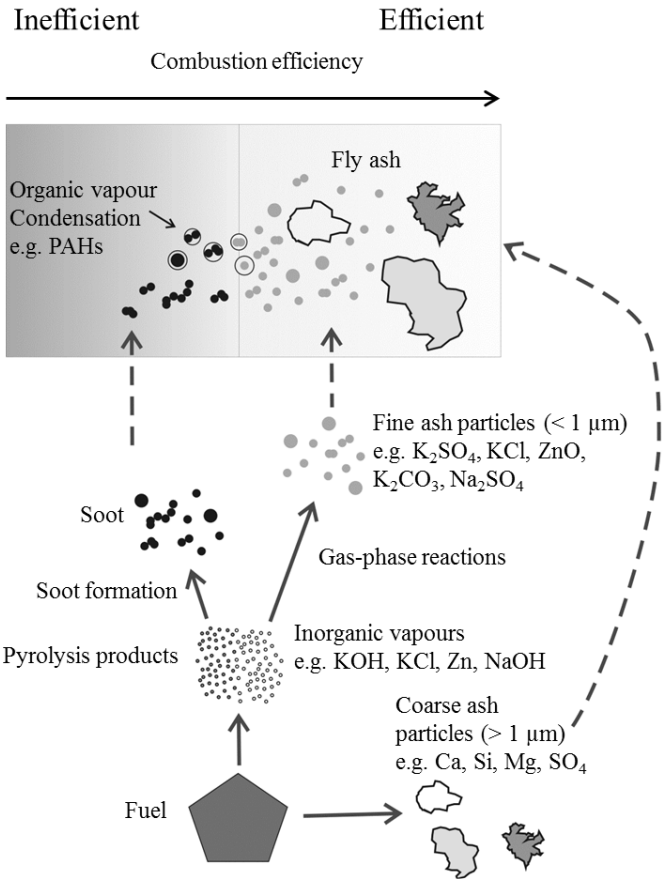


Figure 1. Simplified scheme of particle formation during wood combustion. Modified from Sippula et al. (2009). Abbreviation: PAHs, polyaromatic hydrocarbons.

2.1.2.1 Inefficient wood combustion

Burning wood with a high moisture content, overloading the furnace or providing an insufficient air supply can all cause incomplete combustion i.e. there is a low temperature and high PM emissions (Figure 1.) (Tissari et al., 2008). The emissions emerging from inefficient combustion conditions are dominated by CO, H_2, SO_2, NO_x, partially combusted hydrocarbons, and different solid particles. Inefficient wood combustion produces thousands of different organic compounds including highly oxygenated organic species and PAH compounds (McDonald et al., 2000; Fine et al., 2001; Schauer et al., 2001; Lee et al., 2005; Alfarra et al., 2007; Mazzoleni et al., 2007). The organic compounds can be present either as gases or bound to particles (Tucker, 2001; Kliucininkas et al., 2011). PAHs are formed in the flame when hydrocarbons polymerize (Verhoeven et al., 2013). In addition, the aerosol from inefficient wood combustion includes liquid or tarlike components. Those particles are formed from organic vapors which are cooled down (Pyykönen et al., 2007). Soot particles are the first particles to be formed in inefficient wood combustion. Those particles are formed in the flame from hydrocarbons. The formation of soot is a very complex process but it is believed to happen via PAH clusters, particle inception, surface growth and coagulation (Kozinski & Saade, 1998; Wilson et al., 2013). Soot particles are a typical characteristic of inefficient combustion, and thus while they are likely to be emitted from old wood stoves and boilers (e.g. Tissari et al., 2008), they can be emitted from any appliance under poor combustion conditions (Hindsgaul et al., 2000; Johansson et al., 2003; Wierzbicka et al., 2005; Torvela et al., 2014).

The carbon which is present in wood combustion particles is classified as organic (OC), elemental (EC) or inorganic carbon (IC). The carbon in the emission samples is usually measured using thermal optical carbon analyzer which is based on the principle that different types of carbon-containing particles are converted into gases under different temperature and oxidation conditions (Han et al., 2007). OC is formed from thousands of

organic compounds. EC, on the other hand, is characterized as the carbon that is not organic (Kocbach Bølling et al., 2009). IC is usually calcium carbonate which is subtracted from the EC results (Bisutti et al., 2004). Due to the low combustion temperature, the PM from incomplete combustion is dominated by OC (McDonald et al., 2000; Hays et al., 2003). When the combustion temperature increases and more oxygen is available, more EC is present in emission and there are greater numbers of soot aggregates (Tissari et al., 2008). The size of PM from low temperature incomplete combustion has varied between 50 to 600 nm when measured by electron microscopy (Kocbach et al., 2005; Klippel & Nussbaumer, 2007; Torvela et al., 2014). In contrast, soot aggregates are usually smaller and more homogenous (20-50 nm) (Kocbach et al., 2005; Gwaze et al., 2006; Torvela et al., 2014).

2.1.2.2 Efficient wood combustion

If one wishes to achieve efficient wood combustion, all of the following requirements have to be met: sufficient supply of combustible air to ensure complete oxidation, sufficiently high temperature for chemical reaction kinetics, sufficiently long residence time at high temperature and sufficient mixing of fuel components and air (Sippula et al., 2007a; Tissari et al., 2008). The combustion of wood in NT appliances, e.g. pellet- or wood chips boilers, usually achieves those types of combustion conditions. The emissions from those appliances are dominated by inorganic ash particles (Figure 1.). The most abundant components in efficient combustion PM are potassium alkali salts, sodium, sulfate, chlorides, carbonates and transition metal oxides (Johansson et al., 2003; Boman et al., 2004; Torvela et al., 2014). The content of OC and EC is very low in the PM emitted if there are efficient combustion conditions (Löndahl et al., 2007; Torvela et al., 2014).

In efficient combustion, the particles are formed from vaporized inorganic elements, which originate from wood fuel (Sippula et al., 2007a,b). The combustion temperature has a major impact on the vaporization of inorganic compounds of

wood fuel. Thus, combustion produces more ash particles at a high temperature than at a lower temperature (Davidsson et al., 2002; Knudsen et al., 2004). The most highly volatile inorganic compounds present in wood fuel are potassium, sulfur, chlorine, sodium, zinc and calcium (Knudsen et al., 2004). Thus, the fine fly ash from wood combustion is mainly composed of potassium sulfate (K_2SO_4), potassium chloride (KCl), potassium hydroxide (KOH) and potassium carbonate (K_2CO_3) (Boman et al., 2004; Sippula et al., 2007a; Torvela et al., 2014). The first compound to form in fine ash particles is ZnO; this process occurs during the very early stage of cooling of the flue gas (Sippula et al., 2009; Torvela et al., 2014). The formation occurs due to the oxidation of elemental Zn vapor and its subsequent rapid nucleation due to the very low vapor pressure of ZnO. ZnO formation is followed by the gas-to-particle conversion of alkali sulfates, which are also produced in gas-phase reactions. For fuels with a very low Zn content, it is believed that K_2SO_4 forms the first nuclei into which other chemical species can condense (Sippula et al., 2007b). The formation of vapors of alkali carbonates and alkali chlorides condense later as the temperature continues to decline. The size of the PM from efficient combustion has varied between 25-160 nm when this is measured by electron microscopy (Mavrocordatos et al., 2002; Torvela et al., 2014).

2.2 ADVERSE HEALTH EFFECTS OF WOOD COMBUSTION

In epidemiological studies, exposure to wood smoke has been associated with a range of pulmonary effects, including chronic obstructive pulmonary disease (COPD), decreased lung function, cardiac events, and exacerbation of asthma as well as increased risk of lung cancer (McGowan et al., 2002; Smith et al., 2004; Behera & Balamugesh, 2005; Hernandez-Garduno et al., 2004; Mannino & Buist, 2007; Qian et al., 2007; Noonan & Ward, 2012; Groom et al., 2014; Guarnieri et al., 2014). To confirm the results obtained from epidemiological studies, markers of

induced toxic responses from wood smoke exposure have been studied in human volunteers in chambers as well as in animal models. Moreover, cell cultures have been widely used to reveal the toxic mechanisms of the wood smoke particles (Naeher et al., 2007; Kocbach Bølling et al., 2009). Furthermore, experimental studies offer the potential to reveal information about the mechanisms of toxicity as well as defining the relative toxicities of different emission mixtures and distinct sources. Several mechanisms have been proposed to explain the associations, which have been reported in epidemiological studies between particle exposure and adverse health effects (Squadrito et al., 2001; Anderson et al., 2012; Martinelli et al., 2013). Those effects include particle-induced inflammation, oxidative stress, cytotoxic effects and genotoxic effects (Barregard et al., 2006; Reed et al., 2006; Seagrave et al., 2006; Naeher et al., 2007). Inflammation is considered to be the most important factor and indeed the inflammatory potential of ambient particles has been linked to chronic pulmonary diseases, atherosclerosis and acute cardiac effects (Kofler et al., 2005; Tousoulis et al., 2006; Bai et al., 2007). In addition, the cytotoxicity of particles is involved in tissue damage in the lungs, whereas the carcinogenic risk is primarily linked to genotoxicity (Schwarze et al., 2006; Schins & Knaapen 2007).

2.2.1 Toxic effects of particulate matter

PM exposure triggers a variety of adverse cardiorespiratory health effects. These effects can be clarified by studying the toxicological mechanisms in animal and cell models. Pulmonary inflammation and oxidative stress may be considered as the main toxic mechanisms behind the short-term adverse effects (Riva et al., 2011), whereas cytotoxicity and genotoxicity are the main mechanisms behind the long-term effects (Hogg et al., 2004). The research methods for these main toxicological mechanisms both *in vitro* and *in vivo* are listed in Table 1.

Table 1. *Research methods for toxicological studies*

Endpoint	Marker	in vitro/in vivo	Assay examples
Inflammation			
	Cytokines	both	ELISA, PCR
	Inflammation cells influx	*in vivo*	Cell differentials
	Immune cells surface markers	both	CD-proteins antibody labeling
Oxidative stress			
	Lipid peroxidation	both	MDA detection
	GSH depletion	both	GSH/GSSG relation
	Free radicals	both	DCF-signal, NBT-assay
Cell death and tissue damage			
	LDH	both	LDH-activity assay
	Total protein	*in vivo*	Protein measurement from BALF
	Apoptosis	both	TUNEL-assay, Caspase-3 activation, Annexin A5-labeling
	Necrosis	*in vitro*	PI-staining, Trypan blue
Genotoxity			
	DNA strand breaks	both	Single cell gel electrophoresis
	Structural chromosomal aberrations	both	Micronucleus test

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; CD, cluster of differentiation; MDA, malondialdehyde; GSH, Reduced glutathione; GSSG, glutathione disulfide; DCF, 2'.7'-dichlorofluorescein; NBT, Nitro blue tetrazolium chloride; LDH, Lactate dehydrogenase, BALF; bronchoalveolar lavage fluid; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; PI, Propidium iodide.

2.2.1.1 Inflammation

Inflammation is regarded as the main mechanism activated by PM exposure causing respiratory and cardiovascular effects (e.g. Gold et al., 2000; McCreanor et al., 2007, Anderson et al., 2012). *In vivo* inflammation can be assessed by recognizing different inflammatory cell types from respiratory lavage fluids or by detecting inflammatory changes in histopathological analyses of tissue samples (Oberdorster, 1995). In addition, the levels of inflammatory mediators i.e. cytokines, can be measured from the biological samples including lavage fluids and serum. *In*

vitro inflammation is usually analyzed by measuring these same mediators from the culture media of the exposed cell cultures.

Cytokines are relatively small proteins that are important in the body's inflammatory response. The cytokine class includes chemokines, interferons, interleukins, lymphokines and tumor necrosis factors (Vinatier et al., 1995). Cytokines are produced by many cell types but especially immune and epithelial cells. The most common way to measure those proteins in biological fluids is to utilize enzyme-linked immunosorbent assay (ELISA) where antibodies are used to detect the desired inflammation mediator (Lequin, 2005).

Inflammation occurs when monocytes, macrophages, dendritic cells or epithelial cells become activated by external stimuli to release inflammatory mediators (Silbajoris et al., 2011; Nemmar et al., 2013). The goal of inflammation is to protect the body not only against invading pathogens and other micro-organisms but also any other foreign material e.g. particles (Abbas et al., 2007). Inflammation can be roughly classified into either acute or chronic (Driscoll et al., 1990). The body's acute response to harmful stimuli is characterized by an increased movement of leukocytes from the blood into the site of the inflammation (Oberdorster et al., 1996). This mechanism is mediated by cytokines. Chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation and this condition is characterized by the simultaneous destruction and healing of the tissue (Ferrero-Miliani et al., 2007). This type of inflammatory damage is present in obstructive and long-term respiratory diseases, e.g. COPD and asthma i.e. it is also, related to respiratory tissue damage (Hogg et al., 2004).

2.2.1.2 Oxidative stress

Oxidative stress via reactive oxygen species (ROS) is a well-known and important mechanism activated by particulate exposure in humans (e.g. Sørensen et al., 2005; Anderson et al., 2012). Oxidative stress becomes manifested when there is an imbalance between the amount of ROS and the cell’s ability to

scavenge these radicals or to repair the resulting damage. Disturbances in this balance can cause toxic effects that damage cell proteins, lipids, and DNA (Devasagayam et al., 2004). This causes oxidative stress which is the causative factor in many other adverse toxic mechanisms, including inflammation and genotoxicity. Indeed, oxidative damage is associated with the primary development of asthma and COPD (Vlahos & Bozinovski, 2014). ROS are produced extensively in phagocytic cells as a normal product to fight against invading pathogens. E.g. alveolar macrophages develop a so-called respiratory burst activity, produce reactive oxygen and nitrogen species and release cytokines after particulate exposure (Driscoll et al., 1990). Moreover, ROS may be directly generated from the surface of particles and those radicals can be the source of DNA damage and cell death (Knaapen et al., 2004, Risom et al. 2005).

There are a number of well-established methods with which available to measure intracellular ROS that usually are applicable in both *in vivo* and *in vitro*. Widely used methods involve the detection of oxidation-mediated changes in fluorescent dyes, e.g. dihydroethidium (Zielonka et al., 2012). Nitroblue tetrazolium (NBT) may also be used for detecting the presence of intracellular ROS. NBT can be oxidized into purple-blue formazan compounds that can be detected as a purple-colored precipitate inside the cells (Freeman & King, 1972). In addition, there are several chemiluminescent substances that are very widely used in measuring intracellular ROS (Fingerova et al., 2009).

2.2.1.3 Cell death and cytotoxicity

Long-term exposure to PM results airway remodeling and chronic inflammation, which can lead development of COPD and asthma (Hogg et al., 2004). Asbestos and tobacco smoke are probably the best-known examples of chronic exposures which causes cell damage in lungs (Bartal, 2005; Rastrick & Birrell, 2014).

Cell death can be assessed both *in vivo* and *in vitro*. The detection method depends on which kind of cell death one

wishes to measure. In necrotic cell death, the cells rapidly lose their membrane integrity and release their contents into the surroundings (Fink & Cookson, 2005). This process is uncontrolled and leads to an inflammatory response. If the exposure does not kill the cells immediately, many repair mechanism may be activated. However should, these mechanisms fail, the cells can undergo programmed cell death, i.e. apoptosis. Apoptosis is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation (Fink & Cookson, 2005). This mechanism is a controlled way in which the organism can remove damaged cells from the tissues. There is also a third way in which a cell can die, i.e. pyroptosis. Pyroptotic cells have morphological features resembling both necrosis and apoptosis but pyroptosis is a biochemically district process (Bergsbaken et al., 2009). In particular, pyroptosis is associated with antimicrobial responses evoked by immune cells. In this process, immune cells produce cytokines and die by bursting. Thus, the release of cytokines attracts other immune cells to fight the infection (Fink & Cookson, 2005). Biochemically pyroptosis is related to inflammasome activation and thus interleukin-1 (IL-1) –family cytokines (Kepp et al., 2010).

Cell viability and cytotoxic effects of PM can be assessed by methods, which measure the integrity of the cell membrane. The most common way to measure necrotic or late apoptotic cells is to use dyes, such as trypan blue or propidium iodide (PI). Normally these compounds cannot access the healthy cells. However, if the cell is damaged, those dyes will cross the cell membrane and stain the cell (Lecoeur, 2002). Alternatively, the substances that are normally only present inside cells can be measured from cell culture medium *in vitro* or from lavage fluids from *in vivo* experiments, revealing the impaired integrity of the cell membrane. The compound that is most commonly measured is lactate dehydrogenase (LDH) which is an enzyme present in almost all cell types and tissues. Should a tissue be badly damaged the LDH can be detected in the extracellular matrix (Fotakis & Timbrell, 2006). Cytotoxicity can also be monitored by measuring cellular metabolic activity. This is

usually done by measuring directly or indirectly the adenosine triphosphate (ATP) content of the cells (Weyermanna et al, 2005). Apoptotic cells can be detected by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay where the fragmentation of DNA is detected by labeling the terminal ends of nucleic acids (Gavrieli et al., 1992). In addition, the annexin A5 probe can be used to detect apoptotic cells that express phosphatidylserine and phosphatidylethanolamine on the cell surface (Vermes et al., 1995). Moreover, apoptosis can be detected by measuring proteins, which mediate this proses e.g. one widely detected protein used for this purpose is caspase-3 (Jerome et al., 2003).

2.2.1.4 Genotoxicity

Exposure to PM can result in damage to the genetic information i.e. genotoxic effects. Genotoxicity poses a risk for long term adverse effects in the human body including an increased risk for the appearance of cancers. DNA can be damaged in several ways: e.g., single- and double-strand breaks, cross-linking, base modifications, alkali-labile sites, and loss of excision repair, which may lead to gene mutations and structural chromosomal aberrations (Mazouzi et al., 2014). When a cell's DNA has been damaged, complex pathways are triggered to prevent the formation of permanent DNA changes. One of those repair options is cell cycle arrest where a cell will stop its normal division cycle to allow time repair the damaged DNA (Mahmoud et al., 2011).

The standard *in vitro* genotoxicity test batteries recommended by regulatory agencies to detect genotoxic carcinogens include at least two or three test procedures, such as bacterial reverse mutation test, mammalian cell chromosome damage test and mammalian cell mutation assay (Kirkland et al., 2005). The Ames test (bacterial reverse mutation test) is a commonly used procedure to detect two classes of mutations, base pair substitution and small frameshifts (Mortelmans & Zeiger, 2000) Moreover, single cell gel electrophoresis assay (SCGE) and micronucleus assay which are conducted in

mammalian cell lines are sensitive and frequently used methods to detect the genotoxicity of nanoparticles (Landsiedel et al., 2009; Oesch & Landsiedel, 2012). The SCGE assay measures DNA strand breaks in single cells (Tice et al., 2000). Micronucleus assay detects that an extra nucleus has been formed during cell division (Magdolenova et al., 2014).

2.2.2 Human exposure studies with volunteers

There are still limited numbers of controlled human inhalation exposure studies available which would have investigated the adverse effects of wood smoke (summary in Table 2.). Moreover, all of the summarized experiments have examined only the short-term effects of exposure to wood smoke. In most of those studies, conventional stove or oxygen-restricted conditions have been used as a source of wood smoke emissions (Barregard et al., 2006, 2008; Stockfelt et al., 2012, 2013; Unosson et al., 2013). Generally, the responses detected from volunteers are associated with changes in systemic inflammation, blood coagulation and lipid peroxidation (Sällsten et al., 2006; Barregard et al., 2006, 2008; Danielsen et al., 2008). In addition, increases in the levels of several inflammatory markers have been detected after exposure to wood smoke (Ghio et al., 2012a). Moreover, it is claimed that arterial stiffness is increased after wood smoke exposure (Unosson et al., 2013). Many previously mentioned biomarkers are cardiovascular risk factors. However, several of the human exposure studies have been dominated by negative findings (Forchhammer et al., 2012a; Riddervold et al., 2012; Bønløkke et al., 2014).

Table 2. *Controlled human exposure studies with volunteers*

Author and year	Subjects	Appliance	Exposure	Key findings caused by wood smoke exposure
Barregard et al., 2006, 2008 Sällsten et al., 2006 Danielsen et al., 2008	13 healthy subjects aged 20–56 (mean 34) yr. Exposure to filtered indoor air for 4h on one occasion and to wood smoke for 4h 1wk later.	Conventional cast-iron wood stove. Fuel: hardwood and softwood (50%/50%), moisture content 15–18%. Constant burn rate (fuel added 2 kg wood logs every 40 min).	The $PM_{2.5}$ in the chamber in the range of 240–280 $\mu g/m^3$, and number concentrations were 95000–180000/cm^3. About half of the particles were ultrafine.	• Increased SAA, factor VIII, and the factor VIII/vWf ratio in plasma and isoprostane excretion in urine. • Up-regulated the expression of the DNA repair gene (OGG1). • Significant decrease DNA strand breaks in PBMC. • Increased alveolar nitric oxide 3 h post-exposure while malondialdehyde levels in breath condensate were higher both immediately after and 20 h after exposure. • Increased serum Clara cell protein at 20 h after exposure.
Sehlstedt et al., 2010	19 healthy subjects aged 21-31 (mean of 24) yr. Exposure to filtered air for 3h and 3 weeks, later to wood smoke for 3h. During exposure 15-min intervals of exercise on a bicycle ergometer.	Residential wood pellet burner (15 kW) installed in a boiler. Fuel: softwood pellet/sawdust fuel mixture from pine and spruce (18% moisture). Combustion conditions: Low temperature (700-800°C) reduced air/fuel mixing.	The $PM_{2.5}$ in the chamber was 180-300 $\mu g/m^3$ during each wood smoke exposure. Mean concentration from all exposures was 224 ± 22 $\mu g/m^3$.	• Increase in mucosal symptoms and reduced glutathione in the alveolar respiratory tract lining fluids but no acute airway inflammatory responses.
Forchhammer et al.,2012a Riddervold et al., 2012 Bønløkke et al., 2014	A randomized, double-blinded, study with 20 non-smoking atopic subjects. Exposure to filtered air for 3h and 2weeks later to wood smoke for 3h.	Modern wood stove operated with good practice.	Used mean exposure concentrations were 14, 220, or 354 $\mu g/m^3$.	• Very limited changes after a 3-hour exposure.

Ghio et al., 2012a	Ten subjects. Exposure to filtered air for 2h and to wood smoke for 2h 3 wk later. During exposure the subjects' had 15-minute intervals of exercise and rest.	Wood smoke was generated by heating an oak log on an electric element.	During wood smoke exposure with an average of the mean PM concentration from all exposures was 485 ± 84 $\mu g/m^3$.	• Increased neutrophil count in the blood by 23% after exposure and at follow-up by 17%. • A significant increase in blood IL-1β immediately following wood smoke exposure. • Elevated blood LDH concentrations after wood smoke exposure immediately and at follow-up. • 16.8% decrease in maximal heart rate immediately following particle exposure. • Increased neutrophil numbers in BALF
Stockfelt et al., 2012, 2013	13 healthy adults. Exposed to filtered air followed by two sessions of wood smoke for 3h, 1wk apart. One session used smoke from the start-up phase of the wood-burning cycle, and the other smoke from the burn-out phase.	Conventional cast-iron wood stove. Fuel: hardwood/softwood (50%/50%). In the start-up phase session, smoke was supplied to the chamber for 12–14 min, immediately after the new wood logs were added. In the burn-out phase session, smoke was supplied for 15 min, starting 25 min after wood was added.	Mean particle mass concentrations: 295 $\mu g/m^3$ (start-up phase), 146 $\mu g/m^3$ (burn-out phase), Mean number concentrations: 140 000/cm^3 and 100 000/cm^3, respectively.	Exposure to wood smoke from the start-up caused: • Increase in Clara cell protein 16 in serum after 4h, and in urine the next morning. • Clear diurnal variation Clara cell protein 16. Exposure from the burn-out phase: • Increased fraction of exhaled nitric oxide.
Unosson et al., 2013	14 non-smoking subjects participated in a randomized, double-blind crossover study. Exposed to filtered air or wood smoke 3h during intermittent exercise. There was a 3wk period between exposures.	A common Nordic wood stove (chimney stove) in a controlled incomplete combustion firing procedure. Fuel: Birch wood logs (moisture content 16-18%) were inserted every 5-15 min to maintain a high burn rate with repeated air-starved conditions.	Mean particle mass concentrations was 314 ± 38 $\mu g/m^3$.	• Central arterial stiffness, measured as an augmentation index, augmentation pressure and pulse wave velocity, was higher after smoke exposure as compared to filtered air • Heart rate was increased although there was no effect on blood pressure. Heart rate variability was decreased one hour following exposure.

Abbreviations: BALF, Bronchoalveolar lavage fluid; PBMC, Peripheral blood mononucleated cell; LDH, Lactate dehydrogenase; SAA, Serum amyloid A; vWF, Von Willebrand factor; OGG1, 8-Oxoguanine glycosylase; IL, Interleukin.

2.2.3 Animal studies with wood smoke emissions

The *in vivo* wood smoke exposure studies in animal models may be divided into short-term studies where high PM doses are used or sub-acute and chronic studies with lower exposure concentrations. Wood smoke-induced effects in murine models are listed in Table 3.

Short-term exposure studies usually have a much greater exposure level than those to which the general public is exposed. However, the results from those studies may demonstrate to some extent the effects that could potentially occur as a result of lower level and longer duration exposures (Naeher et al., 2007). In high dose wood smoke exposure studies inflammatory responses are not always detected and in this respect the results differ from experiments conducted with exposure to other airborne pollutants e.g. diesel fumes (Ghio et al., 2012b). Moreover, in many studies conducted with wood smoke, there has been a dramatic reduction in macrophage activity to achieve bacterial phagocytosis and intracellular killing of gram-negative bacteria (Naeher et al., 2007). In addition, short term exposure of animals to wood smoke has been causing oxidative stress (Demling & LaLonde, 1990; Demling et al., 1994; Lalonde et al., 1994; Dubick et al., 2002). These investigations provided the first evidence that short term wood smoke exposure could produce toxic effects and alter lung properties.

Murine, sub-acute and chronic inhalations of wood smoke in concentrations relevant for ambient human exposure scenarios have induced mild inflammatory effects in the airways and also caused systemic effects as well as decreasing lung function (Burchiel et al., 2005; Tesfaigzi et al., 2005; Barrett et al., 2006; Reed et al., 2006; Seagrave et al., 2006; Naeher et al., 2007). Wood smoke PM has also been reported to be able to exacerbate allergic inflammation and allergic sensitization, and decrease the pulmonary macrophage functionality in terms of infection resistance (Tesfaigzi et al., 2005; Barrett et al., 2006; Naeher et al., 2007; Samuelsen et al., 2008; Migliaccio et al., 2013). Overall, the

in vivo experiments indicate that wood smoke PM can induce mild inflammatory responses, cytotoxic effects, genotoxic effects, and oxidative stress, and compromise pulmonary immune defense in a way that leads to an increased susceptibility to infectious lung disease.

Table 3. *Animal exposure studies with wood smoke emissions*

Author	Year	Animal model	Appliance	Exposure	Chemistry	Endpoints	Key findings
Tesfaigzi et al.,	2002	Rat (m, f): Brown Norway rat (6-7wk).	Wood stove. Fuel: *Pinus edulis.*	Whole-body: 1 or 10 mg/m^3. 3h/d, 5d/wk for 30 or 90d. Stable burning.	Impactor: PM mass, OC, EC PAHs, many organic compounds. Gases: NO, NO_x, CO, THC.	Respiratory function tests, LPA. Histopathology: larynx and a part of the trachea, lung, nasal epithelia. BALF: cells, total protein, LDH, β-glucuronidase, IL-1β, IL-6, TNF-α.	• Reduced diffusion at alveolar-capillary membrane (dose 10 mg/m^3). • Increased dynamic lung compliance (both doses). Decreased number of macrophages in the BALF (dose 10 mg/m^3). • Minor histopathological changes.
Tesfaigzi et al.,	2005	Rat (m): Brown Norway rat (6wk). OVA sensitization.	Conventional wood stove. Fuel: mixed black oak and white oak.	Whole-body: 6h/d, 7d/wk for 70d, dose 1 mg PM/m^3. Three-phase burn cycle.	Impactor: PM mass, OC, EC, ions, metals. Gases: NO, NO_2, SO_2, CO, THC.	Respiratory function, BALF cells, LPA, Cytokines. Histopathology: left lung, trachea, larynx nose.	• IFN-γ reduced and IL-4 levels increased in the BALF and plasma. • Inflammatory lesions in the lungs.
Seagrave et al.,	2005	Rat (m, f): CDF(F-344) /CrlBR (10-12wk).	Conventional wood stove. Fuel: mixed black oak and white oak.	Whole-body: 6h/d, 7d/wk for 6mo, dose 30, 100, 300, and 1000 μg/m^3. Three-phase burn cycle.	Impactor: PM mass, OC, EC, ions, metals. Gases: NO, NO_2, SO_2, CO, THC.	BALF: cells, total protein, LDH, β-glucuronidase, alkaline phosphatase, GSH/GSSG, IL-1β, TNF-α, MIP-2.	• Nonlinear responses: decreased total GSH in females, β-glucuronidase and MIP-2 decreased in males and females, minor increase in TNF-α in males.
Burchiel et al.,	2005	Mice (f): A/J (10-12wk).	Conventional wood stove. Fuel: Hard wood.	Whole-body: 30, 100, 300, and 1000 μg/m^3. 6h/d, 7d/wk, for 6mo. Three-phase burn cycle.	Impactor: PM mass, OC, EC, PAHs, metals, ions. Gases: NO, CO NO_2, THC.	Spleen cell mitogenesis assay and surface marker analysis on spleen cells using flow cytometry.	• Increased T cell proliferation in 100 μg/m^3 exposure group. • Suppression of T cell proliferation at < 300 μg/m^3.

Barrett et al.,	2006	Mice (m): BALB/c (8–10 wk). OVA sensitization.	Wood stove Fuel: Oak (mixture of black and white oak).	Whole-body: 30, 100, 300, and 1000 μg/m^3. 6h/d, for 3d. Three-phase burn cycle.	Impactor: PM mass. Gases: NO, NO_2, CO, THC.	BALF: cells, IL-2, IL-4, IL-5, IFN-γ and IL-13. OVA-specific IgG_1, IgG_{2a} and IgE.	• Increase in OVA-induced BALF eosinophils when OVA challenge immediately (24h) preceding wood smoke exposure (dose 300 μg/m^3).
Reed et al.,	2006	Rat (m, f): F344/CrlBR and SHR (6-12wk). Mice (m): C57BL/6 (6-12wk). Mice (m, f):A/J (6-12wk).	Wood stove. Fuel: mixed black oak and white oak.	Whole-body: 30, 100, 300, and 1000 μg/m^3. 6h/d, 7d/wk, for either 1wk or 6mo. Three-phase burn cycle.	Impactor: PM mass, OC, OC ammonium, sulfate, nitrate, metals and associated elements. Gases: NO, NO_2, SO_2, CO, THC.	**F344 rats**: histopathology, clinical chemistry, and hematology. **SHR rats:** cardiac waveform and heart rate analysis. **C57BL/6 mice:** bacterial clearance. **A/J mice**: tumorigenesis.	Mild effects, even at the highest exposure concentration: • Mild responses in organ weights and lung volumes. PM accumulation in lungs. • Increase in platelets, decrease in blood urea nitrogen and serum alanine aminotransferase and alkaline phosphatase.
Danielsen et al.,	2010	Rat (m): F344 (9wk).	Wood stove. Fuel: Beech.	Intratracheal aspiration: 0.64 mg/kg. Exposure 24h. PM from high and low oxygen supply burning.	Impactor: PM mass, PAHs, metals, ions, mannosan, levoglucosan.	BALF cells, DNA adducts, oxidative damage to DNA bases, HO-1, mRNA expression of OGG1, MCP-1 and MIP-2.	• Low oxygen supply increased BALF neutrophils • Wood smoke caused oxidative stress and proinflammatory response.
Migliaccio et al.,	2013	BALB/c mice	Old wood stove. Fuel: mix of local softwoods	Whole-body: 3-15 mg/m^3 for 2h.	PAHs	BALF: cells, total protein. Assessment of bacterial clearance.	• Wood smoke exposure lead to higher bacterial load 24h post-exposure. • Macrophages had decreased neutrophil activation potential.
Ramos et al.,	2013	Guinea pig	Electric incinerator. Fuel: pine.	Whole-body: 3h/d. 2, 4 or 6 exposures.	$PM_{2.5}$, CO, CO_2 and O_2.	BALF cells. ROS marker in serum and BALF. Lung histopathology.	• Increased oxidative stress and antioxidant enzymes. • Increase in BALF cells: macrophages, neutrophils. • Lung and airway damage.

Abbreviations: m, male; f, female; PM, particulate matter; OC, Organic carbon; EC, Elemental carbon; PAHs, Polycyclic aromatic hydrocarbons; LPA, Lymphocyte proliferation assays; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; THC, total hydrocarbon; IL, Interleukin; TNF, Tumor necrosis factor; OVA, Ovalbumin; IFN, Interferon; GSH, Reduced glutathione; GSSG, glutathione disulfide; MIP, macrophage inflammatory protein; Ig, Immunoglobulin; HO-1, Heme oxygenase 1; OGG1, 8-Oxoguanine glycosylase; MCP-1, monocyte chemotactic protein 1.

2.2.4 *In vitro* studies conducted with wood combustion emissions particles

There is still a paucity of toxicological data from highly controlled combustion studies including physicochemical characterization of the emission PM. However, *in vitro* toxicological studies using both human and murine cell lines and primary cells have demonstrated that different toxic mechanisms are activated by emission PM, depending on the biomass combustion efficiency (Kocbach et al., 2008a,b; Danielsen et al., 2009; Jalava et al., 2010; Tapanainen et al., 2011; 2012). It is noteworthy that most of current *in vitro* data on PM originating from small-scale wood combustion appliances is based on commonly used furnaces which usually represent old or conventional combustion technologies.

It has been suspected that particles derived from different combustion conditions may induce differential pro-inflammatory response patterns (Karlsson et al., 2006; Jalava et al., 2010). Moreover, particles that contain high levels of soot and PAH compounds possess a greater potency for cytotoxicity and DNA damage than particles that contain more inorganic compounds (Tapanainen et al., 2011, 2012; Forchhammer et al., 2012b). There are also several studies where wood combustion derived particles have been washed with organic solvents to obtain an organic extract. Incubation of the cells with those organic extracts has led to DNA damage (Danielsen et al., 2009). There is also evidence of immunosuppression induced by particle bound PAH compounds (Tapanainen et al., 2012). The toxic effects induced by wood smoke particles on different cells are collated in Table 4.

Table 4. *In vitro studies conducted with wood combustion emissions particles*

Author	Year	Cells	Appliance	Exposure	Chemistry	End points	Key findings
Karlsson et al.,	2006	A549 Human primary macrophages.	Old wood and new boilers and pellet boiler. Fuel: Wood pellets.	A549 4h to 40 μg/cm^2 (70 μg/ml). Macrophages 18h to 50 μg/cm^2 (100 μg/ml).	PM extraction: Water. OC, EC, ions, metals. Gases: CO_2, CO, TOC.	SCGE, IL-6, IL-8, TNF-α.	• DNA damage (no difference between appliances). • PM from modern wood boiler increased the IL-8 levels.
Kocbach et al.,	2008a, 2008b	THP-1 and a contact co-culture of A549 and THP-1.	Old wood stove. Fuel: Birch.	12h exposure, organic extracts (30 μg/ml) washed particles (140 μg/ml) and native particles (30, 70, 140, 210, 280 μg/ml).	PM extraction: scraped off from filters. OC, EC, PAHs.	LDH, TNF-α, IL-1β, IL-6, IL-8, IL-4 and IL-10.	• Significant increase in TNF-α, and IL-8 by PM. Organic extract significantly increased release of TNF-α and IL-8. • Washed particles: significantly lower IL-8 release than native PM. • Cell death related to organic fraction.
Danielsen et al.,	2009	A549, THP-1	Old wood stove. Fuel: Birch.	Exposure: 24h for LDH analysis, 3h for SCGE assay, (2.5, 25, 100, 200 μg/ml). Washed PM and organic extracts.	PM extraction: scraped off from filters. PAHs, OC.	LDH, SCGE.	• DNA damage with organic extracts.
Jalava et al.,	2010	RAW 264.7	Masonry heater (old) Fuel: Beech.	24h exposure to 15, 50, 150,300 μg/ml. Normal/smouldering combustion.	PM_1 extraction: methanol. Elements, ions, PAHs	Cytotoxicity (MTT-test). TNF-α, MIP-2, cell cycle.	• Smouldering combustion PM caused higher MIP-2, apoptosis and MTT response than normal combustion.
Danielsen et al.,	2011	A549, THP-1	Wood stove. Fuel: Birch.	24h exposure to 0, 2.5, 25, and 100 μg/ml.	PM scraped off from filters using water. Metals, PAHs.	DNA damage, proinflammatory mRNA levels, DNA adducts, ROS.	• Increased 8-oxo-gua levels, but no DNA adducts. • Oxidative, inflammatory and genotoxic responses measured by mRNA.

Ruusunen et al., Tapanainen et al.,	2011 2011, 2012	RAW 264.7, BEAS-2B	Pellet boiler, masonry heater (New and two old) and sauna stove. Fuels: soft wood pellet and birch logs.	24h exposure to 15, 50, 150 and 300 µg/ml.	PM_1 extraction: methanol. Elements ions, PAHs, EC, OC.	Cytotoxicity (MTT-test), cell cycle, TNF-α, MIP-2, SCGE.	• **Sauna stove**: high response in MTT-test, apoptotic cell death, genotoxicity. Moderate MIP-2 response. • **Masonry heaters** (new/old): moderate response in MTT-test, apoptotic cell death. High to moderate genotoxicity. Moderate MIP-2 and TNF-α response. • **Pellet boiler**: moderate response in MTT-test.
Bølling et al.,	2012	A contact coculture A549/ THP-1.	Cast-iron stove. Whole burning-cycle, Fuel: Birch/fir.	12 or 40h exposure to 40 µg/cm^2. Organic extracts and washed particles.	$PM_{2.5\text{-}0.1}$, $PM_{10\text{-}2.5}$ extraction: methanol. PAHs, elements.	LDH, TNF-α, IL-6 and IL-8, cell cycle.	• Washed particles caused cytokine release and organic extract cytotoxicity.
Jalava et al.,	2012	RAW 264.7	Logwood boilers (old/ new), Stoves (old/ new), Tiled stove, Wood chip boiler, Pellet boiler. Fuels: beech, hard-wood.	24h exposure to 15, 50, 150 and 300 µg/ml.	PM_1 extraction: methanol. Elements, ions, PAHs, EC, OC.	Cell death: MTT-test and PI-positive cells. Cell cycle, TNF-α, MIP-2, SCGE.	**Old logwood boiler, old stove:** • High to moderate degrease of cells metabolic activity. • Cell death and genotoxicity. • Moderate MIP-2 response. **Other PM samples:** • Low to moderate responses. • Lowest responses with Logwood and Pellet boiler.
Corsini et al.,	2013	A549, THP-1	Pellet stove. Fuels: Fir or beech pellets.	3–48h exposure to 1–100 µg/ml.	$PM_{2.5}$ extraction: water. Elements, ions, PAHs.	LDH, ROS, SCGE, micronucleus assay, IL-8 and uptake of PM.	**Similar effects by both fuels:** • DNA damage, oxidative stress and IL-8 release.

Abbreviations: PM, particulate matter; OC, Organic carbon; EC, Elemental carbon; PAHs, Polycyclic aromatic hydrocarbons; TOC, total organic carbon; SCGE, single cell gel electrophoresis; IL, Interleukin; TNF, Tumor necrosis factor; LDH, lactate dehydrogenase; MIP, macrophage inflammatory protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, Reactive oxygen species; PI, Propidium iodide.

3 Aims of the study

The overall aim of this thesis was to investigate how the distinctive chemical composition of particulate emissions from new and old small-scale wood combustion appliances influences the toxicity of the emissions.

The specific aims of the individual studies are listed below.

In vivo:

1. To investigate the short-term inflammation and toxic responses in lungs and serum of healthy mouse after exposure to particulate samples derived from old and new small-scale wood combustion appliances (I, II).

2. To identify the potential causative chemical compositions of wood combustion PM inducing the inflammatory and toxic responses in mouse (I, II).

In vitro:

3. To examine toxic responses in mouse macrophage cell line induced by PM emerging from different combustion conditions generated in a novel adjustable biomass combustion reactor (III).

4. To determine the toxic properties in a mouse macrophage cell line of main compounds in the PM emitted from efficient wood combustion (IV).

5. To use a mouse macrophage cell line to investigate the toxic role of Zn in efficient wood combustion PM emissions (V).

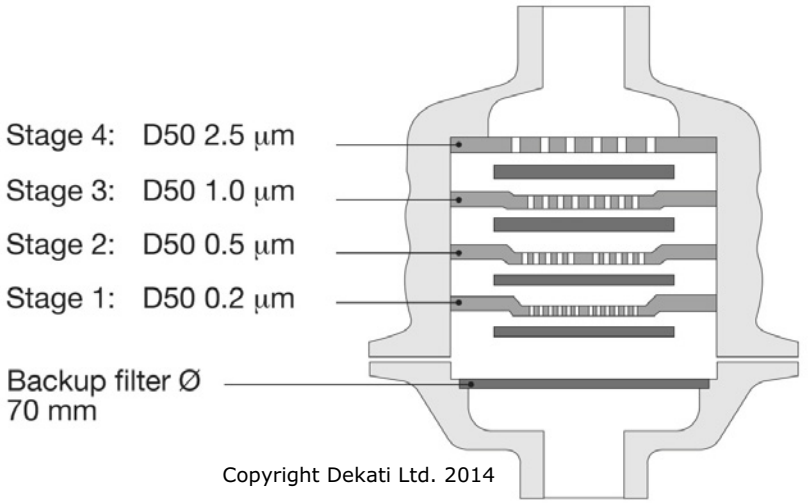
4 Materials and methods

4.1 PARTICULATE MATTER SAMPLES (I-V)

4.1.1 Particulate sampler used in campaigns

The Dekati® Gravimetric Impactor (DGI, Dekati Ltd, Tampere, Finland) was used for PM sample collection in this thesis. It has a high flow rate of 70 l/min and it archives high collection efficiency within relatively a short collection time, yet it is compact in size. In the sample collection setup, a porous tube diluter (PRD) was used to dilute and cool down the sample gas with minimal losses of PM (Ruusunen et al., 2011). In the impactor, the sampled particles will be divided into four size fractions (Figure 2.). In addition, a bottom filter (backup filter) is used to collect the smallest particles. The sampling system and the cut-off points of each stage when using the flow rate of 70 l/min are presented in Figure 2.

Figure 2. The stages of the DGI impactor.



4.1.2 Preparation of filter in the sampling campaigns

The backup filter (Fluoropore™ membrane filter, PTFE, 3.0 μm, 90 mm, Millipore Corp.) was die-cut from 90 mm to 70 mm in diameter. Other 47 mm (Fluoropore™ membrane filter, PTFE, 3.0 μm, 47mm/70mm, Millipore Corp.) filters were unaltered. Before sampling, all PTFE filters were washed with methanol (J. T. Baker HPLC grade, Deventer, The Netherlands), dried at +50 °C for 3 h and weighed in an analytical balance (Mettler Toledo XP 105DR, Mettler-Toledo Inc., Columbus, OH, USA). The dried substrates were packed in petri slides/dishes and the larger petri dishes were sealed with parafilm. The sets of substrates were stored at room temperature prior to DGI sampling.

4.1.3 Particulate sampling campaigns

The small-scale wood combustion appliances and corresponding combustion quality are presented in Table 5. All of the PM samplings from combustion experiments were performed in a laboratory environment. In studies I and II, particle sampling was conducted in Graz University of Technology, Austria. The furnaces represented both old and new wood combustion technologies (Kelz et al., 2010; Brunner & Obernberger, 2009). In studies III-V, PM collections were conducted in University of Eastern Finland, Kuopio, Finland. Study III was done using an adjustable solid-fuel biomass combustion reactor with a moving step-grate burner (Leskinen et al., 2014). Study V was conducted using a pellet boiler (Biotech GmbH, model PZ-RL 25) (Lamberg et al., 2011). The pellet boiler was operated using optimal settings. In study IV, PM was generated by using flame spray pyrolysis (FSP) (Mädler et al., 2002). Table 6 shows the precursors and detected products.

Table 5. *Different small-scale wood combustion appliances and corresponding used combustion situations. Batch combustion in stoves included all batches and burning phases.*

Appliance	Nominal output	Fuel	Combustion quality	Study
Log wood boiler OT	15 kW	Beech	Smouldering	I, II
Log wood boiler NT	30 kW	Beech	Intermediate	I, II
Stove OT	6.5 kW	Beech	Incomplete	I, II
Stove NT	6 kW	Beech	Incomplete	I, II
Tiled stove NT	4.2 kW	Beech	Intermediate	I, II
Woodchip boiler NT	30 kW	Hard-wood	Efficient	I, II
Pellet boiler NT	21 kW	Hard-wood	Efficient	I, II
Moving step-grate burner	40 kW	Wood chips from spruce and broadleaved trees	Efficient, intermediate and smouldering	III, IV
Pellet boiler	25 kW	Sawdust from pine stem wood, added Zn 0, 170, 480 or 2300 mg/kg	Efficient	V

Abbreviations: OT, old technology; NT, new technology.
Note. Combustion quality was determined using appendix 1.

Table 6. *Precursor solution compositions used in flame spray pyrolysis particle synthesis (IV).*

Precursor solute	K-cetylacetonate	Dimethylsulfoxide	Zn-acetate dihydrate
Formula	$C_5H_7O_2K$	C_2H_6OS	$C_4H_{10}O_6Zn \bullet 2H_2O$
Concentration	mmol/l	mmol/l	mmol/l
End product			
ZnO	[-]	[-]	30
K+S+Zn	20	80	0.6
K+S	20	80	[-]
K	100	[-]	[-]

4.1.4 Sample extraction for chemical and toxicological analysis

The sampled PTFE were weighed and subsequently extracted with HPLC grade methanol for 2 x 30 min in a water bath sonicator (FinnSonic m20, Finnsonic Oy, Lahti, Finland) at below +35 °C. The methanol extracts from the particulate-loaded substrates (Stages 2, 1 and backup filter) of each campaign were pooled to form the PM_1 sample and excess methanol was evaporated at +35 °C in a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) attached to a vacuum pump set at 150 mbar. The concentrated suspension was divided into 10 ml KIMAX glass tubes as the defined amount of particulate mass and dried under nitrogen (99.5%) flow. The resultant dried samples were stored at -20 °C prior to the subsequent animal or cell culture studies and chemical analysis. The same procedure as utilized with particulate samples was adopted in the preparation of the corresponding blank filters (Tapanainen et al., 2011). The extraction efficiency was determined by weighing randomly selected substrates and calculating the removed mass from the substrates.

4.1.5 Characterization of the particles

4.1.5.1 PAH analysis

A total of 30 PAH compounds were analyzed by using a gas chromatograph mass spectrometer (6890N GC, equipped with 5973 inert Mass Selective Detector, Agilent Technologies, CA, USA). A HP-17-MS column was used for the separation of the compounds. The equipment was operated with selected ion monitoring (SIM) mode. The analysis was carried out as described by Lamberg et al. (2011). The detection limit of the method was 0.1 ng/mg. The sum of the known genotoxic PAH compounds was calculated according to WHO (1998).

4.1.5.2 Element and ion analysis

In studies I and II, the determination of the chemical composition (Si, Ca, Mg, Mn, K, Na, Zn, S) of PM_1 samples was conducted 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 elemental detection with ICP-OES or ICP-MS. The Cl concentration was measured by bomb combustion in oxygen and absorption in NaOH with ion chromatography (ICS 90 Dionex).

In studies III-V, elements (Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Se, Sr, V, and Zn) and ions (Cl^-, Br^-, F^-, NO_3^-, SO_4^{2-} and NH_4^+) were determined from the PM_1 samples using HF-HNO_3 acid and deionized water. The elements were analyzed with an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700; method EN ISO 17294-2) and ions were analyzed with ion chromatograph (IC, Compact 882 ICplus; anion colon Metrosep A SUPP5-150/4.0; method EN ISO 10304-1/2) system.

4.1.5.3 Carbon analysis

In studies I and II, the contents of different carbon compounds including OC, EC and IC in the aerosol samples were determined with a carbon/hydrogen analyzer (LECO RC-612). The sample was inserted into a quartz tube that was heated to pre-defined temperatures. The temperatures ranged 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 sensors. Carbon released in a temperature range from +200 to +600 °C under an inert atmosphere was designed as OC, carbon released between +600 and +900 °C was considered as IC and carbon detected after switching to oxidizing conditions was defined as EC.

In studies III-V, OC and EC were analyzed from the PM_1 samples collected on the quartz filters from the diluted flue gas. The sampling was carried out using a porous tube diluter and ejector diluter in series, the sampling setup has been previously described in more detail by Torvela et al. (2014). The analyses

were performed with a thermal-optical carbon analyzer (model 4 L, Sun Laboratories Inc.) with the NIOSH-protocol (NIOSH, 1999).

4.1.5.3 Transmission electron microscopy analysis

In studies IV and V, transmission electron microscopy (TEM, JEM 2100F, JEOL Ltd) and energy dispersive X-ray spectrometry (EDS, NS7 Thermo Scientific) were used in the analysis of single-particle morphology and composition. The samples for TEM were collected from diluted flue gas on a holey carbon copper grid (Agar Scientific Inc., S147-400 Holey Carbon Film 400 Mesh Cu) with an aspiration sampler (Lyyränen et al., 2009).

4.2 EXPERIMENTAL DESIGN (I-V)

4.2.1 *In vivo* (I, II)

4.2.1.1 Sample preparation

The dry particulate and blank samples were thawed and stabilized to room conditions for 30 min. Thereafter, 32 µl of 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 (W1503, Sigma-Aldrich Corp., St. Louis, MO, USA) was added and the sample was sonicated for 30 min in a water-bath sonicator (Finnsonic m03, Finnsonic Oy, Lahti, Finland). 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 on the subsequent day. 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 toxicity.

4.2.1.2 Animals

Pathogen-free male C57Bl/6J mice, 8- to 9 week-old (weight 22.3 ± SEM 0.09 g) were used in all of the *in vivo* studies. The animals were obtained from the breeding colony of the Laboratory Animal Center of the University of Eastern Finland. They were transferred from a 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 h light/dark cycle (7 a.m. to 7 p.m.) at room temperature (22 ± 1 °C) and relative humidity of 55 ± 15% (mean ± SD). The National Animal Experiment Board (Eläinkoelautakunta, ELLA) approved all of the *in vivo* experiments and they were carried out in accordance with EU Directive, 2010/63/EU for animal experiments. All the tested samples and toxicological end-points are summarized in Table 7.

4.2.1.3 Exposure method and dose

Prior to intratracheal aspiration exposure, the mice were anesthetized with vaporized 4.5% sevoflurane (Abbott, IL, USA) and placed in a 66° upward bent position with the incisors placed held by thin wire. The administration of particles was performed under visual control with the tongue gently pulled out with forceps to prevent the mouse from swallowing. The sample was delivered onto the vocal folds with a pipette tip. The nostrils were covered forcing the mouse to inspire the particle suspension (1, 3, 10 or 15 μg/kg). No signs of lung overloading with the largest mass dose (15 μg/kg) were observed. Moreover, the doses used in this study were not higher than those usually used to induce inflammatory responses in the rodent lungs (Adamson et al., 1999; Walters et al., 2001; Schins et al., 2004, Gerlofs-Nijland et al., 2005).

4.2.1.4 BALF and blood collection

At the pre-defined time point, the mice were anesthetized with pentobarbital (60 mg/kg) and exsanguinated by cardiac puncture. The collected blood was centrifuged (1900g, 8 min) for the separation (Capiject T-MG, Terumo, MD) of serum which was frozen (-80 °C) prior to subsequent cytokine analysis. The lungs were perfused with sterile saline. Thereafter, the trachea was cannulated with polyethylene tubing and the 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.

4.2.1.5 Experiments

Study I contained both a dose-response screening of the particulate samples with two selected appliances and a time-course investigation of some inflammatory parameters from BALF (Table 7.). In study II, more extensive cytokine and chemokine battery was investigated from the BALF and serum of the mice at two time points (Table 7.).

Table 7. *Used samples and analyzed markers in in vivo experiments*

Study number	Samples	Dose (mg/kg)	n	Time-points (h)	Markers
Dose response					
I	Untreated animal	-	4	4, 18	**From BALF**: Total protein, LDH, IL-6, MIP-2
	Pathogen-free water	50 µl/animal	4	4, 18	
	Blank	10	8	4, 18	
	Diesel*	10	6	4, 18	
	Urban air $PM_{10-2.5}$**	10	3	4, 18	
	LPS	40 µg/animal	3	4, 18	
	Logwood boiler OT	1, 3, 10, 15	6/dose	4, 18	
	Woodchip boiler NT	1, 3, 10, 15	6/dose	4, 18	
Histopathology					
I	Untreated animal	-	6	24	**Histopathology**: Inflammatory changes, particulate matter accumulation
	Blank	10	6	24	
	Logwood boiler OT	10	6	24	
	Logwood boiler NT	10	6	24	
	Stove OT	10	6	24	
	Stove NT	10	6	24	
	Tiled stove NT	10	6	24	
	Woodchip boiler NT	10	6	24	
	Pellet boiler NT	10	6	24	
Inflammation					
I, II	Blank	10	6	4, 18	**From BALF**: Total cell number, cell differentials, total protein, LDH, SCGE **From serum and BALF**: IL-1β, IL-12, IFN-γ, IL-6, KC, IL-10, TNF-α
	Pathogen-free water	50 µl/animal	3	4, 18	
	Logwood boiler OT	10	6	4, 18	
	Logwood boiler NT	10	6	4, 18	
	Stove OT	10	6	4, 18	
	Stove NT	10	6	4, 18	
	Tiled stove NT	10	6	4, 18	
	Woodchip boiler NT	10	6	4, 18	
	Pellet boiler NT	10	6	4, 18	

Abbreviations: PM, particulate matter; OT, old technology; NT, new technology; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; SCGE, single cell gel electrophoresis; TNF, Tumor necrosis factors; MIP, macrophage inflammatory protein; LPS, Lipopolysaccharides; INF, Interferon; KC, Keratinocyte-derived chemokine.
*Diesel from Ruusunen et al. (2011); **Athens PM from Happo et al. (2007)

4.2.2 *In vitro* (III-V)

4.2.2.1 Sample preparation

Half an hour before the exposure, PM_1 samples were dispersed into DMSO (20 µl/mg) (Merck KGaA, Darmstadt, Germany) by mixing with a glass rod. Thereafter pyrogen free water (W1503, Sigma-Aldrich Corp., St. Louis, MO, USA) was added to achieve a final PM concentration of 5 mg/ml. The PM_1 samples were then kept in an ultrasonic water bath (FinnSonic M03, FinnSonic Ltd., Lahti, Finland) for 30 min.

4.2.2.2 Cell line

RAW 264.7 mouse macrophages (ATCC, Rockville, MD, USA) were cultured in a humid atmosphere of 5% CO_2 and +37 °C in RPMI culture medium with 10% heat inactivated fetal bovine serum (Gibco, Paisley, UK), 2 mM L-glutamine (Gibco, Paisley, UK) and 100 U/ml penicillin/streptomycin (Gibco, Paisley, UK). Prior to the exposure experiments, the cells were seeded at a density of 5×10^5 cells/ml in 6-well plates (2 ml/well, Corning Inc., New York, USA) and grown for 24 h. One hour before the exposure, fresh complete culture medium was added to the wells.

4.2.2.3 Experiments

Mouse macrophages were exposed to the same mass doses (15, 50, 150 and 300 μg/ml) of particles for 24 h in each study (III-V). Exposures of the cells to the particulate samples were conducted in three independent experiments. All experiments included DMSO (concentration 0.3 % v/v), blank substrate (dose 150 μg/ml) and the pyrogen-free water (dose 150 μg/ml) controls. After the 24 h exposure, the macrophages were scraped from the wells with a cell lifter (Corning Inc., New York, USA) and a sample was taken for the MTT test. The cell suspension was centrifuged (8000 rpm, 5 min, +4 °C) to separate the cells and particles from the cell culture medium. The supernatant was stored at -80 °C for the analysis of inflammatory mediators. The cells were suspended into 1 ml of PBS (Gibco, Paisley, UK) and were used in the PI-exclusion assay, fixed with ethanol (70% v/v, Altia, Finland) or used in single cell gel electrophoresis. All the used PM samples and toxicological endpoints are summarized in Table 8.

Table 8. *Used samples and end-points in in vitro experiments.*

Study	Samples	Dose (µg/ml)	Time-point (h)	Markers
III, IV, V				
	Water	150	24	PI-exclusion assay, cell cycle, SCGE, MTT ROS, TNF-α, MIP-2
	DMSO	3.6	24	
	Blank	150	24	
	Diesel*	150	24	
III				
	Efficient	15, 50, 150, 300	24	MTT, cell cycle, SCGE, ROS, TNF-α, MIP-2
	Intermediate	15, 50, 150, 300	24	
	Smouldering	15, 50, 150, 300	24	
IV				
	ZnO	15, 50, 150, 300	24	PI-exclusion assay, cell cycle, ROS, TNF-α
	K+S	15, 50, 150, 300	24	
	K+S+Zn	15, 50, 150, 300	24	
	K	15, 50, 150, 300	24	
	Efficient	15, 50, 150, 300	24	
V				
	Native	15, 50, 150, 300	24	PI-exclusion assay, cell cycle, SCGE, ROS, TNF-α, MIP-2
	Zn-low	15, 50, 150, 300	24	
	Zn-medium	15, 50, 150, 300	24	
	Zn-high	15, 50, 150, 300	24	

Abbreviations: DMSO, Dimethyl sulfoxide; PI, propidium iodide; SCGE, single cell gel electrophoresis; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNF, Tumor necrosis factors; MIP, macrophage inflammatory protein.
*Diesel from Ruusunen et al. (2011)

4.3 TOXICOLOGICAL ANALYSIS (I-V)

4.3.1 Tissue damage and cell death

4.3.1.1 LDH and protein measurement (I, II)

In the *in vivo* studies, lactate dehydrogenase (LDH) activity and protein concentration were analyzed from fresh BALF 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). The 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).

4.3.1.2 MTT-assay (III)

In the *in vitro* study the metabolic competence of the RAW 264.7 mouse macrophages was determined using the MTT-assay in 96-well-plates and calculated as a percentage of absorption of exposed cells as compared to unexposed control cells. To ensure methodological reliability, absorptions of emission particles only, vehicle controls (DMSO in pyrogen free water) and blank controls were also measured. The MTT assay measures the colored compound, formazan, which is metabolized from MTT [3(4,5-dimethylthiazol-2-yl)-bromide 2,5-dephenyltetrazolium]. The maximum absorbance was read at 570 nm using Victor^3 Multilabel Counter (PerkinElmer, MA, USA) (Tapanainen et al., 2011).

4.3.1.3 PI exclusion method (IV, V)

In the *in vitro* studies the total amount of PI positive RAW 264.7 cells, was assayed using flow cytometry (CyAnTM ADP Analyzer, Beckman Coulter, CA, USA). The cells were washed once with PBS before labeling them with PI (0.5 ml PBS, 1 μg/ml PI) for 15 min at room temperature in the dark. Thereafter, the cells were immediately analyzed using the excitation at 488 nm and emission at 613 ± 20 nm (channel FL 3). A total of 12 000 cells were analyzed for their PI content using Summit software version 4.3 (Beckman Coulter, CA, USA) (Jalava et al., 2012).

4.3.1.4 Cell cycle analysis (III-V)

In the *in vitro* studies, the cell cycle phase of the RAW 264.7 cells was determined by PI staining of the permeabilized cells. Cells fixed in 70% ethanol were centrifuged (400 g, 10 min), the supernatant was discarded and the cell pellet was re-suspended in PBS. The cell suspension was treated for 1 h with 0.15 mg/ml ribonuclease A at +50 °C before adding PI to a final concentration of 8 μg/ml. The cells were then incubated for another 2 h at +37 °C in the dark before analyzing them with a flow cytometer, excitation at 488 nm and emission at 613 ± 20 nm (Channel FL3, CyAnTM ADP Analyzer, Beckman Coulter, CA, USA). A total of 12 000 cells were analyzed for their PI content using Summit software version 4.3 (Beckman Coulter, CA, USA). Cells that contained fragmented DNA were labeled as apoptotic ($SubG_1$) (Nicoletti et al., 1991; Darzynkiewicz et al., 1992). Possible interference with the method by the emission particles was also tested and found to be insignificant.

4.3.2 Measurement of inflammation (I-V)

4.3.2.1 BALF cells (I, II)

In the animal studies, BALF cells were separated by centrifugation (500 g, 10 min) and the supernatant was removed for further analysis. The separated cell pellet was re-suspended into 220 μl of sterile saline prior to cell counting. The total cell number and share of dead cells were microscopically counted from each sample by using a Bürker chamber and the trypan blue exclusion method. The remaining cell suspension was used for differential counting of cells by cytospin (210 μl, 500 rpm, 8 min; Megafuge, Heraeus Instruments, Germany). The slides were fixed with May-Grünwald–Giemsa dye. In the analysis of cell differential, at least 300 cells 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.

4.3.2.2 Histopathology (I)

The lungs of the animals that were not lavaged for BALF collections were used in the 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. The 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.

4.3.2.3 ELISA method for cytokine analysis (I, III-V)

In *in vivo* study I, interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) concentrations were analyzed from BALF of the animals. Cytokine analysis 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) and determined by interpolation from the standard curve using WorkOut2™ software (version 2.0, Dazdaq, UK). In the *in vitro* studies III and V, MIP-2 and TNF-α and in study IV TNF-α was measured from cell culture medium as described above.

4.3.2.4 Electrochemiluminescence method for cytokine analysis (II)

In the *in vivo* study II, the MSD multiplex Mouse ProInflammatory 7-Plex Assay (Ultra-Sensitive Kit K15012C, Meso Scale Discovery) for mouse IL-1β, IL-12, IFN-γ, IL-6, KC, IL-10, TNF-α cytokines, and chemokines was performed by using Sector™ Imager 2400A. The assay was arranged as follows: calibration curves were prepared in the supplied assay diluent (mouse serum samples) or 0.9% NaCl solution with 1% BSA (BALF samples), in a range of 10,000–2.4 pg/ml. Cytokine and chemokine concentrations in the samples were determined with Discovery Workbench 2006® (3.0.18) software, using the software's curve fitting model.

4.3.3 Genotoxicity and ROS analysis

4.3.3.1 Genotoxicity (I, III, V)

The single cell gel electrophoresis (SCGE) assay was used to determine DNA damage caused by the PM_1 samples both in BALF cells *in vivo* and RAW 264.7 macrophages *in vitro*. The alkaline version of the assay was conducted according to the original version of Singh et al. (1988). The analysis of DNA migration was conducted on ethidium bromide stained microscope slides (100 nucleoids per analysis) using the Comet assay IV (Perceptive Instruments Ltd., UK) image analysis software. The comet parameter used for statistical analysis was olive tail moment (OTM) [(tail mean – head mean) × tail%DNA/100].

4.3.2.2 ROS analysis (III-V)

The intracellular accumulation of reactive oxygen and nitrogen species inside RAW 264.7 macrophages was measured by flow cytometry using logarithmic FL-1 channel. During the last 30 min of the incubation, RAW 264.7 cells were loaded with 1 μM 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Invitrogen Corp. Carlsbad, CA) in PBS. After a 30-min incubation period, the cells were washed with PBS, harvested, centrifuged, and washed one more time with 1 ml of PBS. The cell pellet was suspended in 1 ml of PBS and the fluorescence signal of 2′,7′- dichlorodihydrofluorescein (DCF) was analyzed in a flow cytometer. A total of 12 000 events were analyzed per sample using the Summit software version 4.3. The percentage of DCF positive cells was determined using Summit software's overlay option. The histogram overlay was performed using water treated cells as the negative control.

4.4 STATISTICAL ANALYSIS (I-V)

4.4.1 *In vivo* (I, II)

All the measured values were first analyzed with Levene's test for equality of variances. Statistical differences in the measured BALF and serum parameters between the particulate sample-treated animals and blank sample treated control animals were determined with an analysis of variance (ANOVA) and Dunnett's post-hoc test. In cases where Levene's test gave values < 0.05, the Kruskal–Wallis test was used. The differences in data were regarded as statistically significant at $p < 0.05$. Differences between the heating appliances were tested with Tukey's honest significant difference (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 ($p < 0.05$). The results of the SCGE assay were analyzed using Student's t-test ($p < 0.05$). All the measured values were analyzed with Spearman's rank correlation (two-tailed) to examine the linear relationships between the variables. Correlation coefficients (ϱ) between the different variables were regarded as statistically significant at $p < 0.05$ level. The correlation analysis between chemical constituents and measured responses were conducted at the time point showing the clearest differences between the animal groups. 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).

4.4.1 *In vitro* (III-V)

The measured responses were compared to the control and to the corresponding blank samples with regard to particle doses. Levene's test for equality of variances was used for all the samples before analyzing the data with ANOVA. Dunnett's post hoc test was used when results from the production of the inflammatory mediators or the MTT test ($n = 6$) were analyzed. The results from the SCGE, ROS analysis, PI-exclusion and cell cycle analysis were evaluated by the non-parametric Kruskal-

Wallis test (n = 3). ANOVA and Tukey's post hoc test were used in the analysis of differences between the combustion particles. Differences were considered to be statistically significant at $p < 0.05$. The data were statistically analyzed with IBM SPSS Statistics 19.0 (IBM®, New York, NY).

5 Results and discussion

5.1 *IN VIVO* STUDIES (I, II)

5.1.1 Chemical composition of the particulate samples (I, II)

The small-scale furnaces included in this study were as follows: pellet boiler (NT), wood chip boiler (NT), tiled stove (NT), logwood boiler (NT), stove (NT), log wood boiler (OT) and stove (OT). The chemical composition of the particle samples from animal studies (I, II) is presented in Table 9. The inorganic ash components were enriched in the emission samples from the NT continuous combustion furnaces (pellet and wood chip boiler), which had also the highest combustion efficiencies (Brunner & Obernberger, 2009; Kelz et al., 2010). In particular, there were substantial differences in the Zn concentrations between the pellet boiler and other appliances. In addition, the concentrations of other alkaline (K and Na) and transition metals (Mg, Cd and Mn) were higher in the PM samples from NT appliances. Moreover, the amounts of chloride and sulfur, which usually form metal chlorides and sulfates in combustion particles, were also enriched in the NT samples. In contrast, PAH concentrations were substantially higher in the emissions from OT furnaces. The PAHs followed the OC and EC concentrations in the emission samples.

On the basis of many previous studies, the detected changes in compound concentrations can be considered to be indicative of the combustion efficiency (e.g. Tissari et al., 2008). It is clear that the inorganic ash compounds predominate when there is almost complete combustion whereas organic as well as carbonaceous compounds are present in greater amounts in conditions of incomplete combustion (Tissari et al., 2008; Sippula et al., 2009; Lamberg et al., 2011, 2013).

Substantial differences in the burnout qualities of OT and NT appliances were detected and those also affected the chemical composition of emissions. Modern technology appliances had the lowest PM_1 (mg/MJ) and the old technology appliances had clearly the highest PM_1 (mg/MJ) emissions (I, Table 1.). Many previous *in vitro* studies have demonstrated that wood combustion PM from different combustion conditions induces highly variable toxic effects (Tapanainen et al., 2011, 2012; Jalava et al., 2012). However, there are an extremely limited number of animal studies dealing with toxicity of PM emissions from new technology wood combustion appliances. Thus far, new and old technology wood combustion appliance-derived PM has not been studied as extensively with *in vivo* models as conducted in this thesis.

Table 9. *Amounts of the chemical constituents (ng/mg) in particles emitted from the seven small-scale wood combustion appliances.*

Compound	LogOT	StoveOT	StoveNT	LogNT	Tiled	Woodchip	Pellet
EC	190000	420000	280000	50000	220000	50000	50000
OC	170000	150000	400000	70000	150000	120000	100000
Total PAHs	35000	64000	4000	3300	1800	220	1200
Ca	1140	780	1640	1780	2440	3000	4130
Cd	6	6	17	11	28	22	106
Cl	3820	6640	14400	13900	16500	15000	50300
K	19700	25500	30700	242000	175000	210000	288000
Mg	90	70	160	400	360	690	620
Mn	30	30	30	130	70	830	1830
Na	1960	1710	2910	3490	6380	3330	9720
S	3290	6310	6130	43600	51600	60400	101000
Zn	250	480	590	1520	3230	4450	25900

Abbreviations: OT, old technology; NT, new technology; EC, Elemental carbon; OC, Organic carbon; PAHs, Polycyclic aromatic hydrocarbons.

5.1.2 Pulmonary responses to particulate samples (I, II)

5.1.2.1 Inflammatory mediator responses (II)

The inflammatory responses assessed as cytokine and chemokine concentrations in BALF were detected rapidly after wood smoke PM exposure. At the 4 h time point, pro-inflammatory cytokine IL-6 and chemokine keratinocyte-derived chemokine (KC) responded to woodchip and pellet boiler PM exposure (Figure 3.). At the later time point (18 h), the IL-6 response was already diminishing but KC levels remained at the same or even higher levels as measured at 4 h after the exposure. Moreover, IL-1β concentrations reached a statistically significant level 18 h after the PM exposure from the NT stove and tiled stove. Cytokine responses seemed to be dependent on the combustion efficiency. The PM emitted from the OT stove and logwood boiler did not induce any changes in cytokine concentrations. Instead, almost all of the particles from the NT appliances induced statistically significant increases in cytokine production. The detected inflammatory mediator responses in mouse BALF were at a much lower level than those seen in studies with urban air PM samples (Happo et al., 2007, 2008). However, even these minor inflammatory effects indicate that PM from small-scale wood combustion possesses physicochemical properties that can promote inflammation in murine lungs. Chemical analysis of present PM samples showed that high combustion efficiency leads to an enrichment of metals in emission. Indeed, the NT stove, the woodchip boiler and the pellet boiler samples contained the high concentrations of transition metals (e.g. Cd, Mg, Zn).

Findings of high inflammatory responses with metal rich wood combustion PM samples are in agreement with the previous work of Adamson et al., (1999) and Dick et al., (2003); these works examined the cytokine responses and neutrophil infiltration in the lungs of mice after exposure to ambient air PM containing high metal concentrations. With respect to the single components of emission samples, Zn has been shown to have important role in determining the pulmonary cell reactivity to

inhaled particles (Adamson et al., 2000; Wallenborn et al., 2009) and oxidative stress response in lungs (Gurgueira et al., 2002; Tao et al., 2003). Moreover, soot rich wood smoke particulate samples as well as inhaled wood smoke from conventional stoves have been associated with relatively low inflammatory activity in the *in vivo* studies (Reed et al., 2006; Seagrave et al., 2005, 2006) as well as in *in vitro* experiments (Karlsson et al., 2006; Jalava et al., 2007; Kocbach et al., 2008a,b). This could be due to an immunosuppressive effect caused by the PAH compounds leading to lower inflammatory responses in mouse lungs.

Particle-induced inflammation has been postulated to be one of the important mechanisms for increased human cardiovascular risk (Anderson et al., 2012). Indeed, in this thesis elevated inflammation mediator levels were measured in mice after exposure to PM from NT wood combustion appliances. In particular increased levels of IL-6 could be linked to human exposures of PM (van Eeden et al., 2001; Anderson et al., 2012). Those results clearly indicate that the chemistry of PM is one important factor behind toxic effects. Moreover, insolubility and surface properties of particles may play an important role in determining the types of inflammation response detected. However, sub-acute or chronic toxicity was not evaluated. In those experimental setups, soot rich samples may evoke detectable effects by PM accumulation induced chronic inflammation or genotoxicity.

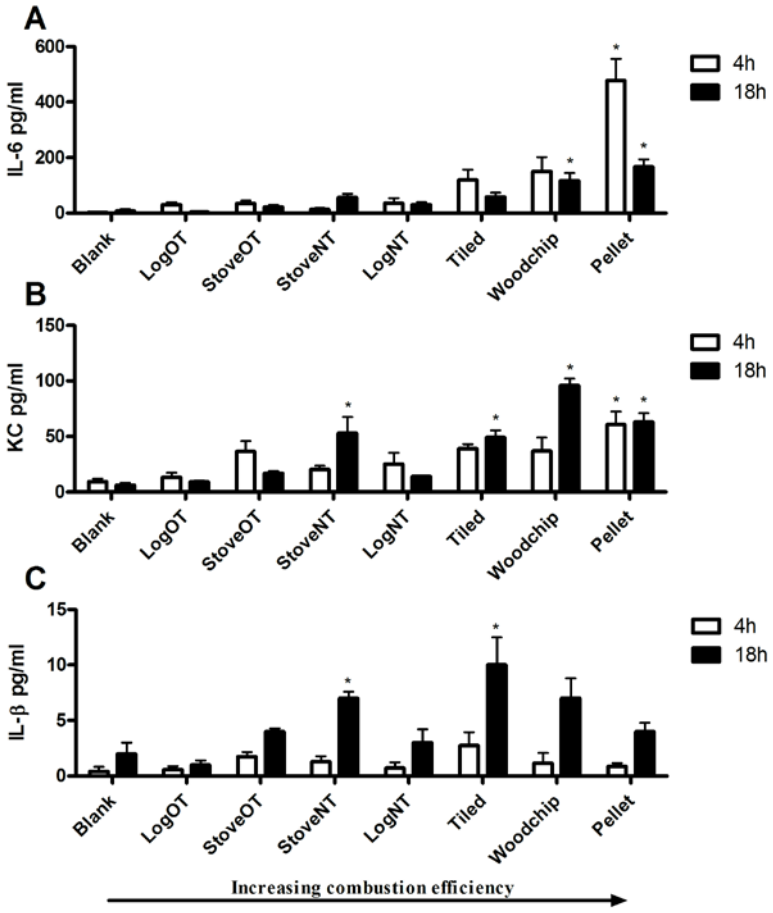


Figure 3. IL-6 (**A**), KC (**B**) and IL-1β (**C**) concentrations in bronchoalveolar lavage fluid (BALF) at 4 and 18 h after intratracheal aspiration of a single dose (10 mg/kg) of particulate samples from heating appliances or the corresponding blank sample in healthy C57Bl/6J mice. Each bar shows mean ± SEM (n = 5–6). The asterisks indicate statistically significant differences from the blank control (Dunnett's C-test, $p < 0.05$). Abbreviations: OT, old technology; NT, new technology; IL, Interleukin; KC, Keratinocyte-derived chemokine.

5.1.2.2 Cells (I, II)

Particles collected from the seven different combustion appliances had no major effect on inflammatory cells present in the lungs of the mice (Figure 4.). However, the numbers of macrophages and neutrophils changed to some extent at the later 18-h time point (Figure 4.). Indeed, the neutrophil counts seemed to increase after dosing PM from the OT and NT stoves as well as from the NT logwood boiler, the tiled stove, the wood chip boiler and the pellet boiler. However, only particles collected from the tiled stove induced a statistically significant neutrophil infiltration in mouse lung (II, Table 3.). At the same time, macrophage numbers were declined with most of the PM samples, and with the pellet boiler sample this decrease reached a statistically significant level (II, Table 3.).

These findings are in line with many previous *in vivo* studies with wood combustion particles, which have observed only a minor influx of inflammatory cells into the lungs after exposure to PM (Seagrave et al., 2005; Tesfaigzi et al., 2005; Danielsen et al., 2010). Moreover, there is evidence that the numbers of macrophages decreased in rat BALF after exposure to wood combustion emissions (Tesfaigzi et al., 2002). In that study, it was hypothesized that macrophages may adhere strongly in the lungs after wood smoke exposure. However, also some other mechanisms may be responsible for the reduction in the numbers of macrophages in BALF e.g. apoptotic or necrotic cell death.

One of the earliest responses encountered 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 (Oberdörster et al., 2002). According to the previous *in vivo* findings on acute phase inflammatory responses, the early 4 h time point was chosen as suitable for assessing the levels of cytokines and cells in BALF after particulate exposure (Happo et al., 2007). However, only slight or negligible cytokine and cell responses were detected in the present 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. It is possible that the soot rich emissions from OT boiler and stove were more insoluble and thus had very local inflammatory activity which was not manifested in this short term study. However, soot has been previously demonstrated to induce oxidative stress in murine lungs in short-term inhalation exposures studies (Chan et al., 2013; Chuang et al., 2013). Endpoints, which could detect oxidative stress, were not measured in the present study. In contrast, samples from NT appliances, which probably contained highly soluble metal sulfates and chlorides as well as insoluble metal oxides were able to induce detectable cytokine and neutrophil influx in mouse lungs. Transition metals are known to be capable of producing ROS on metal oxide surfaces as well as when the metals exist as free ions (Sarkar et al., 2014). It is possible that the solubility differences between PM derived from different combustion conditions were responsible for the differences in cell migration into lungs of mice. Those results support the epidemiological findings where a relationship has been found between ambient air transition metal concentrations and the measured adverse health outcomes (e.g. Lippmann & Chen, 2009).

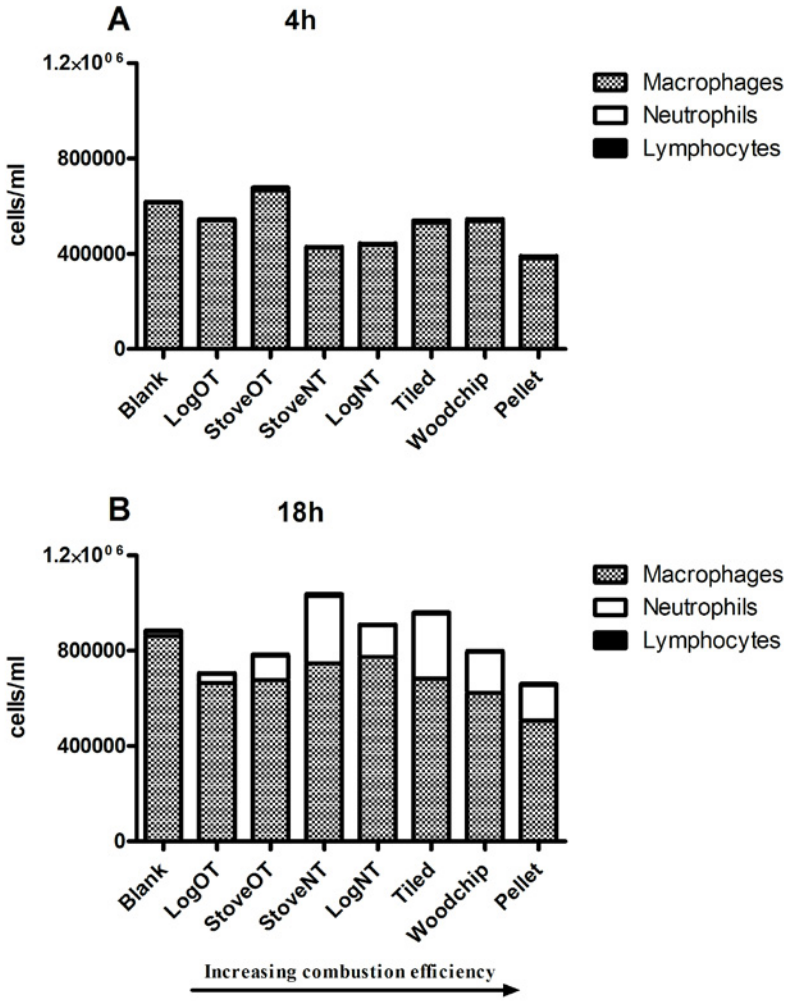


Figure 4. Total cell number in BALF from C57Bl/6J mice at 4 h (**A**) and 18 h (**B**) after intratracheal aspiration of a single dose (10 mg/kg) of particulate samples from seven heating appliances or blank sample. Each bar shows mean (n = 5-6). Abbreviations: OT, old technology; NT, new technology.

5.1.3 Relationships between toxic responses *in vivo* and the chemical composition of the samples

The values of the correlations coefficients (Spearman's ϱ) between the selected chemical constituents of the particle samples and the detected responses in BALF and serum are shown in Table 10. With respect to the PM_1 samples, both positive and negative statistically significant correlations were detected between the chemical constituents and the detected toxicological parameters. The associations between the constituents and the IL-1β or neutrophil counts displayed no statistically significant difference. In general, inorganic elements were associated with increased inflammation as well as genotoxicity. Moreover, the OC concentration which also includes PAH compounds correlated negatively with the inflammatory markers and genotoxicity. The concentrations of two alkali metals (Na and K) and one alkaline earth metal (Ca) as well as transition metals (Mg, Mn, Zn and Cd) displayed positive correlations with those of the inflammatory markers. In addition, the amount of sulfur and chlorine levels, i.e. elements that usually form metal chlorides and sulfates in combustion particles (Tissari et al., 2008; Sippula et al., 2009) had also positive correlation with the levels of inflammation and genotoxic markers. The correlation analysis between chemical constituents and measured responses were conducted at the time point showing the clearest differences between the animal groups. Due to the low response levels, the calculated correlations need to be assessed with some caution. This is particularly the case, with the OT samples, where some measured cytokine values were below the detection limit. Moreover, in some cases, there was a lack of statistical significance between the blank control and the evoked response (e.g. total protein and SCGE). However, most of the correlations detected are good in agreement with those reported in previous studies.

There was a positive correlation with transition metals and SCGE result. Previously, in a study conducted with the same combustion emission particles, it was found that increased

concentrations of PAH compounds were related to significant primary genotoxicity in mouse macrophages (Jalava et al., 2012). In contrast, in the present *in vivo* experiment, the extend of DNA damage was found to correlate with ash related constituents instead of with the level of OC. It has been shown that PAHs can be very tightly bound to carbon black (Borm et al., 2005), and therefore their bioavailability is limited in saline. It has also been reported that tightly bound PAHs may become available to form PAH- DNA adducts in *in vitro*, but no such effect was found in rat lung (Borm et al., 2005). Therefore, it is quite possible that the effective clearance mechanisms present in mouse lung limits the bioavailability time of available PAHs i.e. PAH concentrations are too low to evoke primary direct genotoxic effects in the lungs with these kinds of short-term exposures. Particles can also induce oxidant generation in an aqueous suspension e.g. via a Haber-Weiss reaction, which is catalyzed in the presence of available metals (e.g. Fe, Cu, Cr, and V) (Schoonen et al., 2006; Schins & Knaapen, 2007). This is supported by the fact that in this study the transition metal concentration was higher in the particulate emissions from NT appliance than from the OT furnaces (II, Table 1.). Although, Zn cannot induce ROS production through Haber-Weiss reactions, it can trigger mitochondrial dysfunction via several mechanisms (Rudolf et al., 2005; Rudolf & Cervinka, 2010). Mitochondrial damage is a well-known source of intracellular ROS (Zorov et al., 2014). Moreover, the detected SCGE responses could also be secondary genotoxic effects which are not induced directly by the chemical compounds themselves, but from the reactive oxygen species which are generated during particle-elicited inflammation from activated macrophages and neutrophils (Schins & Knaapen, 2007). Indeed, it has been shown that if neutrophil influx into the lungs is blocked, this will reduce significantly the level of pulmonary oxidative DNA damage (Auten et al., 2002).

The reduced influx of neutrophils has been associated with immunosuppressive effects, most likely because of the PAH-compounds present in urban air particles (Happo et al., 2010).

Moreover, this effect is seen with individual PAH compounds installed in mice (Kong et al., 1994; Jeon et al., 2005). Indeed, high concentrations of PAH compounds or wood smoke-rich particulate samples are associated with reduced inflammatory activity in murine lungs (Seagrave et al., 2006; Happo et al., 2008) as well as with a decrease in the levels of cytokines in BALF (Kong et al., 1994). In addition, PAH-rich samples are known to be genotoxic (Motykiewicz et al., 1990; Sevastyanova et al., 2007) and evoke cell cycle arrest in mouse macrophages (Jalava et al., 2007; Longhin et al., 2013). This cellular damage could be one mechanism for the possible immunosuppression that may explain the low level of inflammatory responses encountered after exposure to the samples gathered from OT appliances. There is also another Ah-receptor (AhR) mediated mechanism which has been postulated to explain how wood smoke can cause immunosuppression (Migliaccio at al., 2013). In this alternative, PAH compounds bind to AhR which activates transcription factor RelB leading to a decreased ability of macrophages to appropriately respond toward pulmonary infections (Migliaccio at al., 2013).

Previously, Ca has been linked with increased inflammatory responses *in vivo* (Happo et al., 2008) with exposure to urban air particles. Moreover, a link between ambient sulfate concentrations and long-term effects on mortality has been detected in epidemiological studies (e.g., Elliott et al., 2007). Other reports (e.g., Abrahamowicz et al., 2003) have challenged this relationship: it has been speculated that the sulfates may act more as a surrogate for other pollutants (e.g. metals) associated with their presence. Indeed, installation of high doses of residual oil fly ash (ROFA) in murine lungs caused acute lung injury and inflammation (Ghio et al., 2002; Marchini et al., 2014). In addition, the Utah Valley PM experiment showed similar results as earlier mentioned ROFA experiments. Utah Valley PM treated with Chelex, an agent that removes cations from solution, produced no chance in the inflammation mediator IL-8. While untreated extraction showed a significant increase in IL-8, when compared to control (Molinelli et al., 2002). These studies

clearly showed that particles with high transition metal concentrations can cause substantial lung injury, but it is not known whether similar phenomena will appear in wood combustion PM since the transition metals in wood combustion derived PM are different from the PM used in the ROFA and Utah Valley experiments. However, zinc that is the most prevalent transition metal in wood combustion emission was shown to cause pulmonary inflammation and oxidative stress in murine models (Ho et al., 2011; Chuang et al., 2014). Indeed, a possible mechanism for the toxic effects of PM transition metals was postulated to be ROS generation (Li et al., 2003). Moreover, it is possible that transition metals may synergize with organic PM components in ROS generation (Saldiva et al., 2002).

Table 10. *Associations between chemical constituents of particulate samples from different heating appliances and the toxicological markers in mice.*

	Serum markers			**BALF markers**					
Compound	IL-1β	IL-6	KC	IL-1β	IL-6	KC	Total protein	Neutrophils	SCGE
OC	0.306	-0.631	-0.739	0.306	**-0.757***	-0.162	-0.234	0.18	**-0.757***
EC	0.630	-0.482	-0.482	0.408	-0.657	-0.259	-0.185	0.074	-0.667
Ca	-0.179	0.714	**0.786***	0.214	**0.857***	0.714	**0.786***	0.464	**0.857***
Mg	-0.286	0.607	0.643	0.107	**0.786***	0.679	0.571	0.393	**0.786***
Mn	-0.334	0.704	**0.815***	-0.037	**0.927****	0.593	0.63	0.185	**0.927****
K	-0.036	**0.893****	**0.857***	0.036	**0.786***	0.536	0.607	0.321	**0.786***
Na	0.036	**0.893****	**0.893****	0.179	0.750	0.429	0.714	0.429	0.75
Zn	-0.036	0.750	**0.857***	0.321	**0.893****	**0.786***	**0.857***	0.5	**0.893****
S	-0.107	0.679	**0.893****	0.250	**0.964****	0.714	**0.821***	0.321	**0.964****
Cl	0.214	0.750	**0.821***	0.536	0.750	**0.786***	**0.964****	0.679	0.75
Cd	0.144	0.739	**0.793***	0.487	0.739	**0.757***	**0.937****	0.667	0.739

The values in this table are Spearman correlation coefficients (ρ). Boldfaced values indicate statistically significant correlations, ** $p < 0.01$, * $p < 0.05$.
Abbreviations: BALF; bronchoalveolar lavage fluid; SCGE, single cell gel electrophoresis; OC, Organic carbon; EC, Elemental carbon; IL, Interleukin; KC, Keratinocyte-derived chemokine.

5.2 *IN VITRO* STUDIES (III-IV)

After the *in vivo* part of this thesis, it was clear that more toxicological information is needed from particles derived from efficient combustion. More specifically, the *in vivo* results indicated that transition metals might be responsible for the short term-toxic effects observed in mouse lungs. Thus, a series of *in vitro* experiments was conducted to investigate the role of the transition metals, especially zinc, in wood combustion PM emissions. It was decided to clarify the toxicological characteristics of PM from three different combustion situations, to study single components from efficient combustion particles and to determine if single components enriched in the efficient combustion PM could enhance toxicity. First, three combustion conditions (efficient, intermediate, and smouldering) were generated using a grate combustion reactor in order to examine whether one could obtain similar results as in the *in vivo* part of the thesis. In the second experimental setup, synthetic nanoparticles (NPs) which represented the main components of efficient combustion PM were made. Based on the results from those experiments zinc was selected for a subsequent single component enrichment study which formed the third *in vitro* study.

5.2.1 PM samples chemical composition

The concentrations of chemical compounds of PM_1 samples from the biomass combustion as well as the composition of the selected synthetic NPs are presented in Table 11. The chemical compositions of emission particles from the three combustion situations differed considerably from each other (Study III). Particles emitted from the efficient combustion contained a much higher fraction of metals than those emitted from the intermediate or smouldering combustion situations. The most abundant transition metals detected in the efficient combustion PM sample were zinc, manganese and chromium. In contrast,

there were more PAH compounds, OC and EC emitted from the intermediate and especially in smouldering combustion situations. The smouldering combustion sample contained the highest concentrations of PAH compounds. Study IV investigated NPs with either zinc oxide or potassium sulfate; their chemical composition is also described in Table 11. Study V examined samples from pellet combustion. The combustion of pellets with added zinc produced the zinc-rich particles is described in Table 11. The chemical composition of the native pellet samples was very similar to the other pellet samples and the efficient combustion sample, with the exception of zinc concentration. Moreover, the synthetic K+S+Zn particle sample contained the same amount of zinc as the native pellet PM sample.

In real life situations, many different combustion conditions are possible. In study III, real life situations were simulated as follows: efficient conditions representing optimal biomass combustion e.g., a modern continuously operated boiler; smouldering conditions e.g. in a conventional batch combustion and intermediate conditions representing a malfunction, a partial load or startup/shutdown situation of a modern boiler (Tissari et al., 2008; Sippula et al., 2009; Lamberg et al., 2011, 2013; Heringa et al., 2011). Those combustion situations represent relatively well those present in the appliances examined in studies I and II. In study IV the synthetic ZnO particles were produced by FSP using an organometallic zinc precursor and ethanol as the fuel. This leads to the formation of ZnO NP (Height et al., 2006) similar to those that have been detected in efficient wood combustion emission PM (Torvela et al., 2014). Moreover, potassium sulfate (K+S+Zn) particles containing a low amount of zinc were synthesized, as they are commonly present in particle emissions from efficient wood combustion (Sippula et al., 2012). In study V, the used pellet materials represented real life situations. Combustion of the native pellet was representative of good quality stem wood pellet burning (Sippula et al., 2007b), whereas the Zn-low sample corresponded to wood residue pellet combustion

(Sippula et al., 2007b; Chandrasekaran et al., 2012; Jones et al., 2014). Finally, the two higher zinc-containing samples would be more commonly encountered in waste incineration (Krook et al., 2006; Jones et al., 2014).

The present result demonstrated that it is possible to generate under experimental conditions PM samples, which resemble real world wood combustion PM emissions. This was done using adjustable combustion devices in a laboratory environment. Moreover, nanoparticles containing defined amounts of potassium, sulfur and zinc were synthesized to study the major components forming inorganic particles in wood combustion. This is important since generally wood combustion emissions are extremely heterogeneous. It is an advantage if one can control many of the potential confounding factors (e.g. appliance, operation practice and fuel) in wood combustion derived particles. Since this can help, in subsequent toxicological analyses aimed at identifying the causative chemical compositions behind the adverse health effects. Moreover, when studying the single components in wood combustion emissions the potential harmfulness of the compound under study can be evaluated.

Table 11. *Concentrations of the chemical constituents (ng/mg) in particulate samples from different combustion cases.*

	Study III			Study IV		Study V			
Compound	Smouldering	Intermediate	Efficient	ZnO	K+S+Zn	Native	Zn-low	Zn-medium	Zn-high
EC	752000	523000	14000	n.a.	n.a.	68000	22000	20000	6000
OC	273000	352000	27000	n.a.	n.a.	21000	13000	22000	12000
Total PAHs	32000	2300	430	3	bdl	200	30	50	30
Cl^-	5800	27600	154000	1400	1100	70000	70000	35000	13000
NO_3^-	bdl	1140	2660	bdl	bdl	6000	3900	2700	bdl
SO_4^{2-}	3400	6000	217000	44600	550000	151000	152000	90000	25000
Ca	4800	bdl	11400	1840	13400	2340	2580	bdl	bdl
Cd	10	20	130	bdl	bdl	10	bdl	10	bdl
Cr	50	90	6000	190	260	60	60	45	50
Cu	50	140	760	40	280	190	180	150	90
Fe	bdl	4400	620	bdl	160	718	1390	846	680
K	16000	64000	300000	4000	182000	180000	129000	84600	30000
Mg	bdl	bdl	1920	bdl	980	980	1060	880	850
Mn	120	100	1260	bdl	120	320	230	180	80
Na	10000	3400	18000	2000	6000	18500	13400	7920	3540
Pb	40	130	3400	bdl	14	120	110	90	50
Zn	4000	9600	58000	560000	8400	9000	162000	348000	828000

Abbreviations: bdl, below detection limit; n.a., not analyzed; OC, Organic carbon; EC, Elemental carbon; PAHs, Polycyclic aromatic hydrocarbons.

5.2.2 Cell death, cell cycle arrest and ROS production

5.2.2.1 Cell death

The cytotoxicity results of RAW 264.7 macrophages as determined with the propidium iodide (PI) exclusion assay after 24 h exposure to selected combustion particles is presented in Figure 5A. The results are expressed as response to zinc concentration in the cell culture medium. All samples except K+S+Zn evoked a statistically significant increase in cell membrane permeability, which is indicative of necrotic or late apoptotic cell death. It is noteworthy that when the free zinc concentration in the cell culture medium exceeded 3 μg/ml (46 μM), all PI exclusion assay responses were statistically significant as compared to the corresponding control. When the zinc content increased to 46 μg/ml (704 μM) virtually all of the cells were dead. In contrast, the K+S+Zn sample did not induce any cell death. Native pellet samples caused some toxicity at the highest PM dose (300 μg/ml). In that case, the zinc content in the cell culture medium was about 1 μg/ml (15 μM).

In previous *in vitro* studies it has been shown that Zn^{2+} ions can induce necrotic cell death in human bronchial epithelial cells (BEAS-2B) as well as RAW 264.7 macrophages (Xia et al., 2008). Moreover, severe cytotoxicity has been detected after treatment of several human and murine cell lines with ZnO NPs (Zhang et al., 2014). Although the Zn^{2+} ion is thought to be responsible for the evoked toxic effects, it has been shown in *in vitro* studies that at least with respect of the ZnO NPs, contact with to cells is required to induce cytotoxicity (Moos et al., 2000; Hsiao & Huang, 2011).

When toxic effects (e.g. oxidative stress) overwhelm cellular defense mechanisms, they will cause damage to many critical macromolecules such as proteins, lipids, and DNA. As a response, the cell will attempt to repair the damage and adapt to the elevated stress. If this fails, the fate of the cell will be to undergo either apoptotic or necrotic cell death (Fink & Cookson, 2005). The present results demonstrated that there was extensive

cell death at Zn concentrations above 8-10 μg/ml in the cell culture medium. This indicates that zinc caused severe failure of cellular protective mechanisms at those concentrations. The *In vivo* results in this thesis revealed decreased macrophage numbers after exposure to PM samples originating from NT combustion appliances that contained high Zn concentrations. This decreased in macrophage numbers may have been caused by cell death.

5.2.2.2 Cell cycle arrest

An experiment was conducted to determine whether particles could disrupt normal cell cycle, this being analyzed with flow cytometry. When the cell cycle of RAW 264.7 macrophages was analyzed (Figure 5B), an accumulation of the cells in S-G_2/M cell cycle phase was noted. This is an indicator of cell cycle arrest in G_2 phase. Cell cycle arrest was detected when the zinc content in cell culture medium was 9 μg/ml (138 μM). The most severe effects on the cell cycle were observed with PM_1 samples containing the highest Zn concentrations.

In line with the present results, Yin et al. (2012) found recently that exposing RSC96 Schwann cells to 8 μg/ml (122 μM) ZnO NPs resulted in an accumulation of cells in the G_2/M phase. Moreover, exposure to zinc sulfate was previously reported to induce G_2-phase cell cycle arrest in human alveolar epithelial cells (A549) at similar Zn concentrations as used in this thesis (Könczöl et al., 2012). In addition, Wong et al. (2008) have demonstrated that Zn^{2+} ion can induce G_2/M cell cycle arrest in human bronchial epithelial cells. The mechanism behind this phenomenon may be oxidative DNA damage induced by Zn (Rudolf & Cervinka, 2011). It is known that damage to DNA may stop cells from passing through the various checkpoints in the cell cycle (King & Cidlowski, 1998). Indeed, ZnO NPs have been reported to evoke the formation of ROS and to trigger apoptotic cell death (Sharma et al., 2012).

5.2.2.3 ROS production

The amounts of intracellular ROS and RNS generation after the exposures of RAW 264.7 macrophages to PM containing various amounts of zinc are shown in Figure 5C. All the PM_1 samples except K+S+Zn and native pellet induced a statistically significant increase in ROS production in RAW 264.7 macrophages, at least when delivered at the highest PM dose (300 μg/ml). The three highest Zn containing PM_1 samples were the most potent in evoking oxidative stress in the macrophages.

Zinc, which is the most prevalent transition metal in the particles emitted during wood combustion (Sippula et al., 2007b), has been linked to ROS mediated depletion of reduced glutathione (GSH) and G_2-cell cycle arrest (Walther et al., 2003; Shih et al., 2008). Moreover, it has been shown that the oxidative potential of ambient PM significantly correlates with the Zn concentration (Zhang et al., 2008; Wang et al., 2013). In addition, it is also known that the ZnO NPs and ionic Zn^{2+} can trigger extensive oxidative stress in a variety of cell lines (Wu et al., 2013). One putative mechanism to explain the generation of ROS inside the cells as well as the other detected effects on cell viability and cell cycle is that Zn^{2+} ions reduce GSSG reductase enzyme activity leading to a decline in the storages of GSH and eventually to compromised ROS homeostasis in the cell (Walther et al., 2000, 2003; Bishop et al., 2007).

Zinc is a ubiquitous contaminant of ambient air that represents an oxidant challenge to the human lung (Wu et al., 2013). The toxicological responses to exogenous zinc range from cell death to cell cycle disruption and these are probably attributable to its oxidative properties. The Zn^{2+} cation is not capable of ROS production by itself and thus the elevated ROS levels must have an endogenous cellular origin, e.g. produced by mitochondria. Moreover, there is evidence that zinc can exert multiple oxidative effects in the lungs as well as cells during *in vitro* incubation (Dineley et al., 2005; Cheng et al., 2010). At the center of these zinc-induced oxidative properties is it's interactions with cellular thiols (Krezel et al., 2007). Thus, the release of ZnO NPs or combustion-generated particles with high

Zn concentrations into the environment would be predicted to have negative health effects.

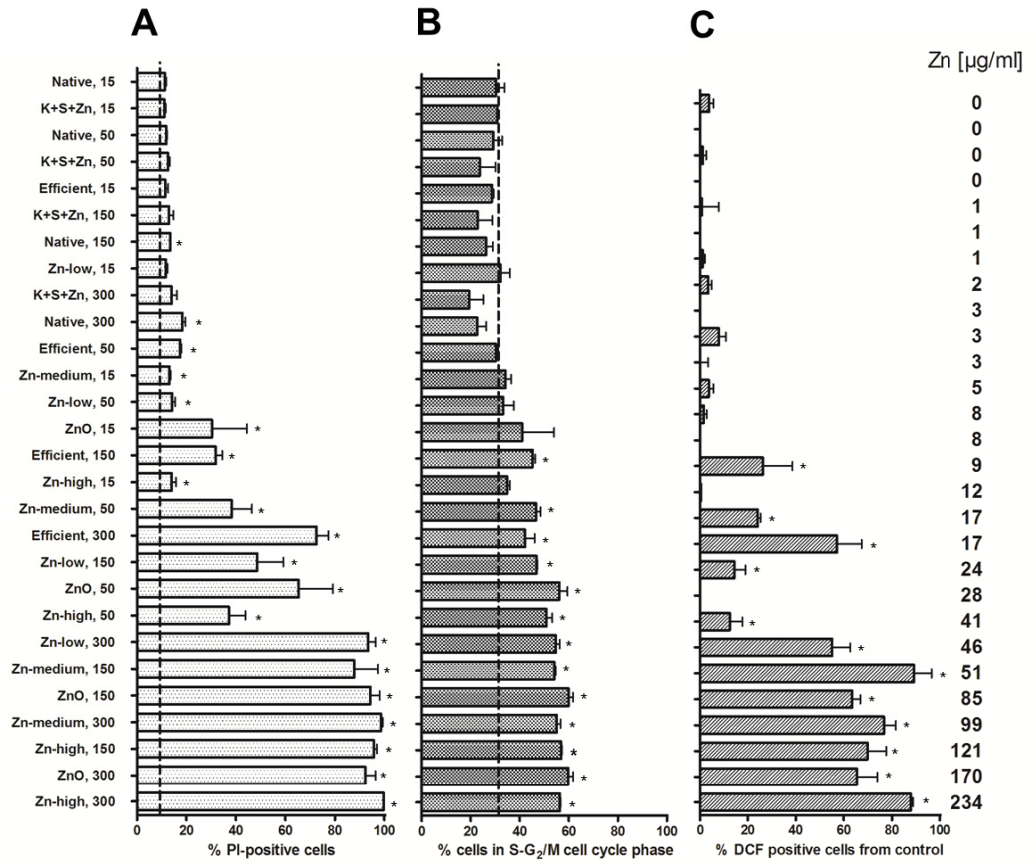


Figure 5. Acute cytotoxicity (**A**), S-G_2/M cell cycle phase (**B**) and reactive oxygen species production inside cells (**C**) after 24 h exposure of RAW 264.7 macrophages to particulate samples from efficient combustion of wood chips and pellets as well as flame spray pyrolysis. The columns represent means, with error bars showing standard errors of the mean (SEM). The asterisks indicate statistical significance compared to the blank substrate control ($p < 0.05$) analyzed by the nonparametric Kruskal−Wallis test. The numbers after combustion samples represent PM doses (15, 50, 150 and 300 µg/ml) and numbers under Zn [µg/ml] represent maximum free zinc ion concentration in the cell culture medium.

Abbreviations: DCF, 2′,7′ - dichlorodihydrofluorescein; PI, Propidium iodide.

5.3 METHODOLOGICAL CONSIDERATIONS

In this thesis, PM samples were collected using a Dekati® gravimetric impactor. After collection, the gathered PM mass was extracted from the filters using methanol and dried using nitrogen flow. Prior to the *in vivo* or *in vitro* exposures, the PM samples were thawed and re-suspended using DMSO and incubation in an ultrasonic water bath. This slight amount of DMSO is needed to detach the dried PM from the sides of the glass tubes. It is known that DMSO can have some cell-protecting abilities due to ROS scavenging and may increase transportation of particles through the cell membranes and surfaces of alveoli (Colucci et al., 2008). In addition, DMSO is known to have both inflammatory and anti-inflammatory effects (Colucci et al., 2008). Those properties were excluded in pilot studies conducted both *in vivo* and *in vitro*. Moreover, DMSO is a standard solvent in PM toxicity studies (e.g. de Kok et al., 2006).

One important issue to be considered is that when PM is collected in an impactor one loses all of the gaseous compounds. Moreover, volatile and semi-volatile compounds are at least to some extent lost during the extraction procedure. Finally, after extraction and PM re-suspension, the particles do not return to their original PM_1 size when *in vivo* and *in vitro* exposures are conducted. However, without direct exposure methods (e.g. at the air-liquid interface) this is the best option available.

An intratracheal aspiration technique was used in the present *in vivo* studies to deliver the PM samples to the lower airways of mice. Naturally, this administration differs from inhalation exposure. However, it was shown that intratracheal aspiration and inhalation exposure can result in similar outcomes, although there were some differences in the intensity of the responses (Costa et al., 2006; Shvedova et al., 2008). It was postulated that in intratracheal aspiration, the particles in the suspension contain agglomerates, which lower their reactivity in comparison with inhalation exposure. In this thesis, the doses used in the *in vivo* experiments may seem relatively high.

However, these doses are no higher than the intratracheal aspirated doses that have been examined previously (Adamson et al., 1999; Walters et al,. 2001; Schins et al., 2004; Gerlofs-Nijland et al., 2005; Happo at al., 2008). The use of relatively high doses is necessary in order to demonstrate statistically significant differences in toxicological endpoints between the particulate samples. This is important especially when small groups of healthy animals are used. The number of mice used in the studies was kept to a minimum for ethical reasons. Mild general anesthesia was used during the intratracheal aspiration exposure of the animals to eliminate any possible pain and discomfort. Overall, the animals appeared to be in good physical condition during the experiments.

Other experiments described in this thesis were conducted with the mouse macrophage cell line, which represents a model to the first line of defense against particles in the lungs. Nevertheless, the *in vitro* model has several limitations when compared to the animal model, e.g. cultured cells cannot undergo interactions with other cell types or receive signals from nerves as well as lacking the clearance mechanism present in the intact animal. It can however, represent a very useful tool for clarifying toxicological mechanisms. More specifically, there are three major reasons behind the selection of this specific cell line. Firstly, there is a large database in our laboratory on the responses induced and on the mechanisms activated in these cells by different inhalable particulates. Thus, the detected responses could be readily compared to previous data. Secondly, a mouse model was used in animal studies. Thus it was possible to compare the responses *in vitro* and *in vivo* in the same species. Finally, wood smoke affects the pulmonary immune defense and thus the lung macrophage cell line can be used to unravel the biological mechanisms participating in this phenomenon.

6 Conclusions

The present thesis has added to our knowledge of the toxic properties of wood combustion derived particles and their association with potentially harmful chemical compositions especially zinc.

The main findings from *in vivo* studies:

1. There were substantial differences in the combustion quality of OT and NT appliances; those also affected the chemical composition of emission PM. The OT furnaces clearly had the highest emissions in terms of total particulate mass. Moreover their emissions were dominated by soot and OC. Instead, with the low emission NT appliances, the following elements were enriched in the particles: Ca, Mg, Mn, K, Na, Zn, S, Cl and Cd.
2. Short-term inflammatory, cytotoxic and genotoxic effects in mouse lungs were seen after dosing of PM from NT appliances. In contrast, OT appliances induced only minor inflammatory responses in the lungs of mice. The levels of inflammatory markers as well as extent of genotoxicity correlated positively with the ash related constituents of particles, whereas OC had a negative correlation with the detected responses, potentially due to the immunosuppressive effect of these organic compounds.
3. The detected responses only represent short-term effects that reflect the acute phase of inflammation. The low inflammatory responses evoked by the samples from OT appliances should not be underestimated since they may disturb the normal foreign particle clearance mechanisms mediated via inflammatory cells. Moreover, there were large differences in PM_1 total mass emissions between the old and new combustion technologies in favor (lower emissions) of the NT appliances.

The main findings from *in vitro* studies:

1. Combustion efficiency had a major effect on chemical constitutions and subsequently on the toxicological properties of the emitted PM_1. Similarly to the *in vivo* results, PM samples derived from efficient wood combustion were cytotoxic, and caused cell cycle arrest and ROS production inside the cells when the emissions contained transition metals. Instead, the particles collected from the inefficient combustion were more potent inducers of programmed cell death and genotoxicity.
2. ZnO NPs displayed a similar toxicity profile as PM from efficient combustion. At the same time, potassium carbonates and sulfates, which are major components of wood combustion particles, were found not to induce any toxic effects.
3. Zn, when it was enriched in emission PM, was significantly toxic. Moreover, there was a similar toxicity profile between the efficient wood combustion PM and ZnO NPs.

In summary, it was observed that short-term inflammatory, cytotoxic and genotoxic activities in mouse lung correlated positively with the transition metal concentrations in the particles whereas the levels of OC correlated negatively with the detected responses. The toxic mechanisms of transition metals were further examined in an *in vitro* model. The mechanisms behind the detected toxic effects seemed to be oxidative stress which damaged the cell's DNA and lipids, leading to cell cycle arrest, membrane damage and ultimately to cell death.

In conclusion, it was shown that the toxic potential of efficient wood combustion PM is likely to be attributable to certain metal species such as zinc, when there are low concentrations of carbonaceous species in the PM. To prevent potential adverse health effects of PM, more attention should be paid to the contents of volatile transition metals in biomass fuels and furthermore the use of efficient combustion should be promoted as a way of lowering the total mass of particulate emissions.

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APPENDIX

Appendix 1. *Classification of combustion conditions based on typical emission factors.*

Combustion condition	Typical emission factors				
	PM_1 (mg/MJ)	**CO (mg/MJ)**	**OGC (mg/MJ)**	**TC (mg/MJ)**	**PAHs (ng/mg particles)**
Efficient combustion[1]	0-20	0-200	0-5	0-2	0-300
Intermediate combustion[2]	20-50	200-1000	5-50	2-10	300-2000
Incomplete combustion[3]	50-100	1000-2500	50-250	10-30	2000-20000
Smouldering combustion[4]	100-	2500-	250-	30-	20000-

Abbreviations: PM, particulate matter; OGC, organic gaseous carbon; TC, total carbon;
PAHs, polycyclic aromatic hydrocarbons.

[1] Complete combustion conditions typically encountered in continuously operating heaters e.g. pellet boilers.
[2] Improved batch combustion appliance operated under good operation practices
(e.g. modern masonry heaters and stoves) or erratic/low load use of continuously operated appliances.
[3] Typical conditions in batch combustion appliances e.g. conventional masonry heaters and stoves.
[4] Batch combustion appliances used with poor operational practices or combustion conditions in an old wood log boiler or sauna stove

Oskari Uski

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Fine particles are one of the most important pollutants present in outdoor air. Domestic biomass combuston makes a substantial contribution to the total worldwide fine particulate emissions. This thesis evaluates the scientific data on fine particle emissions from various combustion appliances and fuels and their relationship to activated toxicological responses. Zinc which is the main transition metal in the emissions from wood combustion, was related in *in vivo* and *in vitro* models to the abilities of the particles to induces toxic effects.

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