Genotoxicity of the A549 cells from the different combustion exposures detected with thermo-ALI-system

Pro gradu -research

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Henri Hakkarainen: Genotoxicity of the A549 cells from the different combustion exposures detected with thermo-ALI-system
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ABSTRACT:

The small-scale wood combustion emits in developing, as well as developed countries a significant amount of air pollution emissions. The toxicity of the emissions is resulted from poorly regulated and the strongly variating combustion conditions. For example, small-scale combustion emissions from smoldering conditions contains dangerous levels the polyaromatic hydrocarbons (PAH) which have proven to cause cancer. Additionally, the diesel engines have been observed to emit hazardous levels of PAH compounds. Huge amount of epidemiological studies has linked the air pollution and multiple different health effects, but the mechanisms of toxicology remain still partly speculative. Therefore, the research concerning air pollution toxicology is and will be crucial.

There is plentiful of different kind of cell studies, formally known as *in vitro* research going on and the use of *in vitro* methods is growing substantially. The strengths of the *in vitro* methods are miscellaneous; price, the ethicalness, repeatability and clarified cellular responses. The obvious weakness is the difficulty of the extrapolate the results to the biology of a whole organism. Addition of that, as concerning research of inhalation toxicology the exposure medium raises many difficulties with widely accepted *in vitro* methods. Therefore, new in vitro methods, i.e. air liquid interface (ALI) have been developed in order to better mimic the exposure in more real life resembling conditions.

In the present study a new kind of thermophoresis-based *in vitro* exposure system was used. The human alveolar type II-like epithelial cells were exposed with multiple small-scale combustion emissions and later the amount of fragmented DNA were measured with genotoxicity analysis called Comet assay. The different combustion fuels were spruce, pine, pellet and diesel engine. Some of the emissions were aged with aging chamber, and from some the PM was filtered out and some were taken to the exposure at the different phases of the combustion.

From the measured genotoxicities all were above the control values and additionally in line with previous studies. The highest genotoxic potentials were measured from the aged spruce and the diesel engine combustion exposures and the underlying reasons for measured genotoxicities might be connected to the PAH compounds. As a conclusion, the thermo-ALI exposure system can be used to detect the genotoxicity caused by the small-scale combustion emissions.

ITÄ-SUOMEN YLIOPISTO, Luonnontieteiden ja Metsätieteiden tiedekunta

Ympäristö- ja Biotieteet
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TIIVISTELMÄ:

Puun pienpoltto tuottaa niin kehittyvissä, kuin teollisuusmaissakin merkittäviä ilmansaastepäästöjä. Pienpolton päästöjen huono säätely ja voimakkaasti vaihtelevat palamisen olosuhteet vaikuttavat huomattavasti päästöjen toksisuuteen. Esimerkiksi syöpää aiheuttavien polysyklisten aromaattisten hiilivetyjen (PAH) määrät voivat olla pienpolton päästöissä vaarallisen suuria. PAH-yhdisteitä on todettu esiintyvän myös merkittäviä määriä etenkin vanhojen dieselmoottoreiden päästöistä, jotka on myös linkitetty keuhkosyöpään monissa eri tutkimuksissa. Monet ilmansaasteiden aiheuttamat terveysvaikutukset on todettu moneen otteeseen epidemiologisissa tutkimuksissa, mutta toksikologiset mekanismit ovat vielä useasti vain spekulaatioita, siksipä ilmansaasteiden toksikologinen tutkimus on ja tulee olemaan erittäin tärkeätä.

Maailmassa käytetään paljon erilaisia menetelmiä solu- eli *in vitro*-tutkimuksissa ja niiden käyttö on koko ajan kehittymässä ja kasvamassa. *In vitro*-tutkimusmenetelmien vahvuudet ovat monet; hinta, eettisyys, toistettavuus sekä vasteiden parempi havaitseminen. Heikkouksena tuloksia *in-vitro*-menetelmistä on todella ongelmallista ekstrapoloida kokonaisen eliön biologiaan. Inhalaatiotoksilogian kyseessä ollessa, ilmansaastealtistuksien tekeminen monilla vanhoilla *in-vitro*-menetelmillä on ongelmallista. Tämän vuoksi uusia, paremmin todellista altistumista vastaavia menetelmiä on kehitetty inhalaatiotoksikologisen tutkimuksen tarpeisiin.

Tässä tutkimuksessa käytettiin uudenlaista *in vitro*-altistusmenetelmää, joka perustuu päästöhiukkasten käyttäytymiseen lämpötilagradienttien välillä. Ihmisen epiteelisoluista johdettua A549-solulinjaa altistettiin kyseisellä altistusmenetelmällä erilaisille pienpolton päästöille ja myöhemmin soluista mitattiin genotoksisuus vahingoittuneen DNA:n määränä, käyttäen komeetta-analyysiksi kutsuttua toksikologista metodia. Tutkimuksessa käytetyt pienpolton päästöt olivat koivun, männyn ja puupellettien poltosta sekä dieselmoottorista. Polttopäästöistä osasta oli lisäksi suodatettu hiukkaset pois, osa otettu polton eri vaiheissa ja osa ikäännytetty ikäännytyskammiossa.

Kaikki mitatut genotoksisuudet ylittivät kontrolliarvot ja olivat linjassa kirjallisuuden kanssa. Suurin genotoksisuus havaittiin ikäännytyillä koivun polttopäästö- sekä dieselmoottorin päästöaltistuksilla. Tulosten perusteella thermo-ALI altistusmenetelmällä voidaan havaita pienpolton päästöjen aiheuttamaa genotoksisuutta *in vitro*.

PREFACE

Writer would like to thank his supervisors Pasi and Tuukka, who have not only taught me how to make and write science but have also helped to achieve the road to lifelong goal of science.

I would also like to thank all my friends during these study years with a special thanks going to the guys of the Handjobstreet, damn what a blast we had.

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ABBREVIATIONS AND DENIFITIONS

Thermophoretic	Kinetic response of the particles to temperature gradient
ALI	Air-liquid interface
In Vitro	Research made on cells
In Vivo	Research made on whole organisms
UFP	Particles that have diameter ≤ 100 nm
PM2,5	Particles that have diameter ≤ 2.5 μm
PM10	Particles that have diameter ≤ 10 μm
DEP	Diesel exhaust particles
DNA	Deoxyribonucleic acid
PAH	Polyaromatic hydrocarbon
ROS	Reactive oxygen species
A549	Human alveolar type II-like epithelial cell lines
SCGE	Single-cell gel electrophoresis
COPD	Chronic obstructive pulmonary disease
NADPH	Nicotinamide adenine dinucleotide phosphate
CO	Carbon monoxide
OGC	Organic gaseous compounds
EC	Elemental carbon

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INTRODUCTION

Air pollution, the one of the most hazardous pollutions in present times with annual death toll counted within millions and effect that nobody can obviate (WHO 2016). Multiple severe health effects have been linked to air pollution on enormous amount of epidemiological studies (Cassee et al. 2013, Ebestein et al. 2016). With the highly complex processes involving the deepest cellular mechanisms, the exact toxicological mechanisms caused by the air pollutant are not fully resolved. Nowadays one of the most concerning sources of the air pollutants is different small-scale combustion processes, which are problem in both developing and developed countries. Therefore, the inhalation toxicity studies concerning small-scale pollution exposures are vital and vastly needed.

The mechanisms of toxicity connected to air pollution exposure are complex and partly still unknown. Many of the mechanisms include oxidative stress caused by reactive oxygen species (ROS) (Sies et al. 1993). The ROS can damage DNA and thus induce genotoxicity which may results in lung cancer. Additionally, the polycyclic aromatic hydrocarbons (PAH) compounds, which have been found from gas and particle phases of the air pollution, have been speculated to be one main mediator for lung cancer (Amstrong et al 2004). With 2.1 million new annual cases (Bray et al. 2018), the lung cancer is one of the most severe health effects caused by the air pollution (Møller et al 2008).

The complexity of the air pollution mixture and the way of the exposure rise a lot of challenges to the air pollution toxicological research. The research made with test subjects, usually animals (*in vivo*) or with cells (*in vitro*) have own major question to overcome. One effective new *in vitro* research system for studying air pollution exposures has been the air-liquid interference (ALI) technique (Aufderheide et al. 2003). In ALI system the cells are cultivated on the membranes through which the nutrients are supplied to the cells, leaving the apical surfaces bare for the exposure.

Aim of this this Master thesis research was to investigate if the different small-scale combustion exposures, ranging from different wood combustions to diesel engine exhaust emissions, have different genotoxic effect to the human alveolar type II-like epithelial cells (A549) cells with new kind of thermophoresis-based exposure system. The combusted wood types used were spruce, pine

and wood pellet. Spruce and pine exhaust gases had filtered and unfiltered exposures. Moreover, the pellet combustion had ignition phase exposure and the constant steady combustion phase exposure. After the exposure the genotoxicity were measured from cells using single cell gel electrophoresis (SCGE) assay which can be used to measure the amount of DNA fragmentation.

1.0 REVIEW OF THE LITERATURE

1.1 INTRODUCTION TO AIR POLLUTION

Pollution is described at The Longman Dictionary of Environmental Science as "any harmful or undesirable change in the physical, chemical or biological quality of air, water or soil,". From all the hazardous pollutions, the air pollution is nowadays the one of the most significant environmental health risks with the death toll almost high as 8 million annually and affecting everyday billions of people around the globe (WHO 2016). Although, knowledge about risks of air pollution has been rising significantly in the past years, with continuously growing urbanization (UN 2018) and thus increasing population density, the future is still far from serene.

The health effects caused by the air pollution are diverse, with the major effects being cardiovascular and respiratory tract related diseases. The one of the most severe air pollution components is the particulate matter (PM) with diameter of 2.5 μm and below (WHO 2016). These include ultrafine (UFP) and fine particles ($PM_{2.5}$) which are responsible for 4.2 million deaths globally annually. There is not really any toxicological threshold value for the $PM_{2.5}$ because even the very low concentrations have been proven to cause health effects (Cassee et al. 2013). Furthermore, it has been estimated that the rise of 10 $\mu g/m^3$ concentration of $PM_{2.5}$ leads to the 6% rise in total mortality and 11% rise in cardiovascular disease mortality (Hoek et al. 2013). In addition to $PM_{2.5}$, the air pollution contains several other hazardous components, including the coarse particles (PM_{10}) and many different toxic agents, for example the PAH compounds (Seinfeld and Pandis 1998). The PAH compounds can be either bonded to PM or being in vaporized form and thus exist in the gaseous phase of the air pollution.

Severity of the global air pollution was well demonstrated in present epidemiological study of Ebestein et al 2016 where the effect of the PM_{10} exposure to the life expectancy was measured from north and south of the Huai river China. The political decision at 1950 resulted in free coal use in northern part of China, which consequently raised the air pollution levels significantly compared to the southern part. In the study of Ebenstein et al 2016 it was concluded that due to effect of air pollution, there were 3.1 years decrease in life expectancy of residents in northern part compared to the southern part. It was also estimated that up to 4.5 billion people live in countries on which the average PM_{10} concentrations are twice as high as the WHO threshold.

The air pollution sources can be roughly separated to those from anthropogenic or natural origin (European Environmental Agency 2013). Natural sources for the PM are wind-blown dust, sea salt, volcanos, wildfires and biogenic material, and for anthropogenic the sources are residential combustion, which includes outdoor and indoor small-scale combustion, e.g. cooking and household- heating, traffic related sources and energy production and industry. Furthermore, for the size distribution and chemical composition of the formed PM depends heavily from the source (Sillanpää et al. 2006). For example, the PM_{10} are usually resulted in mostly from different kind of mechanical wear induced processes whereas, $PM_{2.5}$ is mostly from combustion processes.

The small-scale residential combustion is mostly considered as a development countries problem, but the amount of the premature deaths in Europe due diseases associated with residential combustion, especially wood combustion, is estimated to be over 40 000 each year (Sigsgaard et al. 2015). Indeed, it has been estimated that the small-scale combustion is responsible for up to third of the total PM concentrations in some areas. During the recent years the European Commission has encouraged the residential heating and use of wood as a combustion fuel which has increased the wood smoke emissions significantly (European Commission, 2014). The increased wood combustion is intended to lower the use of oil and other fossil fuels in Europe, but on the other hand also the emissions from the wood combustion are hazardous. For example, the different wood combustion emissions have proven to have different kind of toxicological responses in recent studies (Kasurinen et al. 2017) with the wood combustion PM emissions seeming to be close to that from old technology diesel engines. Additionally, it has been estimated that the concentrations of the $PM_{2.5}$ from wood combustion as being even higher than that from combustion from fossil fuels such as coal (Leskinen et al. 2014).

In Finland, the small-scale residential combustion is the second largest heating method for the residential houses (Offical statistics of Finland, 2015) and it accounts for about 25% of the total $PM_{2.5}$ emissions (Karvosenoja et al. 2008). Additionally, one of the hazardous aspects of the residential combustion is, that at the wintertime it has been estimated to produce much as 20-90% local $PM_{2.5}$ emissions (Boman et al. 2003), as for example, in Denmark in a residential area without the district heating system, wood combustion may result in local PM levels as comparable to those measured on the streets with heavy traffic (Glasius et al. 2006).

Residential combustion emissions especially the PM has been linked to the increased risk of developing chronic obstructive pulmonary disease (COPD), cardiovascular diseases, respiratory symptoms (Brunekreef et al. 2002), childhood asthma (Patel et al. 2009), type 2 diabetes (Eze et al. 2015), autoimmune diseases (Gawda et al. 2017) and neurotoxicological effects (Block et al. 2009). In addition, air pollution and the PM-bound toxic agents have been linked to development of cancer and International Agency for Research on Cancer (IARC) has categorized a carcinogenic potential of PM to group 1, carcinogenic for humans. The toxicological mechanisms how the air pollution causes all abovementioned health effects, stand still with many question marks, but the most theories include oxidative stress induced biological damages which results in cell damage and thus observed health effects (Kelly F.J. 2003).

1.2 FACTORS ACCOUNTING FOR THE PHYSIOCHEMICAL PROPERTIES OF THE AIR POLLUTION EMISSIONS

For the PM emissions, factors like the particle size, morphology, chemical composition and the number and the mass concentrations are vastly depended on conditions at the combustion (Tissari et al. 2007). For the gaseous emissions, the factors affected by the combustion conditions are mainly different combustion related molecules, such as carbon monoxide (CO), elemental carbon (EC) and multiple organic gaseous compounds (OGC). All these different factors at emissions are depended from the combustion appliance (International Energy Agency, 2008), fuel quality and the different operation tunings (Lamberg et al. 2011).

Combustion appliance can account tremendously to the combustion emissions as it was detected at the study from Johansson et al 2004. In this study, the PM concentration at the emissions from old wood boilers were up to 180- times larger than that from the modern wood pellet burner, respectively. Regarding the most common wood combustion appliances in Finland, the masonry heaters and the sauna stoves, the emissions from sauna stoves are notably higher because of too fast pyrolysis and restricted air intake (Tissari et al. 2008). In fact, combustion appliance has a massive effect to the efficiency of the combustion, which can be achieved with a steady air and fuel feed, resulting in significantly lower emissions (Lamberg et al. 2011). An example of such combustion appliance is a modern pellet fueled boiler and the studies have shown that the

emissions from such an appliance are not only significantly lower but have different chemical composition with lower concentrations of toxicological agents.

Combustion conditions can be very roughly divided to the normal combustion and slow heating combustion, called smoldering combustion (Tissari et al. 2008). The gaseous and PM emissions from those two combustion conditions may vary strongly. For example, the CO emissions from smoldering combustion can be up to 3 times higher and the OGC emissions up to 14 times. It seems that also the amount of carbon in the emission has been linked to the conditions of the combustion (Tissari et al. 2007). When the combustion is incomplete the resulted PM emissions are mostly formed from the elemental carbon and different organic materials, whereas from the better combustion conditions the amount of carbon is lower and the inorganic ash components dominate. The carbon is not only indicator of the combustion conditions but also the concentrations of PAH compounds and different alkali metals (K^+, Na^+, Cl- and SO_4^{2-}) depend from the combustion conditions (Lamberg et al. 2011). As for an example, there were high as 10- fold difference in PAH compounds between combustion conditions, with smoldering conditions resulting in higher PAH concentrations. Additionally, the morphology of the produced particles is affected by the combustion conditions (Torvela et al. 2014) with the smoldering combustion resulting in more soot particles and condensed organic compounds on the surfaces of the PM.

Furthermore, the difference to the emissions caused by the combustion fuels have also been detected. In the study of Lamberg et al. 2011 it was found that the emissions from combustion of different pellet materials had different chemical compositions. When concerning wood combustion, the study of Fine et al. 2001 found that $PM_{2.5}$ emissions from combustion of 5 different wood types ranged between 0.88g to 3.5g from an average of 1.8 kg^{-1} of wood burned. Additionally, the concentration of organic compounds might differ between fuels, like in the study of Zou et al 2001, where it was found that PAH concentrations varied between combustions of different wood species.

One other the major source of PM emissions is the old technology diesel engines, which emit more PM than gasoline engines with the size distribution of PM emissions being relatively similar to that of the wood combustion emissions (Leskinen et. al 2015). Additionally, from the emissions of the diesel engines the PM material can be identified as diesel exhaust particle (DEP) and toxicity

of the DEP has been focus on abundant amount of research (Risom et al 2005). The DEP contains carbon nuclei which can absorb several different compounds, for example genotoxic PAH compounds (Draper et al. 1986). Even though the new technology, for example the EURO6 diesel engines produce very small PM emissions (Fiebeg et al 2014), there are still a lot of old technology engines in use, especially in the heavy traffic, construction machinery and agriculture.

There is also evidence that the atmospheric aging of the emissions changes to physio-chemical properties of the emissions (Reid et al. 2005), which will lead to different kind of toxicological effects of the emissions. At the atmospheric aging the oxidation caused by the ozone and the ultra-violet radiation can form so called secondary organic carbon compounds which have been observed to have a different chemical composition which might consequently affect their toxicity (Zhou et al. 2018). In addition, some of the toxicological agents bonded to the PM, for example the PAH compounds can change towards more carcinogenic forms from an effect of the aging (Nordin et al., 2015). Effects of the atmospheric aging can be studied with the measurements from the atmosphere or with artificial aging chambers, in which the emissions are fed and aged with ultra-violet light, changes of temperature and ozone exposures (Leskinen et al. 2015).

1.3 TOXICOLOGY OF THE AIR POLLUTION

1.3.1 Immune system

When concentrating to the toxicology it is essential to underlay first the basic information about the immune system. The immune system can be described as an interconnected system of vessels, organs, cells and processes which main goal is to prevent damage and diseases to the organism (Castelo-Branco & Soveral 2013). To achieve this goal the self-detection of the difference between the own tissues from the transformed cells and outside toxicants is extremely important factor. Following the self-detection, defensive behavior versus the transformed cells and toxicants is highly complex mixture of mechanisms which are either part of innate immunity or adaptive immunity (Lodish et al. 2016)

The innate immunity is non-specific and consists from the surface, chemical and cell barriers in the body, inflammation effect, different kind of defensive cells called leucocytes and the complement system (Lodish et al. 2016). The innate immunity is dominant immunological

defensive system in most of the organisms, including humans (Litman et al 2013). The different mechanisms of the innate immunity system are activated if the toxicants get pass the surface barriers of the body and the activated innate immunity system works non-specific and reacts within minutes to hours to toxicants (Lodish et al. 2016). The detection in the innate system works via receptors such as toll-like receptors which can detect broad numbers of antigen-specific markers, for example, bacterial wall constituents. Many of the cells which work within innate immunity are phagocytic and thus, these cells called macrophages, neutrophils and dendritic cells, can ingest and destroy different antigens entering the host. Phagocytic cells patrol all around body but can also specialize to work within a certain organ. For example, lung macrophages are lung specific and are the first leukocytes to face the external threats which are trying to get in with the air we breathe (Lohmann-Matthes et al. 1994). The complement system is collection of the serum proteins which can bind to microbial and fungal surfaces and thus destroy the membrane of the microbes, mark them for phagocytes or activate different immunological responses with chemokine signaling (Lodish et al. 2016). Furthermore, certain cells as a part of the innate immune system, called natural killer cells can also destroy host's own cells if they are tumorous or infected by viruses.

For the first line of the immunological defense the mechanical and chemical barriers of the body (skin, mucus, chance of pH) are vital part as they prevent external toxicants to get into the host as a first place (Lodish et al. 2016). The exposure area for inhalation is significantly large, with the area of defensive barriers varying from 100 m^2 to 140 m^2 between individuals (Gehr et al 1978). All this large area of surface barriers is exposed daily to variety of different kind of antigens i.e. bacteria, viruses and air pollutants. Therefore, when concerning the research of toxicology, the knowledge of the effects to those vital surface barriers of our body is indispensable.

The inflammation effect is one of the most important defensive mechanisms of organism after the surface barriers (Lodish et al. 2016). Inflammation creates inflammatory responses which includes four signs; redness, swelling heat and pain. Those signs are caused by the leakiness of the blood vessels, attraction of the leukocytes and production of the different soluble mediators. Inflammation is primary part of the innate immune system but also contributes to the adaptive immune system via delivering antigens to secondary lymphoid organs for the differentiation of the lymphocytes. In an inflammation situation the innate immune system introduces the different phagocytes and other kind of leukocytes to the defensive actions which additionally signal with

the cytokines and chemokines to rest of the body about the situation. Even though inflammation is host own way of defending against non-hosts, the chronic inflammatory response can be also damaging to the host and it has been speculated to be underlying cause for development of multiple autoimmune diseases (Murakami et al 2012).

1.3.2 Immune system and the air pollution

Because the way of the air pollution exposure, the innate immunity and especially the surface barriers of the respiratory track play major role in defending the host against the air pollution. Structure and different segments of the respiratory tract itself prevent the larger than PM_{10} particles to reach the alveolar parts of the lungs (Heyder 2004). But in the deepest part of lungs, called the alveolar parts, only epithelial structure of alveolar lining cells with the thickness only about of 0.5 microns works as dividing wall with the inhaled air and the organism's inner system (Hogan et al 1986).

The alveolar epithelium consists from two surface, known as epithelial, cells types, the terminally differentiated squamous alveolar epithelial type I (ATI) and the surfactant producing cuboidal alveolar epithelial type (ATII) (Hermanns et al. 2004). The ATI covers about 93% of the alveolar surface and when the ATII covers only about 7% it constitutes approximately 67% of the epithelial cell numbers (Crapo et al 1982). These alveolar epithelial cells don't only have role as a protective layer in immune system, but they have ability to affect morphology of different lung macrophages via production of cytokines (Chuquimia et al. 2012). For instance, as in the study of Myatt et al. 2011 as the alveolar epithelial cells were exposed with $PM_{2.5}$ they produced proinflammatory cytokine called tumor necrosis-α (TNF).

These surfaces of the airway paths are also protected with multiple different ways; the mucociliary transport system which includes the mucus and cilia, and the different kind of antimicrobial compounds and leukocytes. For the defense of air pollution mediated toxicity, the mucus as viscous solution has an important role as part of mucociliary transport system (Samet et al. 1994). The mucus is moved in the respiratory tract with rhythmic upward beating action by the hair like projections called cilia (Newhouse et al 1976). Thus, it will transport different unwanted inhaled constituents away from respiratory tract to the digestive tract and prevent the toxicological effects

they would have caused (Oberdörster et al. 2005). This system also provides way for removal of alveolar macrophages with their phagocyted content.

The alveolar Macrophages have several important tasks concerning the alveolar parts (Lohmann-Matthes et al. 1994). These cells keep the parts of the alveolar clean and sterile, noticing effectively all the alien objects and reaction to those with phagocytosis or informing about those with the cytokines to other cells and tissues. The alveolar parts of the lungs to have four different kinds of macrophages which are based by their localization; 1) the alveolar macrophage, 2) the interstitial macrophage, 3) the dendritic cell and 4) the intravascular macrophage. The alveolar macrophages are the most unique with being first line of defense against inhaled pollutants and in addition of that, as located within the alveolar surfactant film, being the only species of macrophages exposed directly to inhaled air (Johnsson et al. 1986).

The interstitial macrophages are located in the lung connective tissues and have function as the second line of macrophagic cell defense and antigen presentation (Lohmann-Matthes et al. 1994). The both alveolar macrophages and interstitial have ability to phagocyte. The dendritic cells are specialized to the antigen presentation and have reported to form a network between epithelial alveolar lining cells. The intravascular macrophages are located on the endothelial cell and facing the bloodstream (Warner et al. 1987). Their job has been speculated to remove the unwanted material which enters the lung via bloodstream. As an association with the phagocytosis, the alveolar macrophages are capable producing as an oxygen metabolites different kind of ROS, like hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydrogen radical (OH^-) (Lohmann-Matthes et al. 1994).

1.3.3 Mechanisms of toxicology

The health effects from the air pollution are vast and consequential as a significant amount of epidemiological studies have shown (WHO 2016) and that even a small increase in air pollution concentrations can cause considerable rise in the amount of hospital visits and mortality. Underlying reasons for the toxicological effects of the air pollution have been studied abundantly and for example, it has been observed that the different size fractions of PM can cause different kind of health effects (Brunekreef and Forsberg 2005). For example, the UFP fraction which can pass through lung epithelium to the circulation rapidly and end up in the other organs (Geiser et

al. 2005) thus, contributing to toxicological effects all around the body. Additionally, it has been shown that the UFP size fragmentation can penetrate the cell membranes and therefore cause toxicological effects inside cells organelles or even DNA. Furthermore, not only the size fractions of the PM have been detected to cause different kind of health effects, but the different chemical components in the air pollution have also been linked to different kind of health effects (Kelly and Fussell, 2012).

Although, the different mechanisms behind the air pollution exposure and certain health effects are still partly unknown, there are some propounded biological pathways (Jalava 2008). For example, the speculated mechanisms behind cardiovascular diseases linked to air pollution are related to systematic inflammation which has been proposed to consequence from the effects of the neutrophil recruitment from the bone marrow and liver, originating from the release of the C-reactive protein. (van Eeden and Hogg 2002).

The different pathways for toxicological effects are usually categorized as; inflammatory, cytotoxicity and genotoxicity (Jalava 2008). All these pathways overlap vastly and may be consequent from each other, e.g. the genotoxicity can be resulted from the inflammatory effect (Møller et al 2008). Figure 1. is simplified presentation of above-mentioned pathways for toxicological mechanisms concerning the PM exposure.

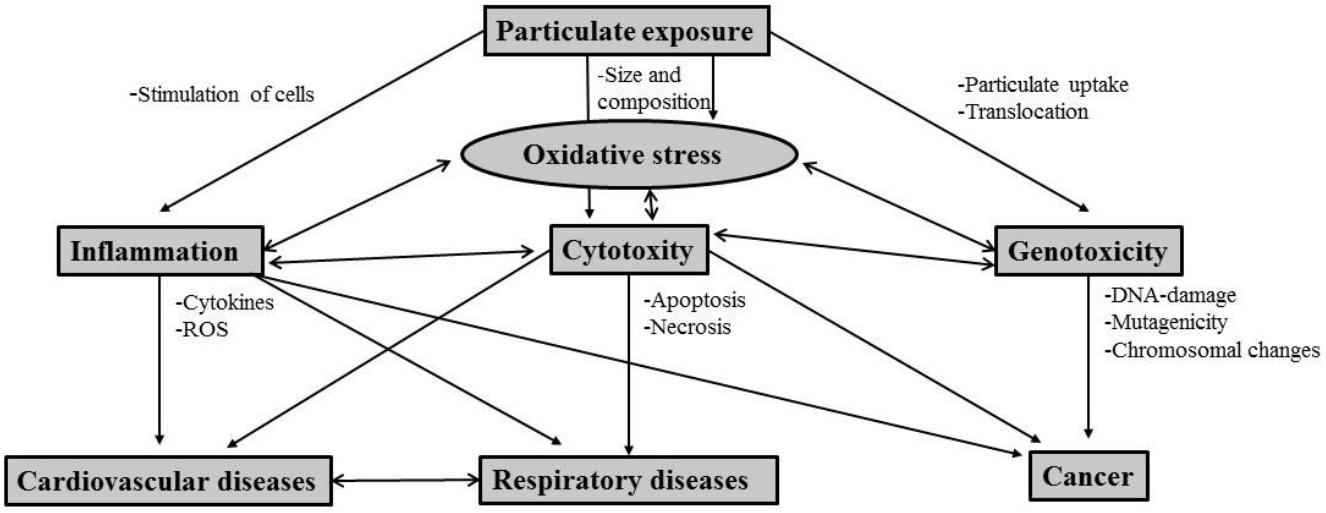

Figure 1. The three main biological pathways established concerning the toxicology of the particulate matter exposure with the explaining factors and consequent health effects linked to certain pathway.

The Oxidative stress is linked to the all different pathways and has been defined as an imbalance in the organism system oxidants due to a vast amount of ROS (Sies et al. 1993). The vast amount of ROS has ability to damage all types of biological molecules of the organisms, including DNA. Example from ROS mediated toxicity is a lung inflammation in which the ROS generated from the macrophage processes may reduce the amount of glutathione which consequence result to chronic inflammation in the lung cells (Rahman et al. 2000).

The pathway of local and systematic inflammation is resulted from the stimulation of the different inflammatory cells (Luster et al. 1999; Barnes et al. 1998). This toxicological response induces different kind of cell transmitters as inflammatory mediators, which can either stimulate apoptosis, recruitment of different macrophages to the inflammation site and increase the phagocytic activity. The chronic inflammation caused by the particulate matter have suggested being the leading mechanism causing the air pollution related cardiovascular and respiratory diseases. (Pope and Dockery 2006).

The second biological pathway is cytotoxicity, cell damage or death caused by PM and/or different components bonded on them. Cytotoxicity is causing either cell death in controlled form, apoptosis or in uncontrolled form, necrosis. In apoptosis, the cell goes through many steps starting from DNA fragmentation which leads to shrinking of cells and in the end the controlled destruction. Apoptosis is occurring mostly independent of the inflammatory responses. Necrosis on the other hand, is usually results from sudden and high toxicity, for example air pollution exposure (Dornhof et al 2017) which leads to lysis of the whole cell and its organelles. Cytotoxicity caused by the inhaled PM exposure has been found to induce chronic respiratory diseases e.g. COPD and asthma (Frampton 2006, Holgate et al. 2006). Addition of that, the apoptosis has been observed to play major role with fibrotic lung diseases (Kuwano et al. 2002).

The third pathway is genotoxicity, which implies the toxicological effects which either destroy or alter the molecules of genetic information, the DNA in the cells. One of the major mechanisms linked to genotoxicity is the oxidative stress caused by ROS (Sies et al. 1993). For the inhaled PM, many of the components abundant on the surface are capable of producing ROS in biological systems. In addition of that, the DNA is considered of being major oxidized target for ROS (Risom et al. 2005). In addition of oxidative stress mediated DNA damage, effect of some organic

compounds has been thought to be important concerning particularly the development of cancer (Borm et al. 2004). ROS production by different macrophages and dendritic cells is also connected to the genotoxicity and translocation of PM for example inside the cells (Risom et al 2005). Genotoxicity causes several different kinds of health effects connected to the DNA, cancer, mutagenicity and chromosomal changes just to name few (Pope et al. 2002).

1.3.4 Mechanism of genotoxicity

Genotoxicity linked to air pollution exposure has been widely observed in different epidemiological studies (Møller et al 2008). The most severe effect from the air pollution associated genotoxicity is lung cancer, which has been the most common cancer worldwide for several decades (Spiro et al. 2005). There were estimated 2.1 million new cases of lung cancer in 2018 which accounted total of 11.6% from all the cancer cases (Bray et al. 2018). Lung cancer also is the most common cause of death from cancer with estimated 1.8 million deaths annually (18.4% of total) with the overall ratio of mortality from lung cancer being high with 0.86.

The genotoxic effects of the air pollution are often linked to components such as PAH compounds, transition metal concentrations and other agents with potential of causing oxidative stress (Risom et al. 2005). How the DNA damage converts to the carcinogenesis, is a process including many steps, beginning at the mutations of the growth-regulatory proto-oncogenes and tumor-suppessor genes to aberrant gene expression leading ultimately to disturbed cell growth and division (Godschalk et al. 2002). These will subsequently lead to mutations, multiple different cell dysfunctions and cancer. There is tremendous amount of research for delineate the underlying mechanisms behind the PM induced genotoxicity (Rahman et al 1998., Xia et al 2006). For example, in multiple studies the PM exposure has been detected to increase by much as sevenfold the activation of NF-kB transcription factor, which can induce gene transcription via pro inflammatory cytokines, enzymes and immune receptors and is therefore linked to genotoxic effects.

Although different DNA repairing pathways exist the vast amount of damage will ultimately lead to cell transformation. As the generation of ROS in the cells is normal mechanism from multiple different metabolic processes involving oxygen, for example from cellular respiration, biotransformation of xenobiotics and phagocyte activation (Azqueta et al. 2009) the excess

generation of the ROS can overwhelm the antioxidant defenses and therefore oxidize cellular biomolecules including DNA (Bjelland et al. 2003). This will result in different oxidative modifications in DNA, such as strand breaks, base oxidation and cross point strands. The oxidation of DNA base guanine is the most typical because of the its lowest oxidative potential from the DNA bases (Kovacic et al. 2001). The typical and most studied oxidative base from guanine is the 8-Oxoguanine which have been shown to affect DNA synthesis *in vitro* (Shibutani et al 1991).

The generation of ROS by air pollution exposure may result from various different mechanisms (Risom et al. 2005). These mechanisms include, direct generation of ROS on the surface of the particles via metabolism of foreign compounds, altered function of nicotinamide adenine dinucleotide phosphate (NADPH) -oxidase and inflammatory effects. In addition of the oxidative stress linked genotoxicity, there exists also non-oxidative DNA damage in a form of DNA adducts (Gerde et al. 2001). The presented pathways of the air pollution linked genotoxicity are roughly presented in figure 2.

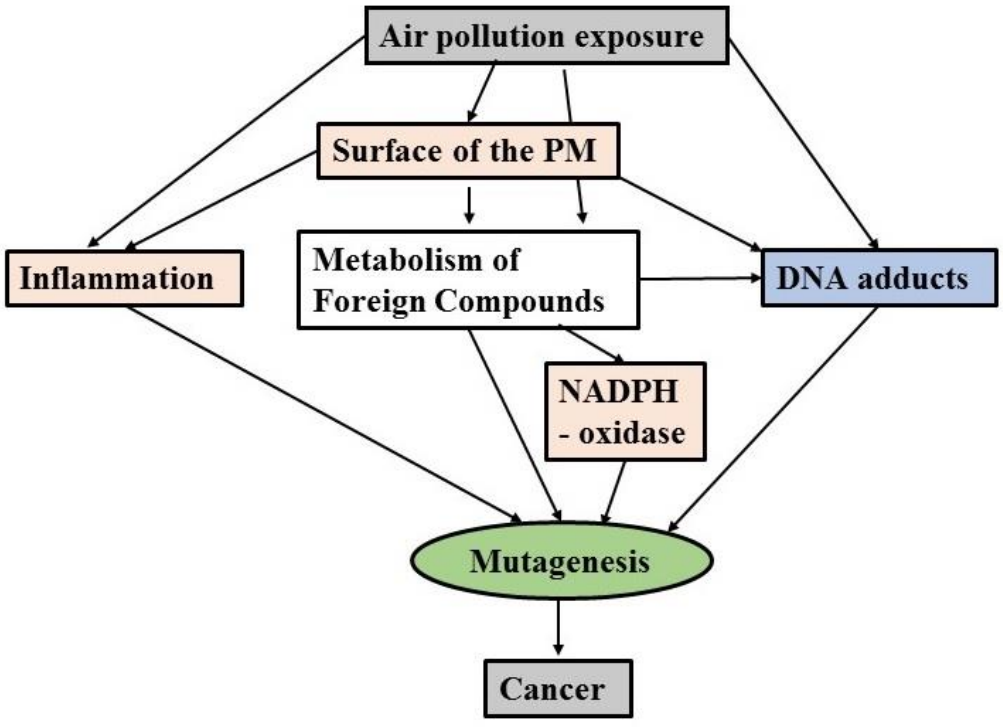

Figure 2. Proposed pathways of air pollution mediated genotoxicity. The boxes with reddish color are linked to oxidative stress related mechanisms.

The mechanism behind direct generation of ROS via PM surface physio-chemicals via metabolism of foreign compounds can include different soluble compounds such as transition metals and organic compounds (Risom et al. 2005). Transition metals can produce ROS through a Fenton type reactions (Halliwell et al. 1999). In these reactions the transition metals work as a catalyst for different kind of chemical reactions which produce ROS. Consequently, multiple studies have shown that transition metals either leaching from PM in to the biological system or presence on the surface of the PM play role in generating ROS in biological systems (Han et al. 2001; Ghio et al. 2000). The DEP has also been shown to contain surface functional groups with the capacity to form iron complex which by accumulation can induce oxidative stress in a form of generating superoxide anions (Han et al. 2001). The organic extracts at DEP has also been reported to form ROS with or without biological activation system (Sagai et al. 1993). The organic molecules absorbed on the surface of the PM are proven to produce ROS but with some different mechanism than the transition metals (Risom et al. 2005). For example, PAH compounds undergo the metabolic activation and the expression of cytochrome P450 enzymes (CYP1A1) which generates ROS and reactive PAH-quinones (Bonvallot et al. 2001).

The PAH quinones caused by the metabolism of PAH compounds can form DNA adducts which cause the genotoxic effects as a part of NADPH-oxidase pathway (Park et al. 2007). In this pathway, the altered function of the NADPH-oxidase is speculated to rise from the oxidation of PAH-*trans*-dihydrodiols to PAH quinones by effect of aldo-keto reductase, which is depended by the NADPH-oxidase (Penning et al. 1999). The PAH quinones may also reduce to catechols thus amplifying the production of the ROS.

Genotoxicity caused by the inflammatory mechanisms is linked to the activation of inflammatory cells which are capable of producing ROS and nitrogen species and is significantly important factor concerning the effects of PM exposure in multicellular organisms (Knaapen et al. 2004) and this bloom of ROS compounds has been observed in multiple studies concerning the air pollution exposure. This autoimmune-kind of effect is perpetrator of the chronic inflammation which may lead to genotoxicity (Risom et al. 2005).

Additionally, genotoxicity might rise from other than oxidative connected mechanisms like with the PM bound benzo[a]pyrene which have been shown induce non-oxidative genotoxicity with the

formation of bulky DNA via binding to the bases of DNA (Gerde et al. 2001). These bulky DNA adducts may results in frameshift mutations, deletions of bases, S-phase arrest and strand breakages (Jung et al. 2013). This non-oxidative DNA damage of PAH compounds may therefore play strengthening role for the genotoxic potential of these compounds.

1.4 *IN VITRO* RESEARCH

In vitro researches are performed outside of the normal biological system, for example to the cells, micro-organisms or biological molecules (Singh et al. 2018). The studies are usually made in laboratory environment and require suitable conditions and tools. Advances for the *in vitro* style of research are better controllability of the experiment setup, more detailed data from the test subjects, ethical reasons as the lesser use of the animal tests, automation and repeatability. The sole disadvantage is that from the *in vitro* research, it is hard to extrapolate the results to the biology of whole organism. When performing *in vitro* research, the test subject is usually manufactured cell lines which are cultured on special cell culture media which contains all the required nutrition for standard viability and grow. Because of the immunological similarities between the mammals it is possible to use parallel cells from different species in *in vitro* experiments. (Rosenthal & Brown 2007). Though, the extrapolation problem must be always taken inconsideration when concluding the risk assessment.

1.4.1 *In vitro* epithelial cell lines

The different categories of cells lines used mainly as epithelial cells are primary and continuous (also known as immortalized or tumor) cells lines. (Hiemastra et al 2018). The primary cell lines, which are obtained directly from tissue are theoretically more ideal for inhalation toxicology studies because they maintain the properties better than continuous cell lines. But because of problems concerning the life span, difficulty and price of the cultivating the primary cell lines, the continuous cell lines are more widely used. The continuous cell lines have been immortalized from certain tissues with using variety of different methods and they usually maintain some properties of the host tissue. The most used continuous cell lines for *in vitro* inhalation toxicology are Calu-3, BEAS-2B, 16HBE14^{o-} and A549.

A549 cell line

A549 cell line was developed by D.J. Giard et al. 1972 through removal and cultivation of the cancerous lung tissue from 58-year-old Caucasian male. A549 cells are adenocarcinomic human alveolar basal epithelial cells which play role for example at diffusion of water and electrolytes across the alveoli of lungs. The A549 cell line can be cultured *in vitro* resulting epithelial kind of layer which attaches to the surfaces. (Lieber et al 1976). A549 cell layer can be detached from the surface with trypsin treatment and light rinsing. These cells are also capable of synthesizing lecithin and for maintaining the membrane phospholipids, contain high levels of unsaturated fatty acids. A549 cell line are widely used as an *in vitro* model for a type II pulmonary epithelial cell model for multiple different uses. (Wu et al. 2018)

1.4.2 Inhalation toxicology and *in vitro*

When concerning to inhalation toxicology and *in vitro* research many troublesome problems arise. The exposures at a such studies are complex gaseous mixture of atmosphere and different substances (Ritter et al. 2001). Hence, it is hard to control the exposure and maintain the homology of the exposure in repetitions. Respiratory track cell tissue is likewise complex and culturing such a cell cultures to mimic complexity of the respiratory track tissue is challenging (Hiemastra et al 2018). Though, feasible inhalation toxicology research has been achieved with mono-cultures or simple co-cultures with the additional support parallel *in vivo* testing (Sandström et al 2005). Still many challenges arise when exposing cells with gaseous compounds. Challenges are mainly focused on between sampling and testing gases under environmental conditions and at the same time maintaining cell viability (Ritter et al. 2001). For example, the humidity of exposure gases in environmental conditions isn't usually suitable for cells, with pH for cell cultures normally adjusted for 5 % of CO_2 but with environmental gases being significantly lower.

Several systems have been developed for exposing cells with air pollutants. Those can be roughly divided into 3 different categories; cells exposed under submerged conditions, conditions changed from direct gas exposure to submerged during the exposure and cultures which are exposed directly to gaseous compounds via air-liquid exposure systems (Ritter et al. 2001). The two firstly listed have several disadvantages when concerning scientific experimental and mimicking the exposure at lungs. The submerged option creates very large interference between exposure gases

and culture medium, not with actual cells and the exposure gases and therefore resulting to high uncertainty with the exposure to the cells itself. The submerged exposure can also affect physio-chemical properties and size of the PM via agglomeration, which both can potentially change the toxicology of the PM (Meissner et al. 2009, Teaguarden et al 2007, Hotze et al 2010). The second option creates large interference between gases and cells, but the frequent change on culture medium creates extra physical stress to the cells which allows only maximum exposure durations of several minutes (Ritter et al. 2001). Additionally, the behavior of the culture medium is hard to control because of adsorption. Third option, the ALI system creates only very thin layer of liquid film between cells and gases (Aufderheide et al. 2005). In this system the results can be reproduced relevantly easily, and the exposure times can be significantly longer.

Consequently, the ALI system has several advantages in inhalation toxicology (Aufderheide et al. 2003). In such system the cells are cultivated on porous membranes mounted on a supporting frame, aggregate which is often called an insert. Nutrients are supplied through these membranes to leave reverse surfaces of the cells in air-liquid conditions to the exposure. Experimental setups also use special kind of exposure chambers where inserts can be setup so that the exposure gases can get in contact with the cells. Several ALI methods have been introduced ranging from solely of the gas exposure (Aufderheide et al. 2005) to exposing with water droplets as an exposure medium (Lenz et al. 2009) to different kind of charged PM exposure systems (Salvi et al. 2008; Sillanpää et al. 2008). However, ALI exposure for certain sized particles is limited with diffusion transport and using the charged exposure systems can affect the cells (Schaeublin et al. 2011).

1.4.3 Thermophoresis based air-liquid interface system

Thermophoresis based ALI system, called Cyto-TP, was first developed by Broßell et al 2013 and it consist emission chamber with 2 inserts mounted at the top of the chamber with cells cultivated on the basolateral side of the inserts semipermeable membrane. The lower and upper side of the chamber is kept at different temperatures resulting the temperature gradient in chamber. The force resulting from temperature gradient, called the thermophoretic force causes the gases and PM lead in the system chamber end up on the cells.

The thermophoretic force implies the effect of the surrounding gas molecules to mobile particles in a change of net momentum of the particles (Huan et al. 2004). As the particles are moving in

the chamber they are constantly being struck by the gas molecules in the system. Warmer the gas molecules are, the higher velocity and therefore, with a higher kinetic energy the molecules strike the particles and causing stronger effect to the net momentum for the movements of particles. Thus, in the system with temperature gradient the effect of warmer gas molecules to the movement of the particles causes the particles shift the movement towards the side with lower temperature. In Broßell Cyto-TP system this thermophoretic force causes particles to strike the basolateral side of the inserts and therefore exposing the cultivated cells to the PM.

At the University of the Eastern Finland (UEF) the thermophoresis-based ALI-system is developed, following Broßell et al 2013 Cyto-TP example (Ihalainen et al. submitted manuscript). -The UEF thermophoresis-based ALI system also utilizes thermophoretic force as described earlier. Although, this UEF system differs from Cyto-TP with several other properties. The temperature gradient in UEF system is higher with system's lower side is at +57°C and upper side at +37°C. (figure 3) whereas respected numbers were +45°C and +37°C for Cyto-TP. The water circulation will keep the temperatures more even compared to the electric heating, which is always undulating due to low thermal buffer capacity and rather slow reaction of the thermostat. Additionally, the flow rate of gases is higher with 150 ml/min at thermo-ALI system compared to the 2 ml/min at the Cyto-TP. Moreover, the UEF system uses six well inserts and has specific cooling units for each of the inserts. For better controllability of the experiment system, the flow rates and humidification of the system can be effectively monitored at UEF's ALI. At UEF ILMARI laboratory environment this ALI system can be fed with several different combustion emissions ranging from different small-scale combustions to car exhaust. ILMARI laboratory also features aging chamber which mimics the effects of the atmospheric aging to the emissions (Tiitta et al. 2016).

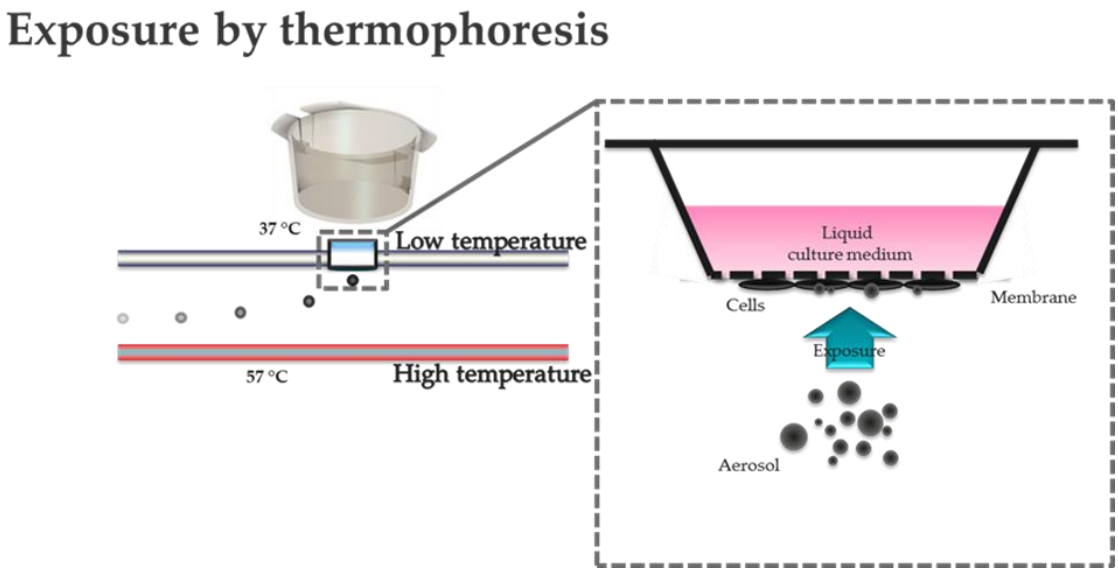

Figure 3. Exposure of the combustion emission to the cell culture on the bottom of the insert with the thermophoresis based air-liquid interference exposure system (Ihalainen et al. submitted manuscript).

1.5 SINGLE-CELL GEL ELECTROPHORESIS

The SCGE, also known as Comet Assay, is relatively simple, rapid and very sensitive assay to detect DNA damage at the level of individual cells. Assay was firstly designed by the Östling and Johanson at 1984 in neutral pH conditions. Later at 1988 Singh et al. used alkaline conditions (pH < 13) to increase the DNA mitigation for significantly increased sensitivity for observed genotoxic effects. The assay can detect DNA damage from double and single strand breaks, cross-linking and alkaline-labile sites to an incomplete excision repair sites.

Assay is based on negative charge of the DNA molecule and therefore the fragmented DNA pieces can be drawn through agarose in electrophoresis separating the DNA fragments from the intact DNA (Tice et al. 2000). The smaller the DNA fragments are, further away they are drawn on agarose and so on creating longer "tail" for the DNA nuclei. When staining the DNA with DNA-specific dye the results from electrophoresis will be visible with microscope in a shape of a comet, from which the name Comet Assay origins.

Single Cell Gel electrophoresis assay have plenty of steps which all are equally important for obtaining reliable results (Tice et al. 2000). When considering the different steps there is also several other matters to take care of for obtaining the best possible results; the sample processing,

solution preparation and usage, and the equipment utilization and maintenance. Once a suspension of cells is obtained, the steps for protocol thoroughly are the following; preparation of microscope slides layered with cells in agarose, lysis of cells to liberate DNA, exposure to alkali (pH >13) to mitigate the DNA for greater sensitivity, electrophoresis under alkaline (pH >13) conditions, neutralization of the alkali followed by staining and visualization of the DNA with microscope and lastly analyzing the pictures of DNA nuclei with software to get the data. See (Figure 4.) for schematic representation of the previously presented steps.

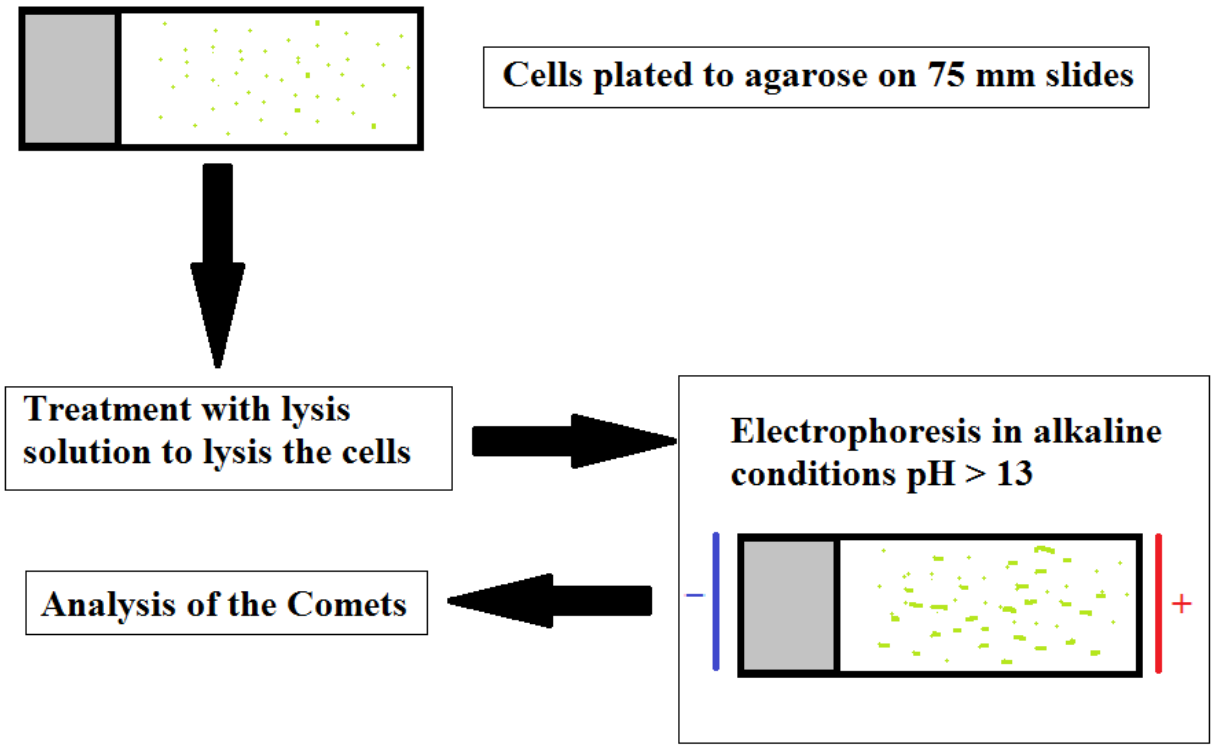

Figure 4. Schematic representation of the different steps in SCGE-assay. Cells are plated on the microscope slides, treated with lysis solution and set to the electrophoresis in the alkaline conditions after which the DNA nuclei are dyed with DNA binding dye and analysed with the microscope and suitable software.

Because of the several positive advantages of the Comet assay, it has been widely accepted as an assay to assess genotoxicity and use of assay has been dramatically increasing during the last decades. The Assay has been used for many different causes, including DNA damage caused by multiple different environmental agents, human biomonitoring, wildlife biomonitoring, effect of aging on gametes and even analysis of the time of death from homicide victims. (Singh et al. 2016;

Johnson et al. 2002). The broad usage of the assay has let to new evolutions that have been designed to make the assay more robust, reliably and meaningfully. For example, different softwires for better and faster visual examinations, image analyses and statistical analyses have been commercially available (Tice et al. 2000) including the fully automated image analyses.

2.0 AIMS OF THE STUDY

Aim of this study was to compare genotoxic responses from A549 cells detected with University of the Eastern Finland thermophoresis based, later thermo-ALI exposure system from different small-scale combustion emissions. Genotoxicity from the cells was measured with SCGE- assay. The comparison between measured genotoxicity from different small-scale combustion exposures and different controls was conducted. Also, the comparison between an incubator control and the thermo-ALI control was essential for concluding the functionality of the thermo-ALI exposure system.

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

Data for this thesis is from HICE campaign which took place in autumn of 2016. HICE, "The Helmholtz Virtual Institute of Complex Molecular Systems in Environmental Health" campaign is cooperation between Helmholtz Zentrum Munich, University of Rostock and University of Eastern Finland. Aim of this campaign was to collect considerable amount of data from different small-scale combustion application's emissions and toxicological effects of the emissions. The measurement part of the campaign was 3 weeks long and, in every week, different kind of combustion applications and fuels were used. All the combustion processes were conducted by the University of The Eastern Finland, Department of Environmental and biosciences, Fine Particle and Aerosol Technology Laboratory (FINE) group. Data for this thesis is from University of The Eastern Finland, Department of Environmental and Biosciences, Inhalation Toxicology (INTOLA) group's toxicological measurements. Every measurement week had 3 days of exposures and during the one measurement week every exposure day had the identical settings. Different combustion exposures and dilution rates can be seen from table 1. Every exposure had exposure time of 1 hour and exposures were made with use of thermophoresis-based air-liquid interface system, later thermo-ALI, exposure system which has been developed at University of The Eastern Finland by INTOLA and FINE research groups. After the exposure cells were carried out through several toxicological analyses to measure different toxicological responses.

Table 1. Combustion exposures and dilution rates used

Week	**The used exposures and dilution ratios**			
1	Spruce (1:30)	Spruce Filtered (1:30)	Diesel (1:10)	-
2	Aged Spruce (1:30)	Aged Filtered Spruce (1:30)	Pellet Ignition (1:10)	Pellet Steady (1:10)
3	Spruce (1:15)	Spruce Filtered (1:15)	Pine (1:15)	Pine Filtered (1:15)

3.2 COMBUSTION APPLICANCES

Combustions of spruce and pine were conducted by a modern heat-storing masonry heater with a staged combustion air supply described in detail at Leskinen et al. 2015 and the pellet combustion was made with the top-feed pellet boiler described in detail at Lamberg et al. 2011.

Filtered exposures were filtered with high efficiency particulate air (HEPA) –filter, therefore leaving only the exhaust gases to expose cells. Aged exposures were aged at the ILMARI laboratory aging chamber described more in detail at Tiitta et al 2016 to mimic the effect of the atmospheric aging.

Diesel engine used for diesel combustion emission was intercooled small industrial 1.123 l IDI (EPA Tier 1/EU stage II) diesel engine (Kubota D1105-T). This engine is described more in detail in the study of Jalava et al 2010. For the road use, it is representing very old technology, but for the construction machinery and non-road vehicles this technology is still widely used.

3.3 CELL CULTURE

Cells used were human alveolar type II-like epithelial cells (A549) (ATCC®, USA) which were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U mL-1 penicillin/streptomycin (all Sigma–Aldrich, USA). Four to six days before start of the exposure, the cells were cultivated 240 000 cells/ml for first two exposure days and 180 00 cells/ml for last exposure of DMEM with 10% FBS, on the basal side of the semipermeable polyethylene terephthalate membrane of 6-well inserts (4.2 cm^2 growth area, 3.0μm pore size; BD Falcon™ Cell Culture Inserts, BD Biosciences, USA). Inserts were kept at submerged conditions on 6-well plates in incubator at +37°C with 5% CO_2.

The cells were allowed to form air-liquid-interface 48 hours before the exposures by changing the cell culture medium to DMEM with 5% FBS and adding it only on the apical side of the inserts. 24 hours before the exposure this cell culture medium was changed to DMEM with 0% FBS. The cells exposed at the first exposure day in the week were exception with changing the cell culture medium DMEM with 5% FBS 24 hours before the exposure. Right before every exposure the cell culture medium was changed to 1.5 ml of DMEM with 25 µM of Hepes buffer (all Sigma–Aldrich, USA). The different exposures are presented in the table 1. The settings for the exposure gases

were flow rate of 150 ml/min and relative humidity of 100%. After the 1-hour exposure the medium was changed to DMEM with 0% FBS and inserts were placed in incubator to +37°C with 5% CO_2 on 6-well plates for 24h to stabilize.

For the exposures, two different kind of controls were used, incubator control and thermo-ALI control. Incubator control were kept at incubator with temperature of 37°C and 5% of CO_2 and with the thermo-ALI control the cells were exposed to purified air sample with the thermo-ALI system.

3.4 TOXICOLOGICAL ANALYSES

After the 24h incubation cells went through the toxicological analyses as described further.

At the start 1 ml of Dulbecco's phosphate buffered saline (PBS) was pipetted to the bottom of the 6-well plate's wells to prevent the drying of the cells. The culture medium was collected from inside the insert wells and deep-frozen at -80°C for the further analysis of the pro-inflammatory markers. Both sides of the insert's membranes were rinsed with PBS and the PBS used to basolateral side of inserts were collected. The PBS from inside of the insert's wells were discarded. Next, cells were detached from the inserts by trypsin treatment with 1 ml of trypsin added to both sides of inserts followed by incubation at +37°C with 5% CO_2. After an incubation period of 5 min, 100 µl of FBS was added to the both sides to inhibit the trypsin. After the trypsin treatment, cells were rinsed to the 6-well plate's wells and the resulting suspensions containing the cells were collected for further toxicological analyses.

Collected cells were resuspended to 1 ml of 10% FBS in PBS and for the cells from 4 inserts the toxicological analyses were following; reduced thiols with VB-48™, AO and PI staining, viability of the cells with DAPI-assay using AO staining, metabolic activity with the MTT-assay, MitoSOX™ (Invitrogen™) for mitochondrial superoxide production, the production of ROS by 2',7'-dichlorodihydrofluorescein diacetate/2',7'-dichlorofluorescein (H2DCF-DA/DCF)-assay and for cell cycle analysis, fixing cells to 70% ethanol (v/v).

For the SCGE-assay, the duplicates of 60 µl cell suspension were mixed to 540 µl of freeze medium ((40% serum (filtered)), 50% RPMI, 10% DMSO) and mixture were deep-frozen to -80°C

for the following SCGE assay. For this master thesis, the SCGE results were chosen for the closer evaluation.

3.5 SCGE-ASSAY

The alkaline version of SCGE assay was applied to this thesis to determine DNA from different combustion emissions. For the positive control, cells were treated with methyl methanesulphonate (MMS) with concentration of 195µg/ml and left in incubator during the thermo-ALI exposures. MMS has previously been shown to cause strong genotoxic response in different cells and therefore it is suitable as a positive control for SCGE-analysis. (Tice et al. 2002)

24 hours before the analysis, 75 mm microscope slides were dipped to normal melting point agarose (1% PBS) and left to dry. The cell suspensions stored at -80°C during the previous toxicological analyses were thawn in water bath and centrifuged (5 min, 8000 rpm, +4°C, benchtop centrifuge, Biofuge Fresco, Heraeus Instruments). After the centrifugation, majority of the supernatant was removed. Cells were suspended and from each duplicate sample, cell suspension aliquots were mixed low melting point agarose (LMPA) (0.5% in PBS) and plated to two 75 mm microscope slides. Therefore, from one sample we got slides A and B. After the plating, cells were lysed at +4°C for 1 h in lysis buffer (2.5 M NaCl, 0.1 M Na2-EDTA, 0.01 M Trizma® - Base, 1% Triton X-100, pH 10).

Followed by lysis the slides were rinsed with the neutralizing buffer (0.4 M Tris(hydroxymethyl)aminomethane, pH 7.5) and they were carefully arranged in the electrophoresis tank. Tank were filled with electrophoresis buffer (0.3 N NaOH, 1 mM Na_2-EDTA, pH > 13) and incubated 40 minutes in the dark. The slides were then objected to 24 V/300 mA for 20 min. After electrophoresis, the slides were neutralized by rinsing them three times with neutralizing buffer, then fixed with ethanol (99% v/v) and left to dry.

DNA on slides were stained with ethidium bromide, which binds between DNA bases and emits fluorescence signal after excitation with wavelength of 605 nm. Thereafter, pictures were taken with fluorescence microscope (Zeiss AxioObserver Z1). The resulting images from DNA nuclei were analyzed with Comet assay IV software (Comet assay IV, Perceptive Instruments Ltd., Suffolk, UK) so that in each slide, 50 nuclei were chosen, resulting to 100 DNA nuclei chosen

from every sample. The Olive tail moment (OTM) ((tail mean – head mean) x tail% DNA/100) was chosen for the parameter to estimate DNA damage, since it has been proven to yield results that are more easily compared between different studies. (Sunjog et al. 2013).

3.6 Statistical analysis

Statistical analyses were performed to ascertain the statistical significance between different samples. Levene's test for homogeneity of variances was used to the samples before using one-way analysis of variance (ANOVA) and Tukey's test. The measured results from exposures were tested against the thermo-ALI system control and each other. All the differences were regarded as statistically significant at $p < 0.05$. The data was analyzed using the SPSS statistics version 25.0 (SPSS Inc. Chicago, IL, USA).

4.0 RESULTS

Results from the SGCE assay are presented in the following 3 figures (figure 5, figure 6 and figure 7). Which compare the measured OTM values from different controls, from all the exposures and additionally represents visually clarified compassion, from which the percentage of the differences can be perceived with greater detail.

4.1 CONTROLS

Figure 5 presents the difference in measured OTM values from two different study controls, the incubator control and the thermo-ALI exposure control, shortly thermo-ALI control. Measured OTMs were 0.48 for incubator control and 0.81 for thermo-ALI control.

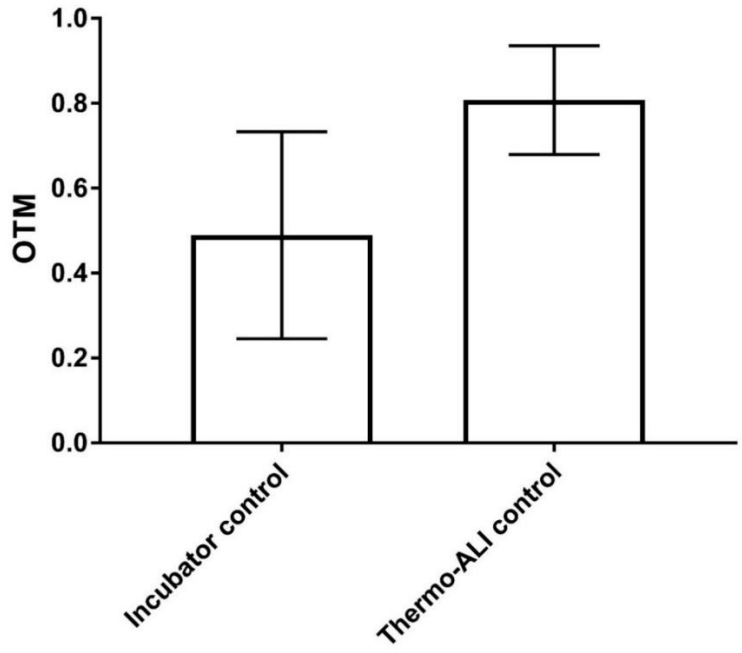

Figure 5. Measured genotoxicity in value of OTM detected with SCGE-assay from A549 cells between incubator control and thermo-ALI clean air exposure control. Error bars present the standard error of mean (SEM).

4.2 COMBUSTION EXPOSURES

The figure 6 presents the measured genotoxicity with value of OTM from incubator control, thermo-ALI control and all the different small-scale combustion exposures.

From different wood combustion exposures, the aged spruce showed the highest genotoxicity in A549 cells with the OTM value of 2.74. The statistical significance compared to the thermo-ALI control value was also found from aged spruce exposure. The lowest responses were detected from the filtered spruce aerosol with dilution of 1:30 with the OTM value of 1 and the second lowest from pine combustion exposure with the OTM value of 1.3. The measured OTM from rest of the wood combustion exposures were following; Spruce 1:30 1.45, aged filtered spruce 1.8, spruce 1:15 1.85, spruce filtered 1:15 1.73 and pine filtered with OTM value of 1.53.

Diesel combustion exposure showed higher genotoxic potential than majority of the wood combustions with the OTM value of 2 and with the statistical significance compared to thermo-ALI control. The pellet combustion aerosol exposures overall showed higher genotoxicity than wood combustion exposures, with OTM value of 1.7 for pellet steady combustion phase exposure and 1.9 for pellet ignition phase.

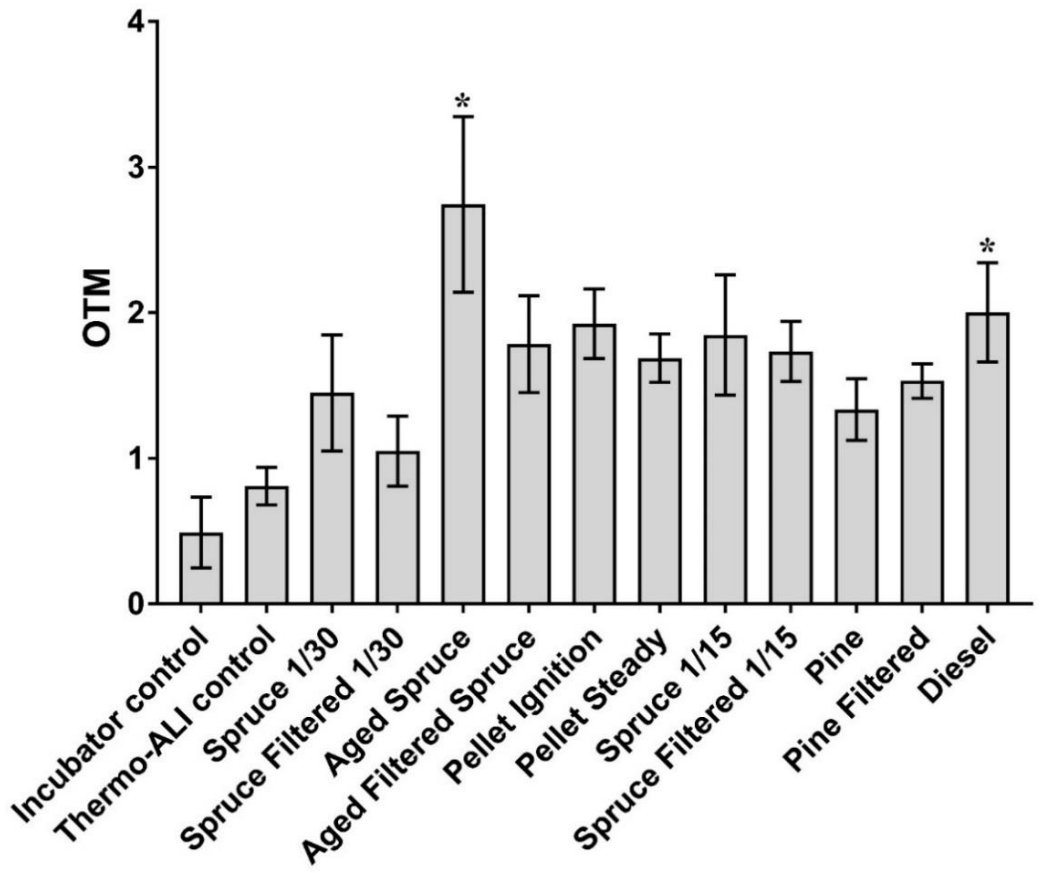

Figure 6. The measured OTM from A549 cells exposed with thermo-ALI exposure system to different small-scale combustion emissions including the different controls. Dilution rates are indicated for different spruce exposures to separate them from each other. Asterisks indicate the statistical significance compared to the thermo-ALI control. SEM is presented with the error bars. n = 12 for incubator control and all the different combustion exposures and n = 36 for thermo-ALI control.

The figure 7 presents fold comparison between the thermo-ALI control and other exposure, including the incubator control. This comparison is made for better visualization in differences of measured OTM between combustion exposures and thermo-ALI control. All the different combustion exposures caused higher genotoxicity than thermo-ALI control. Lowest exposure compared to the thermo-ALI control were spruce filtered with dilution of 1:30 with 1.3-fold higher genotoxicity. The highest genotoxicity compared to the thermo-ALI control was detected from aged spruce with almost 3.5-fold. Overall, majority of the combustion exposures showed above 2-fold difference to thermo-ALI control.

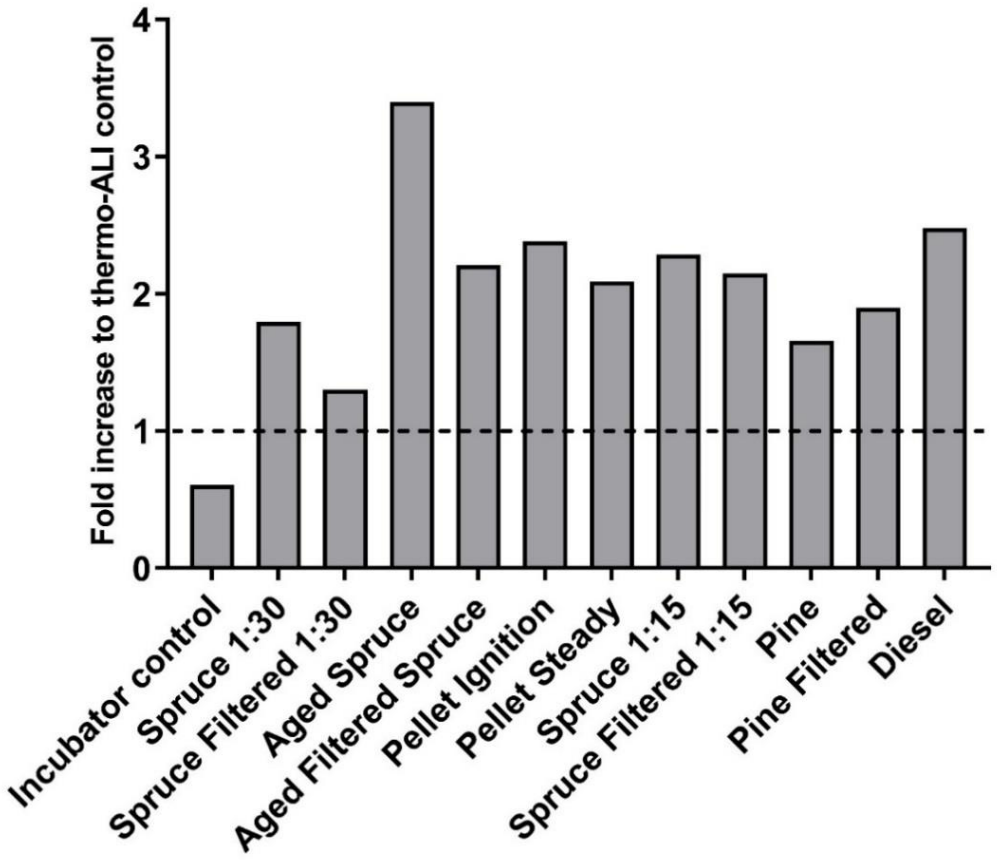

Figure 7. Different combustion exposures and incubator control compared to the thermo-ALI control. OTM value of thermo-ALI control is set as a value 1 and indicated in the graph with a horizontal line. Y-axis indicates the fold increase compared to thermo-ALI control.

5.0 DISCUSSION

Difference between measured genotoxicity from two different controls is about 30%. Thermo-ALI control showed higher genotoxicity which might indicate that the thermo-ALI system itself may affect the cells resulting in genotoxic consequences. Thus, it can be assumed that the observed response is more from the cell membrane and DNA damage due to airflow in the system than being actual genotoxicity in the control. In addition, one has to take into consideration that the thermo-ALI exposure system is very new kind of exposure system which is still under development. Regardless of this, the measured genotoxicities from both controls were lower than from any combustion exposure, which indicates the functionality of the thermo-ALI system as designed. In addition of that, the SEM-value in thermo-ALI control was 2-fold lower than the SEM from incubator control which, in turn supports the good operability and the repeatability of the thermophoresis-based ALI exposure system.

Multiple different previous studies have concluded that the emissions from different small-scale combustion systems cause genotoxicity. In the review study of Risom at el 2005, based on multiple studies ranging from animal experimental models to cell culture experiments, it was concluded that urban particulate matter causes DNA damage via oxidative stress induced reactions. In experimental studies concerning small-scale combustion emissions, it has been concluded that both pellet and spruce combustion exposures cause genotoxicity in A549 cells even with the lowest exposure doses (Kasurinen et al 2017). Results with a similar trend were detected from earlier study of Tapanainen et al 2011 when exposing RAW264.7 cells with emissions from the different combustion applications of masonry heater and pellet boiler. In aforesaid studies, genotoxicity of the exposures was explained with the PAH compounds found from emissions. These compounds have been associated with lung cancer in meta-analyses of Amstrong et al 2004. However, all of the *in vitro* studies mentioned before are conducted with traditional submerged cell culture conditions. Therefore, it was important to study these effects in the air-liquid-interface cell exposure system, where the sample collection and preparation procedures do not change the observed cellular effects.

In fact, different ALI techniques have been used successfully in previous inhalation toxicological studies for wood combustion emissions and cigarette smoke exposures (Steinritz et al. 2013; Rach

et al 2014; Mülhopt et al. 2016). However, these ALI systems differ from thermo-ALI system used in this thesis with several properties. For example, the Vitrocell system used at study of Mülhopt et al. 2016, uses electrical field for PM deposition. This system limits the efficient PM deposition only to charged PM, whereas the thermophoresis-based exposure system doesn't have similar categorizing effect to the deposing PM.

Concerning, in the study of Kasurinen et al 2017 it was concluded that the spruce combustion emissions caused higher genotoxicity than pellet combustion emissions. This finding could be explained with higher oxygenated PAH concentrations found from genotoxic emissions, which have similar toxicological effect *in vivo* with PAHs (Elie et al., 2015). Pine combustion have also been linked to genotoxicity via concentration of the PAH compounds in emissions in study of Avagyan et al 2015 in which the pine combustion resulted in higher PAH emissions than spruce with higher burn rate. Though, with normal burn rate, spruce combustion resulted in higher PAH emissions. Additionally, varying PAH concentrations have been linked to changing genotoxic effects of the emissions from old and new biomass combustion technologies (Jalava et al., 2012). In the results of this master's thesis both the spruce and pine emissions caused genotoxicity, which might be due to the amount of PAH compounds in the emissions. Therefore, results concerning the wood combustion exposures are in line with the previous studies. Consequently, the possible high concentrations of PAH compounds in spruce emissions are most likely the underlying reason behind the highest genotoxic potential of spruce combustion aerosol compared to other samples in this study.

Results from the pellet ignition and pellet steady state combustion showed relatively high genotoxicity compared to the previous studies (Tapanainen et al 2011, Kasurinen et al 2016). One explanation may be the different exposure technique compared to the previous studies. In addition, the aerosol exposure from the ignition state of pellets caused slightly higher genotoxic response than that from the steady combustion phase. In previous studies the emissions from pellet ignition phase have been higher than those from the steady combustion phase (Heringa et al 2012, Lamberg et al 2011) because of the smouldering combustion conditions at the ignition. Additionally, the exposure from emissions at smouldering combustion phase been studied to be more carcinogenic than from steady combustion (Uski et al 2014). Therefore, results concerning pellet combustion

exposure are in line with previous studies, with the smouldering combustion at ignition state emitting more genotoxic emissions than exposure from steady pellet combustion state.

The DEP genotoxicity has been studied abundantly (Attfield et al 2012; Silverman et al., 2012; Guo et al., 2004; Garshick et al., 2008). Genotoxic effects of the DEP are connected to the structure of the DEPs, which consist elemental carbon core which can absorb on its surface; PAH compounds, transition metals, acids and quinones (Dybdahl et al 2004). In the results of this thesis, diesel exposure caused overall higher genotoxicity than the wood smoke exposures, excluding the aged spruce, with differences ranging from close to 50% with spruce filtered 1:30 to close to 8% for spruce 1:15 with statistically significance compared to the control. These results are therefore in line with the plentiful previous studies and indicates that the DEP consist in this exposure also compounds with genotoxic potential and therefore induces DNA damage in A549 cells. In addition of that, in the previous study of Jalava et al 2010 in which the same diesel engine was used, significant genotoxic responses were measured from RAW264.7 cells even with the lowest exposure concentrations. Most of the previous diesel exhaust studies have been conducted with old technology engines, which is well in line with our non-road machinery engine. However, far reaching conclusions on the diesel exhaust based on old technologies cannot be drawn to current road traffic due to stricter regulations and better particle filtration technologies.

In the experiments, HEPA filtration was included to remove particulate phase of the emissions in selected cases. Filtered exposures showed lower genotoxic response in all the different spruce exposures with the highest difference after aging of the emissions. Interestingly, with the pine exposures the results were opposite. The difference between filtered and non-filtered emissions are potentially explained by carcinogenetic compounds distributed in the gas to particle phases in the emissions. This indicates for example how much of the different PAH compounds in certain emissions are in the gas phase or bonded to the surfaces of the particles. The gas to particle balance of PAH compounds have several different reasons ranging from size of the particles to temperature of the gas and chemical components present (Turpin et al. 1999, Tsapakis and Stephanou 2005). Especially dilution of the exhaust gases and thus, decreasing the vapor pressure of the gas, affects the gas-particle distribution of the PAH. The study of Hytönen et al 2009 concluded that the gas to particle distribution of PAH compounds can be divided in to three groups defined by the molecular size of the compounds. The lighter PAH compounds staying primary in the gas phase

and heavier in particle phase. In addition of that, the combustion conditions also affected the amount of PAH compounds in emissions. The smouldering combustion conditions resulting in more PAH compounds and additionally, shifting the gas-particle distribution towards the particle phase. In these results, the non-filtered spruce combustion exposures caused higher genotoxicity than filtered, the differences were minor in spruce combustion exposures with dilution of 1:15 but up to 20% in spruce with dilution rate of 1:30 and up to 30% in aged spruce combustion exposures. Therefore, filtered emissions itself caused close to the same genotoxicity than non-filtered which indicates that the particles in emissions didn´t have such a significant genotoxic effect and the majority of the genotoxic PAH compounds were most likely in gas phase of the emissions. In the aged sample, the aging process has probably led to more reactive compound on the particle surface, resulting in a higher genotoxic response.

The opposite results in pine combustion aerosols exposures are interesting with filtered exposures causing higher genotoxicity than non-filtered. Possible underlying reason for these results might be explained by the lack of PM resulting to lack of PM bound PAH compounds. Which in turn will result more freely available PAH compounds to expose cells and therefore cause genotoxic effects. Interesting is why this speculated effect only took place in pine combustion emissions. One possible reason is that the PAH composition is different, resulting in more volatile compounds in gaseous fraction of the emissions.

The aging of the emissions was made in aging chamber described in study of Leskinen et al 2015, to mimic the effects of the atmospheric aging of the emissions. Aging of the emissions affected the genotoxicity notably with close to 35% difference between filtered and unfiltered aged spruce exposures being almost 10% higher when compared to the differences in other spruce combustion exposures, respectively. Proportionate differences for spruce exposure with dilution of 1:15 were less than 7% and for pine exposures close to 13%. In several previous studies (Tiitta et al. 2016; Joylleys et al. 2012) aging of the emissions, in aging chambers and in the atmosphere, have shown to affect the emissions in different ways. For example, aging has been shown to affect the concentrations of different PAH compounds in the emissions and especially concentrations of the genotoxic PAH compounds (Nordin et al. 2015). The aging of the emissions has speculated to turn the particles more volatile due to gaining (semi)volatile material from gas phase. Additionally, the less volatile compounds can degrade to more volatile, similarly to PAH photodegradation

(Leskinen et al. 2007). The secondary carbon products have also been shown to form with the aging and these secondary components of the air pollution have different toxicological effects compared to the initial primary emissions. Formation of secondary organic aerosols have been shown increase the concentrations of PM bound reactive oxygen species (PB-ROS) in emissions (Zhou et al. 2018). These different effects of the aging might explain the highest genotoxic potential found from results of aged spruce exposure. The relatively high difference between the aged spruce filtered and non-filtered emissions might indicate that the aging have affected the genotoxic potential of the PM in emissions, therefore filtering the genotoxic PM from exposure significantly lowers the genotoxicity.

6.0 CONCLUSIONS

As a conclusion, in the results of this master's thesis all the different exposures caused higher measured genotoxicity to the human alveolar type II-like epithelial A549 cells than respective thermo-ALI control. This indicates that the different small-scale combustion and non-road diesel engine exhaust emissions caused genotoxicity *in vitro* when cell cultures were exposed with thermophoresis-based air-liquid interference system. Level of genotoxicity varied between different exposures from the lowest 1.3-fold to thermo-ALI control to the highest being 3.5-fold. Results are in line with previous studies done with submerged conditions, with the exposures linked to high genotoxic potential causing the highest genotoxicity also in this study. Reasons behind the genotoxicity could be explained by the different oxidative stress causing agents in the exposures, for example the PAH compounds. The aging of the emissions seemed to shift the genotoxic agents towards the particle phase of the emission, which was observed with higher difference between filtered and not filtered results from aged spruce exposure. With the data from physiochemical measurements of the emissions and therefore knowing concentrations of different compounds in emissions, these speculations will be verified in later studies.

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