KU LEUVEN

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Contribution of bacterial endotoxins and transition metals to the inflammatory potential of particulate matter (PM): method development

Bijdrage van bacteriële endotoxines en transitiemetalen tot de inflammatoire activiteit van fijn stof: methodeontwikkeling

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Abstract

Background: Particulate matter (PM) is a global and pressing problem, linked to cardiovascular and respiratory morbidity and mortality and more recently also to neuro-degeneration. Although often treated as a uniform pollutant, PM has a highly complex composition, which is strongly associated with its toxicity. Endotoxins and transition metals are important components linked to the ability of PM to elicit a pro-inflammatory response and oxidative stress, which are believed to lie at the base of the observed adverse health effects. However, to provide conclusive evidence of the importance of these components new and optimized methods are necessary for standardization of the experimental set-ups and to provide additional mechanistic insights.

Materials and Methods: Several techniques were evaluated and optimized over the course of this project. First of all, for the collection of PM samples the Coriolis® µ air sampler was used. The collected samples were subsequently analysed on their composition by the recombinant Factor C assay, to determine their endotoxin content, and inductively coupled-plasma mass spectrometry (ICP-MS) and cyclic voltammetry, to provide information on their transition metal content. The biological activity of the samples was evaluated with the use of three *in vitro* models, more specifically the HEK-Blue™ hTLR4 cell line, to check for TLR4 stimulation, and two macrophage cell lines, THP-1 and U937, to test for expression of pro-inflammatory cytokines with qRT-PCR. Finally, also the possibility of using nasal respiratory epithelium samples, isolated from human volunteers, as a non-invasive *in vivo* alternative to estimate PM toxicity was investigated.

Results and Discussion: First and foremost**,** an optimized protocol was developed for PM sampling and the recombinant Factor C assay, while the use of the ICP-MS technique did not require additional optimization. Furthermore, important difficulties were encountered with the application of cyclic voltammetry, and future work is necessary to estimate if this technique could be applied in PM research. The use of the HEK-Blue™ hTLR4 cell line, however, did show promise for its application in PM research, as preliminary results showed measurable TLR4 activation upon exposure to the PM samples. Moreover, contrary to what is known in literature, an down-regulation of pro-inflammatory cytokine expression was observed upon exposure of the THP-1 and U937 cell line to the PM samples, which likely results from a suboptimal assay protocol. Lastly, promising results were obtained for the first steps in method development for the use of the nasal respiratory samples.

Conclusion: The evaluation of several techniques in this project has indicated future work necessary for, and research questions which could be investigated after optimization of the used techniques.

Samenvatting

Achtergrond: Fijn stof is wereldwijd een nijpend probleem dat gelinkt wordt aan cardiovasculaire en ademhalingsaandoeningen, en recentelijk ook aan neurodegeneratieve ziekten. Ondanks dat fijn stof vaak behandeld wordt als een uniforme luchtvervuilende stof, heeft het een erg complexe samenstelling, die sterk gerelateerd is aan zijn toxiciteit. Endotoxines en transitiemetalen zijn twee belangrijke componenten die geassocieerd worden met het vermogen van fijn stof om een ontstekingsreactie en oxidatieve stress op te wekken. Van deze twee mechanismen is geweten dat ze aan de basis liggen van de geobserveerde gezondheidseffecten van fijn stof. Desondanks, om sluitend bewijs over het belang van deze componenten te leveren heeft men nieuwe en geoptimaliseerde methoden nodig voor standaardisatie van de experimentele opstellingen en om aanvullende mechanistische inzichten te verwerven.

Materiaal en methode: Tijdens dit project, werden verschillende technieken geëvalueerd en geoptimaliseerd. Vooraleerst, werden fijn stof stalen verzameld met behulp van de Coriolis® µ air sampler, om vervolgens geanalyseerd te worden op hun samenstelling. Hierbij werd de endotoxine concentratie bepaald door middel van de recombinante Factor C test. Informatie over de metaal inhoud werd bekomen aan de hand van inductief gekoppelde plasma massa spectrometrie (ICP-MS) en cyclische voltammetrie. De biologische activiteit van de stalen werd geëvalueerd met behulp van drie *in vitro* modellen, namelijk de HEK-Blue™ hTLR4 cellijn, om stimulatie van TLR4 te achterhalen, en twee macrofaagcellijnen, meer bepaald THP-1 en U937 om expressie van pro-inflammatoire cytokines te testen met qRT-PCR. Ten slotte werd ook de mogelijkheid bestudeerd om stalen van het nasale respiratoire epitheel geïsoleerd van vrijwilligers als een niet invasief *in vivo* alternatief te gebruiken voor het inschatten van het toxische karakter van fijn stof.

Resultaten en discussie: Tijdens het project werd een geoptimaliseerd protocol ontwikkeld voor het nemen van fijn stofstalen en voor de recombinante Factor C test, terwijl het gebruik van de ICP-MS techniek geen verdere optimalisatie vereiste. Verder werden er moeilijkheden ervaren bij het toepassen van cyclische voltammetrie en toekomstig werk zal moeten uitwijzen of deze techniek gebruikt kan worden voor het fijn stof onderzoek. Aangezien de eerste resultaten van de experimenten met de HEK-Blue™ hTLR4 cellijn meetbare TLR4 activatie aangaven, lijkt dit wel een beloftevolle techniek te zijn voor gebruik in het fijn stof onderzoek. Daarnaast werd na het blootstellen van de THP-1 en U937 cellijnen aan de fijn stof stalen een down-regulatie van pro-inflammatoire cytokine expressie geobserveerd, die waarschijnlijk te wijten lag aan een suboptimaal protocol. Tenslotte, ook

bij de eerste stappen in methodeontwikkeling voor het gebruik van de nasale stalen werden beloftevolle resultaten verkregen.

Conclusie: De evaluatie van de verschillende technieken gebruikt in dit project heeft het nodige toekomstige werk aangegeven nodig voor, en de onderzoeksvragen die onderzocht zouden kunnen worden na optimalisatie van de technieken.

List of abbreviations

Abbreviation	Significance
AA	Ascorbic acid
ACS	American Cancer Society
AD	Alzheimer's disease
AGI-30	All glass impinger 30
AhR	Aryl-hydrocarbon receptor
AP1	Activator protein 1
APHEA	Air pollution and health: An European approach
AQG	Air quality guidelines
BaP	Benzo-a-pyrene
BBB	Blood-brain barrier
CD14	Cluster of differentiation 14
CINC	Cytokine induced neutrophil chemo attractant
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
D_{50}	Particle diameter with 50% collection efficiency
DEP	Diesel exhaust particles
EEA	European environmental agency
EU	European Union
GSH	Glutathione
HR-ICP-MS	High-resolution inductively coupled plasma mass spectrometry
IDT	Integrated DNA Technologies
IKK	IκB kinase
IL	Interleukin
IRAK	Interleukin1 receptor associated kinase
IRF3	Interferon regulatory factor 3
IκB	Inhibitor of NFκB
JNK	c-Jun N-terminal kinase
LAL	*Limulus* Amoebocyt lysate
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase

MD-2	Myeloid differentiation factor 2
MIP-2	Macrophage inflammatory protein 2
MS	Mass spectrometry
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor κ B
PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PM	Particulate Matter
$PM_{0.1}$	Fraction of PM of particles smaller than 0.1 μm
PM_{10}	Fraction of PM of particles smaller than 10 μm
$PM_{2.5}$	Fraction of PM of particles smaller than 2.5 μm
PMA	Phorbol-12-myristate-13-acetate
pNPP	Para-nitro phenyl phosphate-13-acetate; also known as 4-NPP
qRT-PCR	Quantitative real-time polymerase chain reaction
RF	Radio frequency
rFC	Recombinant Factor C
RNSA	Réseau National de Surveilance Aérobiologique
ROS	Reactive oxygen species
SEAP	Secreted embryonic alkaline phosphatase
TAK	Transforming growth factor β activated kinase
TANK	TRAF associated NFκB activator
TBK1	TANK binding kinase 1
TIRAP	Toll-IL 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TRAF	TNF receptor associated factor
TRAM	TRIF related adaptor molecule
TRIF	Toll-IL 1 receptor domain containing adaptor inducing interferon β
TRIS	Tris-hydroxymethyl-aminomethane
UFP	Ultrafine particles
VMM	Vlaamse mileu maatschappij (Flemisch environmental agency)
WHO	World Health Organisation

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Introduction

Over the last decades, our awareness of human influence on the environment has grown. We now face several pressing environmental issues, for which there is no straightforward solution. Air pollution is one of these topical and global problems, affecting our health, climate, ecosystems and economy. Particulate matter (PM) is the component in polluted air that has the strongest association with adverse health effects, with even low exposure doses harming our health, as suggested by the World Health Organisation (WHO) (WHO 2013). Various epidemiological studies have provided strong evidence for associations between PM exposure and respiratory and cardiovascular morbidity and mortality. Furthermore, research now also points to a range of other effects, including adverse effects on the central nervous sytem (CNS), liver, reduced insulin sensitivity, cancer and low birth weight (Brook et al. 2013; Grigg 2013; Lewtas 2007; Terzano et al. 2010). Indeed, the influences on the CNS are becoming an important part of PM research. Recent studies have indicated possible associations of PM exposure with stroke, Alzheimer's disease, Parkinson's disease and neurodevelopmental diseases (Genc et al. 2012).
Due to these severe health effects, the PM problematic has gained attention outside the scientific world and awareness is growing in the general public. Headlines of recent articles from popular Belgian newspapers read as follows:

"*Te veel fijn stof en ozon voor 90 procent van stedelingen in EU*"- De Morgen, 15 October 2013 *(" Too high levels of particulate matter for 90 percent of EU urban population")*

"*Strijd tegen fijn stof moet dringend worden opgevoerd*"- De Morgen, 18 October 2013
("Fight against particulate matter must be raised urgently")

"*Smog legt miljoenenstad in China lam*"- De Morgen, 21 October 2013
("Smog paralyzes megacity in China")

"*In 2012 stierven 7 miljoen mensen door luchtvervuiling*"- De Standaard, 25 March 2014
("*In 2012, 7 million people died because of air pollution*")

Jacqueline McGlade, European Environmental Agency (EEA) Executive Director, stated the Eurobarometer survey showed that the impact of air pollution is something European citizens feel strongly about. She also noted that the decision to designate 2013 as the Year of Air

reflects both on the economic seriousness of the problem and also its impact on humans. However, despite our extensive knowledge of the consequences of air pollution, little to date is known about the mechanisms by which it exerts its effects. A good understanding of the contribution of PM components to their toxicity, the mechanisms by which the particulates act, and if there are possible synergistic effects between components would greatly enhance our approach in dealing with PM in the future.

Particular PM components of interest are endotoxins, potent pro-inflammatory mediators derived from the Gram-negative bacterial cell wall and ubiquitous in PM. Secondly also the transition metal content of PM has been suggested to be an important factor of PM toxicity. However, in the research for these components, it is necessarily to develop new methods or optimize existing methods to provide better standardization throughout experimental set-ups and provide alternative approaches to help elucidate the responsible mechanisms involved in PM related health effects.

This was the main goal of this project, the investigation and optimization of several methods, which could be applied in the investigation of the importance of endotoxins and metals in PM toxicity. Therefore, techniques for PM sampling, determination of the endotoxin and metal content of the samples, and for the evaluation of their biological activity were tested and discussed in this project.

Chapter 1.
Literature study

1.1. Particulate matter

1.1.1. What is particulate matter?

Particulate matter (PM) is the general term used for a mixture of solid and liquid particles suspended in the air, being heterogeneous in size and composition (C. A. Pope III & Dockery 2006). The variability of PM may be attributed to a range of factors, including particle sources, weather conditions, season and many others factors (EEA 2013a).

PM is often categorised in different **fractions** depending on its size range, namely coarse (2.5-10 µm), fine (≤ 2.5 µm) and ultrafine (≤ 0.1 µm). The size of the particle is a main determinant of how deep in the respiratory tract the particle will reach when inhaled, as shown in figure 1.1. Particles smaller than 10 µm (**PM_{10}**) are known as respirable particles as they settle in the bronchi and lower lung tissues whereas larger particles tend to be filtered by nose and throat cilia and mucous. The 10 µm particle size does not represent a strict boundary between respirable and non-respirable particles, but has been agreed upon for monitoring of airborne PM by most regulatory agencies (Seinfeld & Pandis 2006).

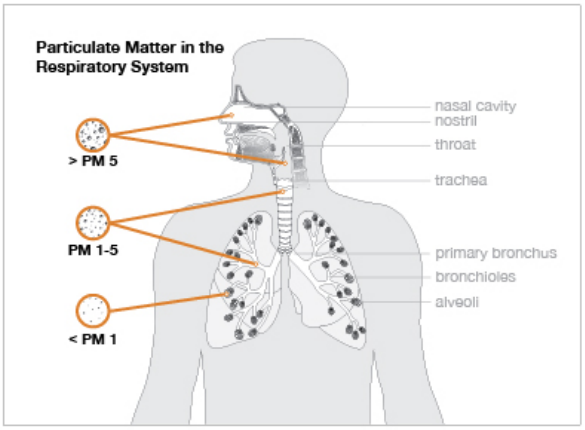

Figure 1.1: Deposition of different size fractions of PM in the respiratory tract. Reference: Alen (2014)

Studies have investigated the sources of the particles in these size fractions. Larger particles often have a natural source, such as sea salt, dust, pollen and volcanic ashes (EEA 2012). Meanwhile, the smaller particles (**$PM_{2.5}$ and $PM_{0.1}$**) often have an anthropogenic source (as a result from human activity) such as fuel combustion for thermal power generation, incineration, domestic heating for households, or vehicular fuel combustion (EEA 2013a). Furthermore, airborne particles may undergo various chemical interactions and transformations. Distinction can therefore be made based upon the particle's origin, namely **primary or secondary particles**. Primary particles are emitted directly in the air, and are

found in the atmosphere in the same chemical composition as they were emitted, whereas secondary particles are formed in the air by chemical reactions such as oxidation or transformation of precursor gases after their emission. Important sources of these secondary particles are SO_2, NO_x, NH_3 and volatile organic compounds (EEA 2013a). The formation of these particles depends on a variety of chemical and physical factors such as the concentration of the precursor gases, reactivity of the atmosphere and the meteorological conditions (e.g. solar radiation, relative humidity and cloud cover) (EEA 2013a). This highlights one of the means by which the weather conditions can have an influence on the ambient PM composition, in this case by the formation of secondary particles.

Additionally, **weather and season** can influence the concentration and composition of PM in other ways. For instance, rainfall can have a strong influence on ambient PM concentrations. Precipitation has the effect of pulling down the particles with it, decreasing the PM concentration (Stephenson et al. 2004; KNMI 2001). Furthermore, meteorological and topographic factors also strongly influence the transport of air pollutants (EEA 2013a).

Studies have also shown an important seasonal effect on PM composition. It is logical that particles derived from combustion sources are more numerous in cold seasons, due to residential heating (EEA 2013a), while emission from terrestrial vegetation will increase in spring (Long et al. 2001; Rylander 2002) and summer (EEA 2013a; Farina et al. 2013).

1.1.2. PM Composition

The highly variable nature of PM is often not recognised in monitoring, studies and legislation. Instead, PM is treated as a uniform pollutant, characterised by its aerodynamic diameter and mass concentration, which are an imperfect estimate of the biological activity (Borm et al. 2007). The toxicity of PM is, however, strongly dependent on the characteristics of its components and therefore the PM source and composition (Frampton 2006). There are three essential factors that contribute the toxicity of PM, namely (i) its **physicochemical characteristics** including shape, size and density (Brown et al. 2001; Frampton 2006), (ii) its **capacity to elicit oxidative stress** by acting as redox catalysts (redox-active metals and quinones) (Ayres et al. 2008; Frampton 2006);(iii) its ability to **trigger innate immune responses** (Monn & Becker, 1999; Osornio-Vargas et al., 2003; Schins et al., 2004).

These factors strongly influence the capacity of PM to induce inflammation and oxidative stress, which are thought to play a key role in mediating PM induced damage (González-Flecha 2004). Inhalation of a toxic air pollutant can cause **cell and tissue injury**, which triggers an **inflammatory response**, necessary for clearing cell debris and initiating healing. Consequently, inflammatory markers may be considered an indicator of the damage resulting from PM exposure (Frampton 2006). On the other hand, the inhaled particles themselves can also directly activate and modulate the innate immunity response (Frampton 2006;

Gangamma 2012). The subsequent inflammatory response causes cellular injury, making tissue injury the consequences of the inflammatory response instead of its cause (Frampton 2006). Therefore, studying the characteristics of various PM components driving these responses, could lead to a better understanding of how specific components contribute to PM toxicity. This knowledge can in turn be used to develop regulations targeting concentrations of the more harmful components (Grahame & Schlesinger 2007; Nel 2005). An overview of several important PM components is listed below.

1.1.2.1. Diesel exhaust particles (DEP)

DEPs comprise a complex mixture of combustion derived components with a central **core of elemental carbon** and **adsorbed organic compounds** such as polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs, quinones (Yu & Xu 1987), and small amounts of metals, sulphates and nitrates (Kocbach et al. 2006; Bérubé et al. 1999; United States Environmental Protection Agency 2002). Subsequently, carbon black may be used as a model for DEP without the adhered compounds (Ema et al. 2013). These particles are commonly found in the $PM_{2.5}$ and ultrafine fractions (Ema et al. 2013).

1.1.2.2. Transition metals

Transition metals (elements capable of forming cations) form another important PM component, contributing substantially to PM's oxidative capacity. Every element has its own specific sources and health effects. The **origins** of transition metals in PM are very widespread and range from the combustion of fuels, vehicular wear and industrial processes to suspension of mineral dust (European Commission 2001; Manalis et al. 2005; Schauer et al. 2006; EEA 2013a). As a consequence, the fine fraction of PM (consisting largely of particles from anthropogenic origin) was shown to be enriched in metals as compared to the coarse fraction (Monn & Becker 1999). The variability between various elements is also reflected in the **observed health effects**, which include carcinogenic, neurological, respiratory, haematological and reproductive effects (European Commission 2001; EEA 2013a; Manalis et al. 2005; Schauer et al. 2006). An overview of the sources and health effects of some important transition metals in PM can be found in annex.

Both *in vivo* and *in vitro* research has correlated transition metal content in PM with its capacity to induce **oxidative stress**(Frampton et al. 1999; Ghio & Devlin 2001; Ghio et al. 2012; Valavanidis et al. 2008; Mukhtar & Limbeck 2013; Kelly & Fussell 2012; Kelly & Mudway 2007). Two important variables determining the oxidative burden posed by the transition metals in PM are their **bioavailability and their redox activity** (Costa & Dreher 1997; Dominici et al. 2007; Roemer et al. 2000). The latter can be explained by ability of redox active metals, such as iron (Fe), copper (Cu), manganese (Mn), vanadium (V) and

nickel (Ni) to participate in redox cycling reactions, creating reactive oxygen species(Kelly & Fussell 2012; Ghio et al. 2012; Valavanidis et al. 2008). Nonetheless, also non-redox active metals, like zinc (Zn), aluminium (Al) and lead (Pb) can influence the resultant oxidant burden, likely by exacerbating or decreasing free radical production, or possibly depleting antioxidants (Ghio et al. 2012; Kelly & Fussell 2012). These principles are also shown in figure 1.2.

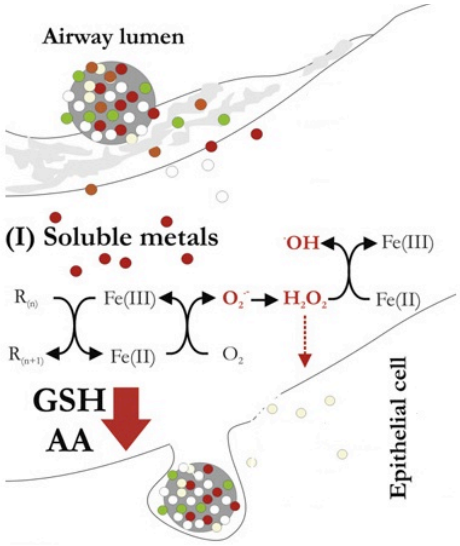

Figure 1.2: Mechanism for induction of oxidative stress in the lung by the transition metal component of particulate matter. By redox cycling, redox active metals can generate reactive oxygen species such as the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (°OH). Furthermore, a depletion of antioxidants such as glutathione (GSH) and ascorbic acid (AA) can result from interactions with the metal component of PM. Adapted from Kelly & Mudway (2007)

Fortunately, according to measurements of the EEA, airborne metal concentrations throughout Europe are generally low (EEA 2013a). However, metal concentrations may build up in food chains and accumulate over time in the body, due to **bioaccumulation** (EEA 2013a).

In attempt to restrict their airborne concentrations, **target or limit values** have been implemented for arsenic, cadmium, nickel and lead. The significant health effects of Lead (Pb) have motivated the EU to restrict the maximal allowable airborne lead concentration to an annual limit value of 500 ng/m^3. The World Health Organisation (WHO) considers blood levels below 100 µg/l not to be dangerous to our health. Some suspect, however, that lower concentrations can still have an effect on children's intelligence, and ask for a lowering of the limit value (Van Steertegem 2012). For arsenic, cadmium and nickel, target values were designed at the level of 6ng/m^3, 5ng/m^3 and 20ng/m^3 respectively (EEA 2013a).

1.1.2.3. PAH's

Polycyclic aromatic hydrocarbons (PAH) are a growing health concern in Europe and are often associated with airborne particles, like DEP (EEA 2013a). These compounds have

mutagenic and carcinogenic properties (Mesquita et al. 2014), and can cause irritation to eyes, nose, throat and bronchial tubes. Recently, PAHs have been linked to cardiovascular morbidity and mortality, although these effects cannot be disentangled from the effects of the particles (EEA 2013a).

PAHs are derived from the (incomplete) combustion processes of both natural sources such as forest fires (Mesquita et al. 2014), and anthropogenic sources such as the heating of buildings, traffic and industrial combustion processes for energy generation (Mesquita et al. 2014; Vlaamse milieumaatschappij 2013). Due to their strong affinity for carbonous cores, they are primarily found in smaller size fractions of PM (Miguel et al. 1998; Zhou et al. 2005). PAHs do not easily degrade in the environment and are prone to long distance transport (Mesquita et al. 2014). Strong seasonal influences can be found regarding PAH concentrations, giving higher concentrations in winter than in summer (Vlaamse milieumaatschappij 2013). This observation is to be expected since residential heating is a major source for PAHs.

PAHs comprise a range of molecules, seven of which are suspected to be carcinogenic (Vlaamse milieumaatschappij 2009). Of these, **benzo-a-pyrene (BaP)** is used as a reference for which the EU government has set a target value of 1 ng/m^3 (Vlaamse milieumaatschappij 2013). Since the concentrations of different PAHs seem to be strongly correlated, the BaP concentration is used as an indicator for the total PAH concentration (Vlaamse milieumaatschappij 2013; EEA 2013a).

A mechanism by which PAHs can influence cellular physiology is by influencing the **aryl hydrocarbon receptor (AhR)**. After translocation into cells, PAHs can bind nuclear receptors, including AhR in the cytosol resulting in translocation of the complex to the nucleus where it can modulate transcription of certain target genes. It is suggested that this AhR activation by PAHs precedes the proliferative, oxidative and carcinogenic effects observed with PAH exposure (Mesquita et al. 2014).

1.1.2.4. Biological components

In addition to previously discussed components, biological materials are also important PM components. These 'bioaerosols' may include **organic dust particles, *Actinomycetes* and other airborne microorganisms, microbial endotoxins, pollen and fungal spores** (Timm et al. 2009). Biological PM components of particular interest are endotoxins, discussed more extensively in section 1.3.

Bioaersosols are commonly investigated in occupational settings such as the farming industry (Eduard et al. 2001; Gladding et al. 2003; Heldal et al. 2003; Skórska et al. 1998) Occupational health effects, especially respiratory symptoms, have been reported during hay handling, in swine confinement houses and poultry farms (Timm et al. 2009). In these

environments, a variety of microbial components can cause inflammation in the respiratory system after inhalation (Douwes 2003; Eduard et al. 2001; Mandryk et al. 2000; Moore et al. 2004; Williams & Ownby 2005). Nonetheless, occupational exposure limits are commonly aimed at the concentration of a single contaminant, such as bacterial endotoxins (90 EU/m^3) (Michel et al. 1997). During the development of a granulocyte assay used to determine the total inflammatory potential of bioaerosols, Timm et al. (2009) found the strongest responses for bacterial endotoxin, fungal spores, β-d-glucan (a component of yeast cell membranes) and mesophilic *Actinomycetes*. The presence of these structures in PM could thus be one of the factors contributing to the harmful effects.

1.1.2.5. Ultrafine particles (UFP)

This fraction, rather than component, of PM has been suggested by literature to be crucial in evaluating the physiological effects of the whole PM mixture. UFPs are commonly derived from combustion processes, and have a variable chemical composition (Terzano et al. 2010). Despite this heterogeneity, it seems that for these tiny particles their **size** is the most important feature to determine their toxicity. Generally the smaller the size of the particles, the more harmful they may be to our health (de Hartog 2003; Donaldson et al. 2002). Compared to larger particles at similar mass concentrations, UFPs have a larger surface area, enhanced oxidative capacity, a greater inflammatory potential and higher pulmonary deposition efficiency (Oberdörster et al. 1995; Brown et al. 2001; Li et al. 2002; Chalupa et al. 2004; Daigle et al. 2003). Besides this, toxic compounds can adhere to their surface making them more toxic (Don Porto Carero et al. 2001). This adhesion leads to the ability of ultrafine particles to grow rapidly, forming larger complex aggregates, resulting in a very short UFP lifetime. Typically these aggregates remain part of $PM_{2.5}$ (C. A. Pope III & Dockery 2006).

As mentioned before, the ambient levels of PM are most often described in terms of its mass concentration. This, however, neglects ultrafine particles (UFPs) since they are far better described by their **number** than their mass. Nano-sized particles will make up only a small portion of the particulate mass but still might be very numerous. It appears their numbers might even exceed the numbers of larger particles in some urban and industrial areas (Terzano et al. 2010). This once again, shows the limitations of only monitoring mass concentrations of ambient PM.

Importantly, UFPs interact differently with our bodies than larger PM components. They can be breathed deeply into the lungs, reaching the alveolar spaces. Coming in contact with lung cells, UFPs appear to diffuse through the lipid cell membrane and gain access to the cytoplasm without being enclosed in a membrane vesicle (Geiser et al. 2005). As a result, they can penetrate alveolar epithelium and enter the pulmonary interstitium and vascular space, being absorbed directly into the **bloodstream** (Stearns et al. 1994).

1.1.3. Particulate matter in Europe and Belgium

1.1.3.1. Regulation of PM concentrations

PM is associated with very diverse and serious health effects. Research has supplied mounting evidence for some of these adverse relationships, which has caused governments globally to draft and implement laws aiming to reduce the emissions of primary and secondary PM sources. With the **2008/50/EC directive,** the European Union (EU) extended its air pollution regulations, restricting the maximum allowed PM concentrations in the member countries (EU 2008). These values and the stricter **WHO air quality guidelines** (WHO AQG) (WHO 2006) are listed in table 1.1. Nonetheless, long-term studies have shown correlations between morbidity and mortality with PM at concentrations well below the WHO recommended limit values (EEA 2013a), stressing the need for stricter legislation. However, research hasn't shown a threshold below which health effects can be avoided (WHO 2013), complicating the search for a good limit value. This highlights the need to gain more knowledge about the mechanisms through which PM causes its health effects, in order to shift to a more targeted approach.

Table 1.1: European directives and WHO AQG for PM concentrations

Organisation	PM fraction	Limit value	Time frame
EU	PM_{10}	40 µg/m^3	Annual
	$PM_{2.5}$	25 µg/m^3 (a)	Annual
	PM_{10}	50 µg/m^3 (b)	Daily
WHO	PM_{10}	20 µg/m^3	Annual
	$PM_{2.5}$	10 µg/m^3	Annual
	PM_{10}	50 µg/m^3	Daily
	$PM_{2.5}$	25 µg/m^3	Daily

(a) Target value

(b) Not to be exceeded more than 35 days per year

1.1.3.2. Current situation in Europe

Due to substantial efforts, emissions of the major air pollutants have declined over the last decade (EEA 2013a). Nevertheless, PM and ozone remain problematic pollutants in terms of effects on human health in Europe (EEA 2013a), with Belgian particulate air pollution being one of the highest of western Europe (Remy et al. 2010). According to the 2013 report of the European Environmental Agency (EEA) on European air quality, EU limit and target values were exceeded widely in 2011.

The stricter WHO guidelines for annual mean PM_{10} and $PM_{2.5}$ were exceeded at most of the monitoring stations across continental Europe. The situation seems particularly bad in urban

regions. 33% of EU urban population and 49% of the population in EEA-32 countries (the 32 countries represented in the European Environmental Agency) was exposed to PM_{10} concentrations above the EU limit value in 2011. Comparing the 2011 **PM_{10} concentrations** to the WHO guidelines, a staggering 88% was exposed to a PM_{10} concentration exceeding these limit values. The same EEA report also evaluates **$PM_{2.5}$ concentrations** in Europe. Due to limited $PM_{2.5}$ stations, it is problematic to make general conclusions. It seems that on average, between 2006 and 2011, $PM_{2.5}$ concentrations declined at traffic and other, mostly industrial type monitoring stations, and increased for urban and rural background stations. Nevertheless, there are several countries that registered an increasing trend of $PM_{2.5}$ annual mean concentrations at one or more stations types, including Belgium. It was estimated that in 2011, about 15% of the urban population in the EU and EEA-32 countries, was exposed to $PM_{2.5}$ concentrations at or above the level of the EU target value (25 $\mu g/m^3$), which will become a limit value in 2015. Figures 1.3 and 1.4 show the annual PM_{10} and $PM_{2.5}$ concentrations across Europe in 2011. These figures show that Belgium exceeds the WHO air quality guidelines for both fractions, and does not meet the $PM_{2.5}$ EU target value.

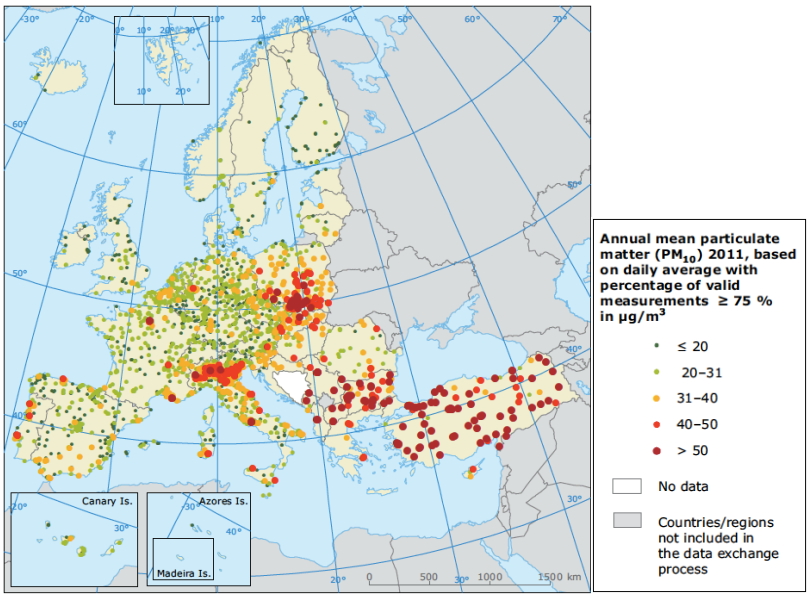

Figure 1.3: Annual PM_{10} concentrations in Europe in 2011. Dark red and red dots imply an exceedance of the EU limit value, while orange and light green dots imply exceedances of the WHO AQG. Reference: EEA (2013a)

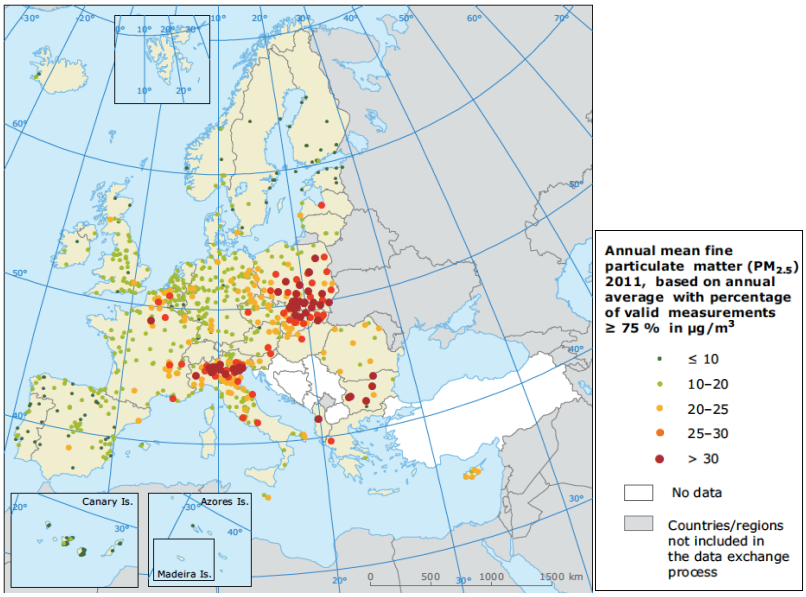

Figure 1.4: Annual $PM_{2.5}$ concentrations in Europe in 2011. Dark red and red dots imply exceedances of the EU target value while orange and light green dots imply exceedances of the WHO AQG. Reference: EEA (2013a).

1.1.4. Additional consequences

While the devastating effects of PM on our health are certainly the most severe, the repercussions of PM extend even further, as briefly outlined below.

Firstly, PM can have important environmental impacts, influencing the quality of water, soil and the ecosystems they host (EEA 2013a). Aquatic environments act as a natural sink of PM, as a result of atmospheric deposition and soil run off (Mesquita et al. 2014). For example, the main responsible contributor to the global distribution of PAHs in lakes, rivers, estuaries, and seas is atmospheric deposition (Mesquita et al. 2014). Heavy metals and toxic metalloids can also be an important threat to sensitive ecosystems, because of their environmental toxicity and their tendency to bioaccumulate (EEA 2013a).

Secondly, **climate forcing** is another environmental consequence of PM pollution Components contributing to global warming include carbon black, while other PM components have a cooling effect on climate. PM can also indirectly cause climate forcing by changing the properties of clouds, such as cloud reflectivity, cloud distribution, cloud formation, and precipitation (EEA 2013a).

Finally, indirectly because of all these effects, PM also has a profound impact on the **economy,** especially the consequences of PM on health. Premature deaths, increasing medical costs, reduced quality of life, and reduced productivity through lost working days pose a large economic burden with PM at its origin. Although epidemiological studies and risk analysis often only include severe health outcomes, affecting only a small portion of the population, the overall cost of less severe health impacts might be higher, since they affect a much larger portion of the population (EEA 2013b)

1.2. Health effects of PM exposure

The hazardous impact of air pollution became blatantly obvious and began to gain much attention a few decades ago when **episodes of extreme air pollution** caused strong effects on the population's health. The extreme fog in the Meuse valley in Belgium in December 1930 was one such example (Firket 1931; Nemery et al. 2001). Later in the 1990's, several **epidemiological studies** also found unexpected associations between daily mortality and daily changes in PM concentrations at low to moderate PM concentrations. Since then, a vast amount of epidemiological studies have been published discussing the health impact of exposure to PM, both in terms of mortality, which reflects a reduction in life expectancy, and morbidity, which reflects the occurrence of illness, including both minor effects such as coughing and more severe effects that require hospitalisation (EEA 2013a). Based on these observations mechanistic studies were set up, researching the underlying causes of these health effects. Clinical studies, animal and *in vitro* models, were applied to provide insight to the body's responses to PM. Today these mechanisms are still not fully elucidated and remain an important topic of research, together with the importance of the various PM components.

1.2.1. Effects on mortality

In estimating the effects of PM exposure to health, epidemiological studies often turn to mortality rates and correlate these to PM concentrations. Numerous articles have reported on the **short-term effects** of PM exposure. Together these have established that PM exposure, especially of fine particles, contributes to acute mortality (C. A. Pope III & Dockery 2006). Important examples of these short-term studies are the "Air pollution and health: a European approach" **(APHEA) study** and its follow-up APHEA-2. The latter found an increase in risk for total, cardiovascular and respiratory mortality of 1.2%, 1.5% and 1.2% respectively, with a 20 $\mu g/m^3$ increase in the ambient PM_{10} concentration (Analitis et al. 2006). Regarding these short-term studies, it is important to keep in mind that it is remarkable that statistical evidence for an effect on mortality could be found at all, since these only observed small effects (C. A. Pope III & Dockery 2006).

Studies investigating the **long-term effects** of PM exposure are much less common since they are costly and time consuming (C. A. Pope III & Dockery 2006). These **long-term studies** provide additional information on the degree of life shortening, pollution effects on longer term mortality rates, and the role of pollution in inducing or accelerating the progress of chronic diseases (McMichael et al. 1998). Examples of important studies of this kind were the **Harvard Six Cities** study and the study by the **American cancer society (ACS),** and their extended follow-up studies. Compelling evidence was provided from these studies on

mortality effects of long term fine PM air pollution(C. A. Pope III & Dockery 2006). Importantly, the observed mortality effects were much higher than in their short-term counterparts. The extended Harvard Six Cities study showed as increase in risk of mortality for all causes, cardiovascular causes and lung cancer of 16%, 28% and 27% respectively, for an increase in ambient $PM_{2.5}$ of 10 $\mu g/m^3$ (Laden et al. 2006). For the same exposure increment, the extended ACS study found an increase in risk of mortality for all causes, cardiovascular causes and lung cancer of 16%, 28% and 27% respectively, for an increase in ambient $PM_{2.5}$ of 10 $\mu g/m^3$ (Laden et al. 2006). For the same exposure increment, the extended ACS study found in increase in risk for all cause, cardiopulmonary, cardiovascular and lung cancer mortality of 6.2%, 9.3%, 12% and 13.5% respectively (Pope et al. 2004a; Pope III et al. 2002). Woodruff et al. (1997) specifically investigated **post-neonatal infant mortality** and found particle air pollution to be associated with mortality for all causes, respiratory causes and sudden infant death syndrome. They reported a near doubling of the risk of post-neonatal death because of respiratory causes for each 10$\mu g/m^3$ increase in $PM_{2.5}$ concentration.

1.2.2. Respiratory effects

Early research on the health effects of PM mainly discussed its **impact on the respiratory system** (Dockery & Pope 1994; Souza et al. 1998). Reductions in lung function and symptoms of bronchitis have been observed in children and adults upon exposure to PM, and often traffic related PM (Kelly & Fussell 2012). For instance, respiratory symptoms and illness in children, including cough, wheeze, bronchitis, runny nose, allergic rhinitis and decreases in lung function have been associated with traffic density (Frampton 2006). Additionally, the development of childhood allergies has also been implicated following PM exposure (Kelly & Fussell 2012). Besides this, a strong link between **asthma** and PM exposure has been demonstrated (Frampton 2006; C. Pope III & Dockery 2006). PM exposure has not only been associated with asthma exacerbations, and a subsequent increase in asthma related hospitalisations, but even with the onset of asthma (Kelly & Fussell 2012; Frampton 2006). For instance, DEP has been shown to cause asthma in susceptible people by contributing to allergen sensitization, an early phase in developing allergic asthma (Frampton 2006). Additionally, a similar association between PM exposure and chronic obstructive pulmonary disease (**COPD)** has been reported. Studies have shown a heightened severity of symptoms and hospital admissions for COPD as a result of PM exposure and again a role for PM in the development of COPD (Kelly & Fussell 2012).

The potential of PM to induce inflammation and oxidative stress is thought to lie at the root of these observed problems. Activated neutrophils and macrophages play a central role in the pathogenesis of COPD. These cells of the immune system are recruited to the airways in

case of inflammation and release proteases and reactive oxygen species (ROS) upon activation, which causes lung parenchymal destruction, leading to emphysema. Therefore, PM could enhance the progression of, or the risk for COPD by increasing neutrophil influx into the airways of people susceptible to this disease (Frampton 2006).

1.2.3. Cardiovascular effects

Next to effects on the respiratory system, epidemiological studies showed that PM could also have a profound influence on the circulatory system. PM exposure has been associated with ischemic heart disease, dysrhythmias and heart failure (Samet & Dominici 2000; Peters et al. 2001; Mann et al. 2002; Ware 2000; Pope et al. 2004b; C. Pope III & Dockery 2006).

These acute cardiovascular events are the result of underlying chronic pathologies, which can arise due to various factors, including PM exposure. Firstly, the link between PM exposure and **atherosclerosis** (thickening of artery wall and reduction of elasticity) has been investigated. It is thought that PM exposure promotes atherogenesis or the formation of atherosclerotic plaques, the progression of the already existing plaques, an increase in their vulnerability for rupture, and ultimately atherothrombosis (Franchini & Mannucci 2009; C. Pope III & Dockery 2006; Mills et al. 2009). These observations are assumed to be driven by a pro-inflammatory mechanism (Franchini & Mannucci 2009; Libby et al. 2002). Hypothetically, long-term chronic PM exposure brings about low to moderate inflammation, which initiates and accelerates atherosclerosis, while short term exposures contributes to the acute thrombotic complications resulting from rupture of plaques, clotting and precipitating acute cardiovascular or cerebrovascular events (hearth attack or stroke) (C. Pope III & Dockery 2006).

Furthermore, research suggests that PM exposure also promotes blood coagulation and **thrombus formation** in other ways. The prothrombic effects of PM include an increase in expression of tissue factor on endothelial cells, shown both *in vitro* and in *vivo*, and accumulation of fibrin and platelets on endothelial surfaces (Gilmour et al. 2005; Sun et al. 2008; Khandoga et al. 2004). Additionally, PM has been hypothesised to influence **vascular tone** and reactivity, as studies observed increased vasoconstriction and blood pressure, and an increase in circulating levels of vasoactive peptide endothelin in specific models (C. Pope III & Dockery 2006).

To explain the occurrence of health effects outside of the primary organ exposed to PM, two pathways, illustrated in figure 1.5, leading to the previously discussed cardiovascular effects were proposed. In the first pathway, termed the **"classical pathway"**, it is not the PM itself that causes direct damage to the cardiovascular structure, but the soluble pro-thrombotic and pro-inflammatory factors released into the bloodstream resulting from pulmonary inflammation (Seaton et al. 1995). The pulmonary response to PM leads to elevated levels in

the blood plasma of pro-inflammatory mediators and eventually systemic inflammation. Examples of these pro-inflammatory mediators include granulocyte-macrophage colony-stimulating factor and interleukins (IL) such as IL1β and IL6, which can be released upon interaction of PM with alveolar macrophages and airway epithelial cells (van Eeden et al. 2001; Fujii et al. 2002). Besides this "classical pathway", it has been proposed that inhaled, insoluble fine PM and UFPs can rapidly translocate into the circulation, crossing the alveolar-endothelial barrier, which is referred to as the **"alternative pathway".** When present in the bloodstream, these particles could interact with the vascular endothelium or cause local oxidative stress and pro-inflammatory effects similar to those seen in the lungs (Mills et al. 2009).

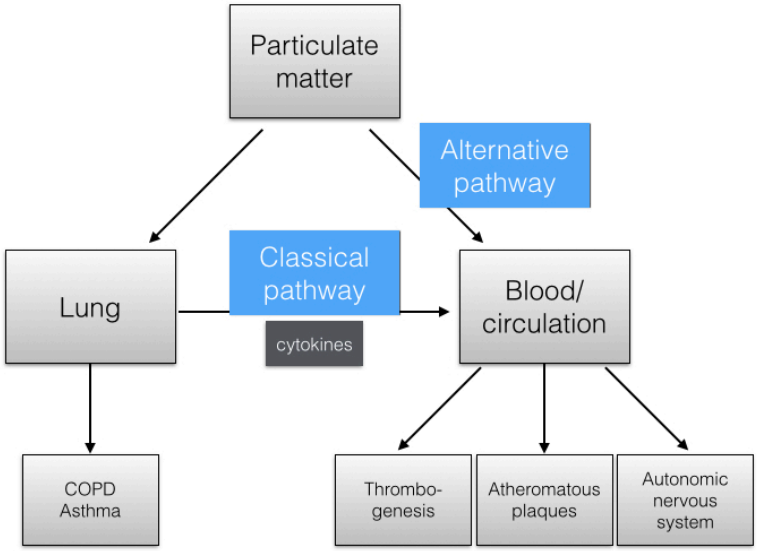

Figure 1.5: Classical and alternative pathway for cardiovascular effects of particulate matter.

1.2.4. Neurological effects

Although the respiratory and cardiovascular systems were the first organ systems researched for PM related health effects, accumulating evidence now points to the central nervous system (CNS) as an additional important target organ system (Oberdörster et al. 1995; Block et al. 2012; Block & Calderón-Garcidueñas 2009).

1.2.4.1. Epidemiological, post-mortem and clinical studies

Over the last decade, epidemiological studies have established a strong link between PM exposure and **Alzheimer's disease (AD), Parkinson's disease (PD) and stroke** (Block & Calderón-Garcidueñas 2009; Block et al. 2012). Additionally, PM exposure has also been associated with autism spectrum disorder, lower IQ in children, multiple sclerosis, decreased cognitive function and lower neurobehavioral development in the first four years of life (Block et al. 2012). These studies indicate a higher vulnerability to effects of PM in young life

stages, especially foetal life and early childhood, which are characterized by rapid growth, cell differentiation, organogenesis and network development in the brain (Block et al. 2012). These findings have been supported by clinical and post-mortem studies, which found evidence of neuro-inflammation, an altered immune response, blood-brain barrier (BBB) damage, protein accumulations associated with AD pathology, endothelial cell activation and brain lesions in children, young aldults and dogs upon exposure to heavily polluted air (Calderón-Garcidueñas, Mora-Tiscareño, et al. 2008; Calderón-Garcidueñas, Solt, et al. 2008).

1.2.4.2. Mechanisms

Although the exact mechanisms that drive the CNS pathology following PM exposure are not yet fully understood, it is expected that neuro-inflammation and oxidative stress again play a significant role, together with microglial activation and cerebrovascular damage (Genc et al. 2012). Nonetheless, important pathways responsible for cerebral vascular damage, neuro-inflammation and neuro-degeneration have been identified (Block & Calderón-Garcidueñas 2009). As seen with the cardiovascular system, systemic inflammation arising from the body's response to particles elsewhere in the body is an important mechanism driving PM-related CNS pathology (Block & Calderón-Garcidueñas 2009; Genc et al. 2012). Increased levels of **circulating cytokines** associated with systemic inflammation can enter the brain through diffusion and active transport (Dantzer et al. 2008), where they can cause neuro-inflammation, neurotoxicity and cerebrovascular damage (Qin et al. 2007; Perry et al. 2007; Ling et al. 2006; Manousakis et al. 2009).

Additionally, particles can also exert their adverse neurological effects following deposition from the bloodstream into brain. However, this requires **passage through the BBB**, which limits transport to the brain parenchyma. Nonetheless, several studies have shown PM entry into the brain (Calderón-Garcidueñas et al. 2002; Calderón-Garcidueñas et al. 2004; Peters et al. 2006) Active transport and a leaky BBB have been proposed as mechanisms granting $PM_{2.5}$ and UFPs access to the brain, while UFPs can also reach the brain parenchyma by diffusion (Oberdörster et al. 2004; Geiser et al. 2005). Furthermore, it seems that PM itself can alter physical and chemical barrier BBB function, including alterations in transporter expression and function and a decreased expression of various tight junction proteins (Hartz et al. 2008). A second major pathway, by which fine and ultrafine PM can be deposited into the CNS, is referred to as **the nasal pathway**. PM deposited on the olfactory epithelium can be internalized into dendrites of olfactory nerves and transported to the olfactory bulb by slow axonal transport (Elder et al. 2006; Oberdörster et al. 2004; Block & Calderón-Garcidueñas 2009; Block et al. 2012; Genc et al. 2012). An overview of these pathways is given in figure 1.6.

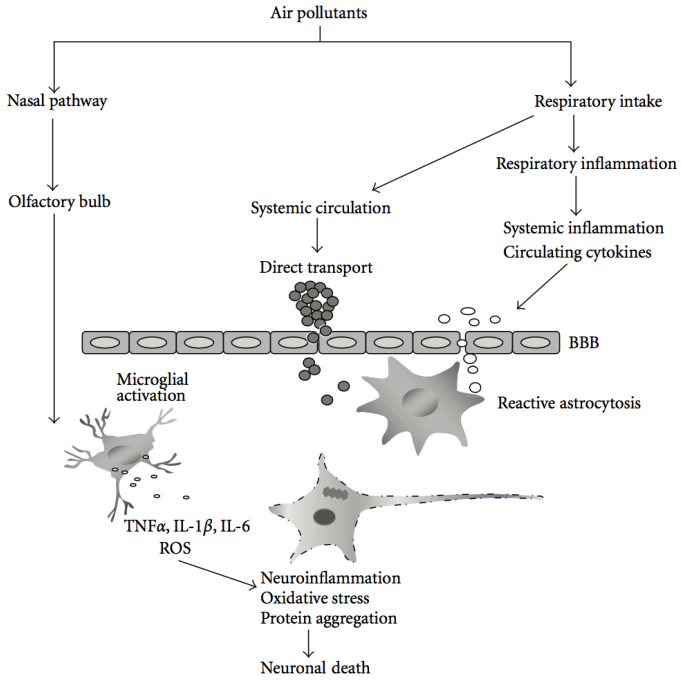

Figure 1.6: Key pathways involved in CNS pathology in response to PM exposure. Adapted from Genc et al., (2012)

Microglia, sometimes referred to as the macrophages of the CNS, are the predominant regulators of neuro-inflammation, and are one of the key players mediating the response to PM in the brain. PM can activate these cells either directly or indirectly through the induction of systemic inflammation or neuronal damage (Block et al. 2007). If excessive or chronic microglial activation occurs, this can trigger reactive microgliosis, where microglial activation leads to neuronal damage and vice versa, creating a cycle of neuronal death.

1.3. Endotoxin

1.3.1. Characteristics and structure of endotoxin

Potent pro-inflammatory biological PM components that have gained attention are endotoxins. Endotoxin can in fact refer to any microbial cell associated toxin, but mostly, as used in this dissertation, it refers to **lipopolysaccharide** (LPS) (Morgenstern et al. 2005). LPS is a major component of the cell wall of most Gram-negative bacteria. While smaller amounts of these molecules are shed during cell growth, huge amounts are released in the environment when the cells are dying off (Ryan 2008; Madigan et al. 2010; Liebers et al. 2008). Furthermore, because of their extreme resiliency, thermostability and relatively low sensitivity towards pH changes, these molecules are able to persist in the environment for long periods of time (Ding & Ho 2010). Consequently, this molecule is as abundant in our environment as the bacteria from which they originate.

LPS is an **amphipathic** molecule with a very hydrophobic lipid group (Lipid A), covalently bound to a long and complex polysaccharide tail. The LPS structure is represented in figure 1.7. **Lipid A**, responsible for most of the biological activity, consists of two phosphorylated glucosamines, coupled to various fatty acids. This part anchors the LPS molecule to the bacterial cell membrane and provokes an immune response. The long hydrophilic polysaccharide tail consists of a conserved **core polysaccharide**, and variable **O-specific side chains**, which form the O-antigen. LPS molecules have a net negative charge and tend to form large aggregates in aqueous solution. As a result of their hydrophobic character, they have a strong affinity for hydrophobic materials such as plastics (Ryan 2008).

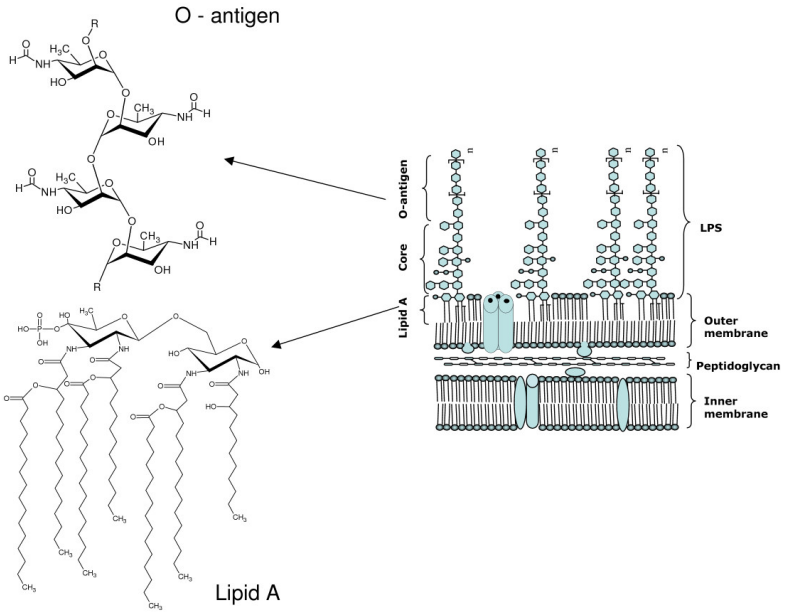

Figure 1.7: Structure of Lipid A, the O-antigen and a schematic illustration of the Gram-negative cell wall with associated LPS. Reference: Cardoso, Macedo, Azevedo & Oliveira (2006)

1.3.2. Biological response to endotoxin

Our innate immune system has been trained to detect endotoxin in picomolar concentrations (Gioannini et al. 2004), as it represents a pathogen-associated molecular pattern (PAMP), alerting the body of an invasion of Gram-negative bacteria. The receptor responsible for this recognition is **Toll-like receptor 4** (TLR4), a member of the TLR family, able to interact various PAMPs (Lu et al. 2008). Activation of TLR4 by endotoxin will trigger an inflammatory defence against the invading bacteria. On the down side, this powerful response can induce massive inflammation and cause acute sepsis or chronic disorders when excessive activation occurs (Lu et al. 2008). Due to the strong pro-inflammatory character and the ubiquitous nature of LPS or endotoxin, this component has gained much attention in several research fields, including the cellular pathways it activates, the health effects it causes and its possible role in PM toxicity. However, because of some difficulties in this research area, as discussed in section 1.3.3.5, exposure to airborne endotoxins remains a challenging topic, in need of more research.

1.3.2.1. LPS recognition

TLR4 is the primary receptor responsible for LPS recognition and its cellular response. The extracellular LPS recognition is chaperoned by several accessory proteins that facilitate the response to LPS, including **LBP** (LPS binding protein), **CD14**, and **MD-2** (myeloid differentiation factor 2) (Jerala 2007).

The most conserved part of the highly variable LPS structure, the Lipid A moiety, is targeted by the proteins responsible for LPS recognition (Jerala 2007). However, even in this most conserved portion, structural diversity exists including variability in length and number of the acyl chains and the conjugation of the phosphate group of Lipid A (Jerala 2007; Ding & Ho 2010). It has been found that sometimes even subtle variations in the structure can have a large impact on ability to stimulate the TLR4 receptor and consequently on the resultant immunological response (Paramo et al. 2013).

1.3.2.2. Intracellular TLR4 signalling pathways

In trying to elucidate the signalling pathway downstream of TLR4 activation, the first adaptor characterised as an essential component for activation of the innate immunity by all the TLRs was **MyD88** (myeloid differentiation primary response gene 88) (Takeda & Akira 2004). Following the discovery of MyD88, several other components of the associated pathway were identified, including members of the interleukin-1 receptor-associated kinase (IRAK) family and tumour necrosis factor receptor associated factor 6 (TRAF6) (Lu et al. 2008) Activation of MyD88 and the associated proteins, resulted in activation of nuclear factor

kappa-B **(NFκB)** and the mitogen-activated protein kinase (MAPK) pathway leading to the production of **pro-inflammatory cytokines** (Lu et al. 2008). Later on, studies using MyD88 knockout mice found that although these mice did not produce pro-inflammatory cytokines after stimulation with any of the TLR ligands, stimulation of TLR4 did lead to a delayed activation of NFκB and JNK (c-Jun N-terminal kinase). This, however, did not result in the production of pro-inflammatory cytokines (Kawai et al. 1999). These findings suggested the existence of one (or more) MyD88-independent pathways that lead to NFκB and JNK activation, which was demonstrated in further studies (Takeda & Akira 2004). This MyD88 independent pathway was shown to be responsible for induction of **Type I interferons** and interferon-inducible genes (Lu et al. 2008)

Since excessive activation of the TLR4 pathways can have very detrimental effects, the innate immune system has evolved to integrate various systems that can regulate TLR4 activation and its downstream signalling cascades. This **regulation** can occur at multiple levels of the TLR4 pathways, by inhibition or activation, but also by influencing transcription and translation of the pathway components (Lu et al. 2008). A schematic representation of the most important components of LPS recognition and the TLR4 pathways is given in figure 1.8.

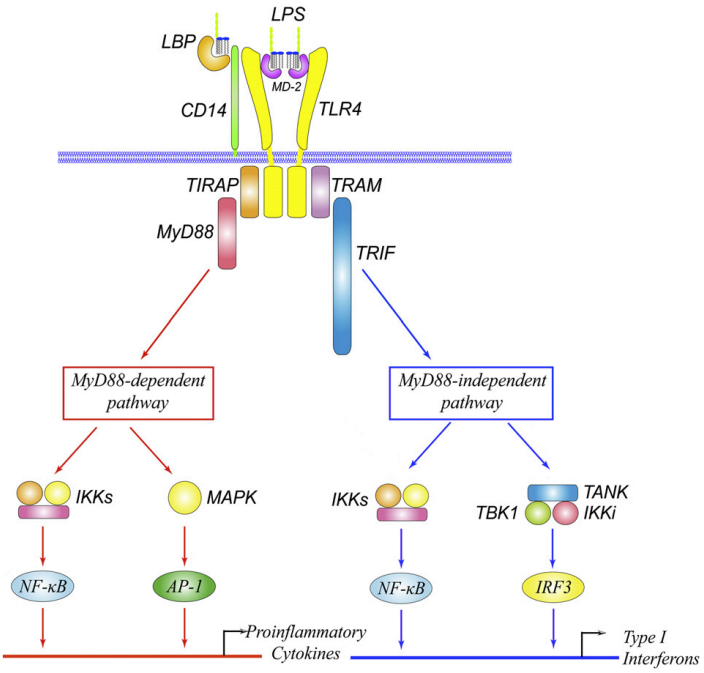

Figure 1.8: Schematic representation of LPS recognition and intracellular TLR4 signalling pathways, with some important components indicated. Components previously not introduced include TIRAP (Toll-interleukin 1 receptor domain-containing adapter protein), TRIF (TIR-domain-containing adapter-inducing interferon-β), TRAM (TRIF-related adaptor molecule), IKKs (IκB kinases), TANK (TRAF associated NFκB activator), TBK1 (TANK binding kinase 1), AP-1 (activator protein 1) and IRF3 (Interferon regulatory factor 3). A more detailed overview of the TLR4 signalling cascades, including these components, can be found in Lu et al. (2008). Adapted from Lu et al. (2008)

1.3.3. Endotoxin as a component of PM

Over 30 years, health effects of endotoxin inhalation have mostly been studied in occupational settings and in house dust (Rylander 2002). However, the suggestion that endotoxin might be an important pro-inflammatory agent in outdoor PM (Becker et al. 1996; Becker & Soukup 2003; Dong et al. 1996; Long et al. 2001), gave rise to the research of ambient endotoxin concentrations in other settings, such as outdoor air.

1.3.3.1. Sampling for airborne endotoxin

A primary step in every study investigating airborne endotoxins, involves the collection of air samples. However, due to the lack of a well-established and **standardized protocol**, this remains a challenging task. Moreover, only a few studies have put effort in optimization of sampling methods for endotoxin (Kujundzic et al. 2006). Although some standard methods suggested by standardization or occupational health institutes exist, none of these protocols have been adopted at an international level. Additionally, they differ between each other, leave out important details and are primarily aimed at sampling of endotoxin in occupational environments which can contain endotoxin levels several thousand times higher than non-exposed environments such as outdoor air (Madsen 2006; Duquenne et al. 2013) Occupational studies investigating endotoxin levels most often use pyrogen-free glass fibre **filters**, combined with a three-piece closed-faced filter cassette (Duquenne et al. 2013). Some studies investigating endotoxin concentrations in outdoor air have used impaction based filter samplers like the Anderson impactor (Heinrich, 2003; Huang, Cheng, Lee, Huang, & Chan, 2002; Schins et al., 2004; Traversi, Alessandria, Schilirò, & Gilli, 2011) or the Harvard impactor (Long et al. 2001; Ning et al. 2000; Morgenstern et al. 2005; Nilsson et al. 2011), combined with various types of filters. The choice of sampler and filter material can significantly influence the efficiency of endotoxin sampling as the physical performance of various samplers differs, and the filter material will affect the ability to release endotoxins during extraction from the filter. An additional source of variation between different studies is the applied method for extraction of the PM from the filters (Kujundzic et al. 2006; Duquenne et al. 2013) Moreover, the true efficiency of endotoxin recovery by extraction from filter is not well known. Furthermore, substances released from the filter material during extraction (Milton et al. 1990) or agents improving extraction efficiency, can possibly interfere with downstream assays such as endotoxin quantification (Duquenne et al. 2013). Another drawback in the use of filter based sampling is the lack of information regarding the biological efficiency, i.e. effect of the residence time on the filter while sampling, which has never been tested (Duquenne et al. 2013).

An alternative to filter based methods is the use of a **liquid impinge**r type sampler such as the all glass impinger 30 (AGI-30) or the biosampler. This sampling technique is based on the induced collision of airborne particles with an agitated surface of collection fluid, concentrating the particles in the fluid (Duchaine et al. 2001). This technique offers the advantage of eliminating the extraction process, minimizing recovery artefacts (Kujundzic et al. 2006) and losses of endotoxin during filter extraction (Duquenne et al. 2013), while providing a liquid sample that can be readily used for analysis, such as endotoxin quantification. An important disadvantage of the use of liquid impingers is the risk for evaporation and loss of collected particles by re-aersolation (Lin et al. 1999; Han & Mainelis 2012). Some studies have evaluated the efficiency of liquid impingers to collect endotoxins, compared to filter based sampling methods. Overall, these studies showed higher endotoxin levels in samples collected by the liquid impinger type sampler (Duchaine et al. 2001; Kujundzic et al. 2006; Stephenson et al. 2004; Zucker & Müller 1998) supporting the use of liquid impingers for endotoxin sampling.

A novel sampler type, the **Coriolis® µ**, which shows some resemblance to the liquid impinger, was used in this project. This sampler was designed specifically for the collection of biological particles larger than 0,5 µm (D_{50} < 0,5 µm), into a liquid sample. The applied principle for sampling is shown in figure 1.9.

The Coriolis is suggested to be adapted to any specific microbiological research area, including endotoxins. The ability of the sampler to efficiently collect endotoxins was validated by preliminary data of the Réseau National de Surveillance Aérobiologique (RNSA) laboratory in France (Bertin technologies 2008; Bertin technologies & Réseau National de surveillance Aérobiologique 2009). Currently, this relatively new technique has not been widely applied, and implementation in other studies evaluating airborne endotoxin concentrations could not be found. Currently, the application of the Coriolis micro in research has been restricted to the sampling of bacteria in specific environments (Bonifait et al. 2014; Bellanger et al. 2012; Le Goff et al. 2010), while showing to be promising as a sampling technique in comparison to other samplers (Langer et al. 2012; Blais Lecours et al. 2012; Ahmed et al. 2013).

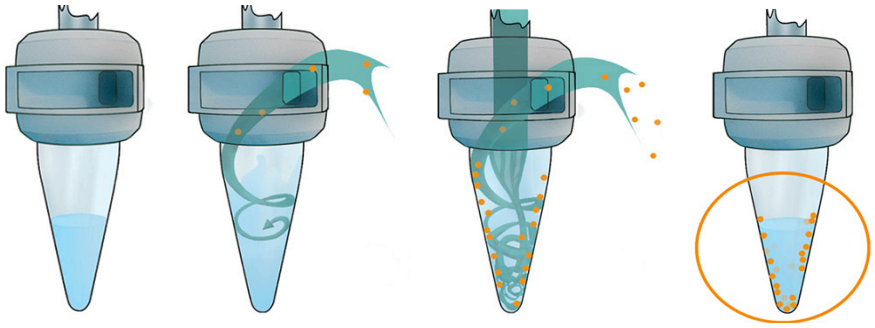

Figure 1.9: Scheme clarifying the Coriolis® µ air sampling principle. A sterile cone containing the collection fluid is screwed into the sampler device. During sampling, air is sucked into the collection cone and suspended particles are pulled into the collection fluid, through the centrifugal force of the swirling air. After collection, the particles are contained inside the cone. Reference: Bertin technologies (2008)

1.3.3.2. Airborne endotoxin concentrations

Among numerous studies quantifying endotoxin in PM samples, a few were aimed specifically to characterise the **spatial variation** of endotoxins in cities and rural and other areas (Nilsson et al. 2011; Morgenstern et al. 2005; Traversi et al. 2011; Menetrez et al. 2009; Mueller-Anneling 2004). Some of their findings are represented in table 1.2. An important first observation made in several studies is that higher endotoxin concentrations are present in the larger fractions of PM (Nilsson et al. 2011; Morgenstern et al. 2005; Guastadisegni et al. 2010; Mueller-anneling 2004). However, some studies do not support this observation, finding higher endotoxin concentrations in the smaller fractions (Kroll et al. 2013; Behbod et al. 2013). Furthermore, analysis of the spatiotemporal patterns of ambient endotoxin concentrations showed associations with humidity and season, measuring higher concentrations in spring and summer (Carty et al. 2003). A link between traffic density and endotoxin concentrations could not be found (Morgenstern et al. 2005; Guastadisegni et al. 2010).

1.3.3.3. Determination of endotoxin concentrations

The golden standard for endotoxin quantification is currently the **Limulus amoebocyt lysate (LAL) assay**, being widely applied over the last 30 years, primarily in the pharmaceutical and healthcare industry. As the name suggests, this assay is based on the lysate of amoebocytes of the Horseshoe crab (*Limulus polyphemus*), which contains coagulation factors including three serine protease zymogens, Factor C, Factor B and the pro-clotting enzyme, and a clottable protein, coagulen, which interact in a cascade reaction leading to coagulation upon activation by LPS (Muta & Iwanaga 1996; Ding & Ho 2010; Lonza 2009).

Table 1.2: Ambient endotoxin concentrations described in studies investigating spatiotemporal patterns of airborne endotoxins.

Site	Fraction	Mean (EU/m3)	Standard deviation (EU/m3)	Minimum-maximum (EU/m3)	Study by
Stockholm, Sweden (urban)	PM10	0.050(*)	0.025	0.020-0.107	Nilsson et al., (2011)
	PM2.5	0.015(*)	0.017	0.005-0.064	Nilsson et al., (2011)
Munich, Germany (urban)	PM2.5	0.020 (*)		0.009 - 0.213	Morgenstern et al., (2005)
	PM10	0.081(*)		0.041 - 0.141	Morgenstern et al., (2005)
Torino, Italy (urban)	PM10	0.512	0.043		Traversi et al., (2011)
Riva, Italy (rural)	PM10	0.331	0.137		Traversi et al., (2011)
Fiano, Italy (rural)	PM10	1.424	0.100		Traversi et al., (2011)
California (various)	PM10	0,44 (*)		0.03- 5.44	Mueller-Anneling (2004)
Denver, CO (urban)	PM2.5	0.0057			Menetrez et al. (2009)
Orange County, CO (rural)	PM10	0.051			Menetrez et al. (2009)
	PM2.5	0.023			Menetrez et al. (2009)

(*) Geometric mean

Factor C, sensitive to LPS, is the first step in the coagulation cascade and will activate Factor B upon LPS stimulation. Factor B will in his turn activate the pro-clotting enzyme, which in turn will trigger the clotting of coagulen. The LAL assay is based on this principle, detecting the presence of LPS by exploiting the activity of the pro-clotting enzyme to form a gel clot, creating a turbidimetric assay, or alternatively to cleave a chromogenic substrate, creating a chromogenic assay. Figure 1.10 gives an overview of assay principle. Notwithstanding the popularity of the assay, it does have some important **disadvantages**, including a differential sensitivity due to batch-to-batch variances in LAL preparations, various interfering substances, problems with specificity, and overharvesting of the horseshoe crab leading to an alarming drop in their population. An important specificity issue is caused by yeast 1-3 β-D glucans, which stimulate the cascade through Factor G, an activator of Factor B (Lonza 2009; Ding & Ho 2010). Furthermore, since the assay is based on the interactions of various enzymes, the pH, temperature, and ionic strength can significantly influence the activity of

these enzymes and therefore the end result. Furthermore, the quantification of endotoxins from environmental samples, such as with PM analysis, may contain various unknown variables which may interfere with the enzyme cascade

These issues generated a growing need for a more reliable, sensitive and specific assay to replace the LAL, which could be produced without the use of animal tissues. This led to the development of the **recombinant Factor C (rFC) assay**, a simple, rapid specific and sensitive assay for the detection of LPS (Ding & Ho 2010), based on a recombinantly produced Factor C. Upon stimulation by trace levels of LPS, the proenzyme rFC gains full enzymatic activity to hydrolyze a synthetic substrate, such as a fluorogenic substrate, as illustrated in figure 1.10. A comparison of the rFC and LAL assays under the same assay conditions showed a lower background reading and a more sensitive response to endotoxin of the rFC assay (Ding & Ho 2010; Thorne et al. 2010) Furthermore, since the rFC assay uses only one enzyme, interferences by inhibiting or activating substances and/or conditions are substantially reduced. Furthermore, the production of the assay does not suffer from batch-to-batch or seasonal variance and reduces the ecological burden on the horseshoe crab. However, because of the relatively recent introduction of this assay, to our knowledge no studies have investigated the performance of the rFC assay on urban PM samples.

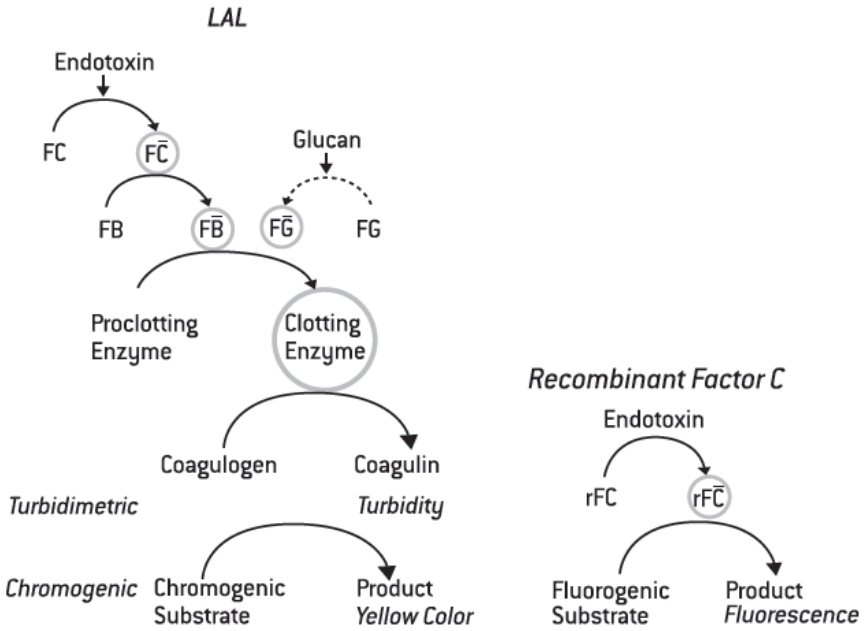

Figure 1.10: Cascade reactions involved with the LAL and rFC assays and their end results. Reference: Lonza (2009)

1.3.3.4. Health effects of endotoxin

Due to the strong pro-inflammatory character of endotoxin, several studies have aimed to determine whether or not the endotoxin level in PM is an important factor contributing to the pro-inflammatory capacity of PM mixtures. Results of several examples of these studies are listed in table 1.3. Some studies indicated that **synergistic effects** were likely to influence PM toxicity, for example between endotoxin and metals. It was suggested that metals could act as inducer of toxic effects, while most of the pro-inflammatory responses were due to endotoxin (Alfaro-Moreno et al. 2002). Other studies demonstrated the effect of endotoxin as a priming agent, altering the response to future exposures (Degobbi et al. 2010).

Since the effects of endotoxin are mediated by **TLR4**, the ability of PM to interact with this type of receptor has been investigated, as shown in table 1.3. However, TLR4 activation does not necessarily reflect activation by endotoxin, since also metals such as nickel, cobalt and palladium (Rachmawati et al. 2013), and danger-associated molecular patterns like heat shock protein 70 resulting from cell damage (Becker, Dailey, et al. 2005; Lafferty et al. 2010) can activate TLR4. Additionally, PM can also alter the ability of TLRs to respond to its ligands, by priming the cells for future stimuli (Bauer et al. 2012) or affecting TLR receptor expression (Becker, Mundandhara, et al. 2005; Williams et al. 2007).

Although endotoxin exposure is often discussed in a context of adverse health effects, some **beneficial effects** have also been described, especially in the case of allergy development (von Mutius et al. 2000; Braun-Fahrländer et al. 2002; Eduard et al. 2004) and atopic asthma (Remes et al. 2003; Rylander & Michel 2005) in early life, and in a lesser extent concerning a decrease in cancer risk (Lange 2000; Mastrangelo et al. 2005). These studies support a role for exposure to endotoxins in the development of an intact immune system, reducing the risk for atopic disease (Liebers et al. 2008; von Mutius et al. 2000; Liu & Redmon 2001; Wong & Chow 2008) and in compliance with the hygiene hypothesis. It seems likely that the beneficial or adverse character of the response to endotoxin exposure not only depends on the dose, but also on timing of exposure, co-exposure with other environmental factors, and genetic predisposition (Degobbi et al. 2010; Vandenbulcke et al. 2006).

Table 1.3: Studies investigating contribution of endotoxin in PM

Reference	Biological system	Exposure to	Biomarkers	Observation
Lai et al. (2013)	Three murine models	Low dose inhaled endotoxin	Gene expression profiling	Similar gene expression patterns in all three models. Activation of genes and pathways important in asthma and COPD.
Osornio-Vargas et al. (2003)	J77A.1 cells [a]	PM10 en PM2.5	Cytotoxicity Tumour necrosis factor α (TNFα) and IL-6	All samples induced cytotoxicity. $PM_{2.5}$ samples showed highest cytotoxicity and this was not dependent of endotoxin concentration PM_{10} samples that showed the highest levels of endotoxin, elicited the strongest cytokine induction, and this was endotoxin dependent.
Finnerty et al. (2007)	Mice	PM_{10} and PM_{10} + endotoxin	Pulmonary histology Plasma and pulmonary TNFα and IL-6	Mild to moderate pulmonary inflammation was observed. Endotoxin enhanced the inflammatory response, and triggered a systemic effect. Observed effects were not due to endotoxin alone.
Dong et al. (1996)	Rat alveolar macrophages	Urban air	TNF α, IL1, IL6 and CINC [b], MIP-2 [c]	Cytokine expression was prevented by endotoxin neutralising factor.
Ning et al. (2000)	Murine alveolar macrophages	$PM_{2.5}$ with trace endotoxin	TNFα and MIP-2	MIP-2 production was 28 fold higher than predicted by endotoxin content alone, suggesting a synergistic effect with other components. Neutralisation of endotoxin caused decrease in production of 80% for TNFα and 40% for MIP-2
Huang et al. (2002)	RAW 264.7 [a]	PM_{10} and $PM_{2.5}$	TNFα	TNFα expression was associated with endotoxin content. Endotoxin neutralisation lead to 42% and 32% decrease in PM_{10} and $PM_{2.5}$ induced TNFα production
Behbod et al. (2013)	Healthy adults	Concentrated PM_{10} and $PM_{2.5}$	Sputum cell counts, venous blood total leukocytes, neutrophils, IL-6 and CRP [d]	Coarse and fine PM independently induced acute systemic inflammatory responses. Endotoxin contributed to the inflammatory role of PM.
Bonner et al. (1998)	Rat alveolar macrophages	PM_{10}	PGDF [e] receptor and IL1-β	Metals and endotoxin present in PM induced IL-1β and PDGFα-receptor expression.
Monn & Becker (1999)	Human monocytes	PM_{10} and $PM_{2.5}$	IL 6 and IL8 production Cytotoxicity	Significant toxicity and cytokine production by PM_{10}, not by $PM_{2.5}$. A metal chelator inhibited cytotoxicity, but not cytokine production. Cytokine production was completely inhibited by neutralising endotoxin, and cytotoxicity was partly inhibited.

Reference	Biological system	Exposure to	Biomarkers	Observation
Schins et al. (2004)	Rats	$PM_{2.5-10}$ and $PM_{2.5}$	Glutathione, TNFα, lactate dehydrogenase, MIP-2 and neutrophils in broncho-alveolar lavage fluid, TNFα and IL-8 production from whole blood	PM_{10} induced neutrophilic inflammation in the lungs. No severe lung toxicity was observed. Effects of samples were associated with endotoxin content and the ability to induce TNFα and IL8 in whole blood. Metals did not explain the observed effects.
Guastadisegni (et al. 2010)	RAW 264.7 (a)	$PM_{2.5-10}$ $PM_{2.5-0.1}$	Arachidonic acid TNFα and IL-6	PM_{10} elicited the largest responses. Pro-inflammatory response was related to the endotoxin and metal content. A metal chelator inhibited the release of arachidonic acid, while an endotoxin neutralising factor partially inhibited TNFα production.
Manzano-Léon et al. (2013)	THP-1 (f) and J774A.1 (a) cells	PM fractions separated by source	TNFα and IL-6 expression	Pro-inflammatory effects were strongly correlated to the fraction containing elements linked to soil and endotoxin. Endotoxin added to the effects but did not explain them all.
Imrich et al. (1999)	Rat and human alveolar macrophages	Urban PM, concentrated $PM_{2.5}$ Priming with endotoxin	TNFα production	LPS priming amplified TNFα production in response to $PM_{2.5}$ and urban PM, in both models.
Long et al. (2001)	Rat alveolar macrophages	$PM_{2.5,}$ priming with endotoxin	TNFα production	Significant induction of TNFα with unprimed macrophages. Priming further increased the observed effects.
(Becker et al. 2002)	Transfected CHO cells (g)	$PM_{2.5-10}$	TNFα and IL6 production	TLR2 and TLR4 contributed to response to $PM_{2.5-10}$
(Shoenfelt et al. 2009)	Murine peritoneal macrophages (h)	$PM_{2.5}$ PM_{10}	TNFα and IL6 production	Secretion of cytokines was induced by a TLR2-dependent mechanism in response to $PM_{2.5}$, high in redox metals and low in endotoxins, and by a TLR4-dependent mechanism in response to PM_{10}, high in endotoxins.

(a) Murine monocyte/macrophage cell line
(b) Cytokine induced neutrophil chemoattractant
(c) Macrophage inflammatory protein 2
(d) C-reactive protein
(e) Platelet derived growth factor
(f) Human monocyte/macrophage cell line
(g) Chinese hamster ovary cells transfected with CD14, TLR2/TLR4
(h) isolated from $TLR2^{-/-}$, $TLR4^{-/-}$, and $MyD88^{-/-}$ mice

1.3.3.5. Problems with endotoxin research

Although the investigation of the biological effects of PM already is a challenging task, research on the contribution of endotoxin therein poses some additional difficulties. Consequently, studies have shown varying results, rendering it difficult to make general conclusions.

Firstly, the **lack of a standardized and optimized protocol** at an international level for sampling and analysis of airborne endotoxin is an important obstacle. Moreover, details of the used materials, methods and even reagents in some cases, can be insufficiently stated in published studies, leaving much to the interpretation of the reader. As a result, a great variation exists among the used protocols and methods from one study to another (Duquenne et al. 2013; Spaan et al. 2007; Liebers 2006).

Furthermore, the immunological response elicited in response the endotoxin challenge is strongly dependent on the biological system. It can be expected that with ***in vitro* research**, the observed immunological response is strongly dependent on the used cell line and the expressed receptor levels, resulting in a variable sensitivity towards certain compounds such as endotoxin (Allermann & Poulsen 2002; Sharif et al. 2007). Additionally, when investigating endotoxin effects *in vivo,* the use of **mice** is not an optimal model for estimating effects on humans. Studies have reported humans as the most sensitive model of all models for endotoxin challenge (Martich et al. 1993; Copeland et al. 2005), with an effective dose at 4 ng/kg bodyweight (Boujoukos et al. 1993; Martich & Danner 1991; Martich et al. 1993; Suffredini et al. 1999). In a study by Copeland et al. (2005), it was reported that exposure to a 250 times greater endotoxin concentration was necessary to obtain a similar response level of plasma IL-6 in mice as in humans. It should be noted that studies using LPS inhalation in healthy volunteers as model for airway inflammation, higher doses were required, but these could be reduced if delivery to the lung was enhanced. An example is the study of Janssen et al. (2013), which reported a dose of 2µg LPS sufficient to induce significant neutrophilic airway inflammation.

Moreover, when investigating health effects of endotoxin inhalation *in vivo*, the dose and timing of exposure, other environmental factors and genetic predisposition all influence the responses elicited in the study object increasing the amount of variables that need to be taken in to consideration when analysing the obtained results and decreasing the reproducibility of the study (Vandenbulcke et al. 2006).

Chapter 2.
Introduction to experimental work

Air quality is the main research theme of the Department of Bioscience Engineering at the University of Antwerp. My promotor, Sarah Lebeer, only recently joined the department and is responsible for starting up research on microbiological and immunological aspects of air pollution and PM. Serena Moretti, my supervisor, is the first PhD student on this topic. Therefore, my master thesis project was aimed at the evaluation and optimization of several methods previously not applied in PM research and to estimate their feasibility and added value. Hereto, significant effort was put into the development or adaptation of protocols for the determination of the pro-inflammatory capacity of PM samples and the relative importance of microbial endotoxins therein, together with gaining experience in working with this complex sample type. An overview of the used methods based on the aspects of PM research they provide insights on can be found in figure 2.1.

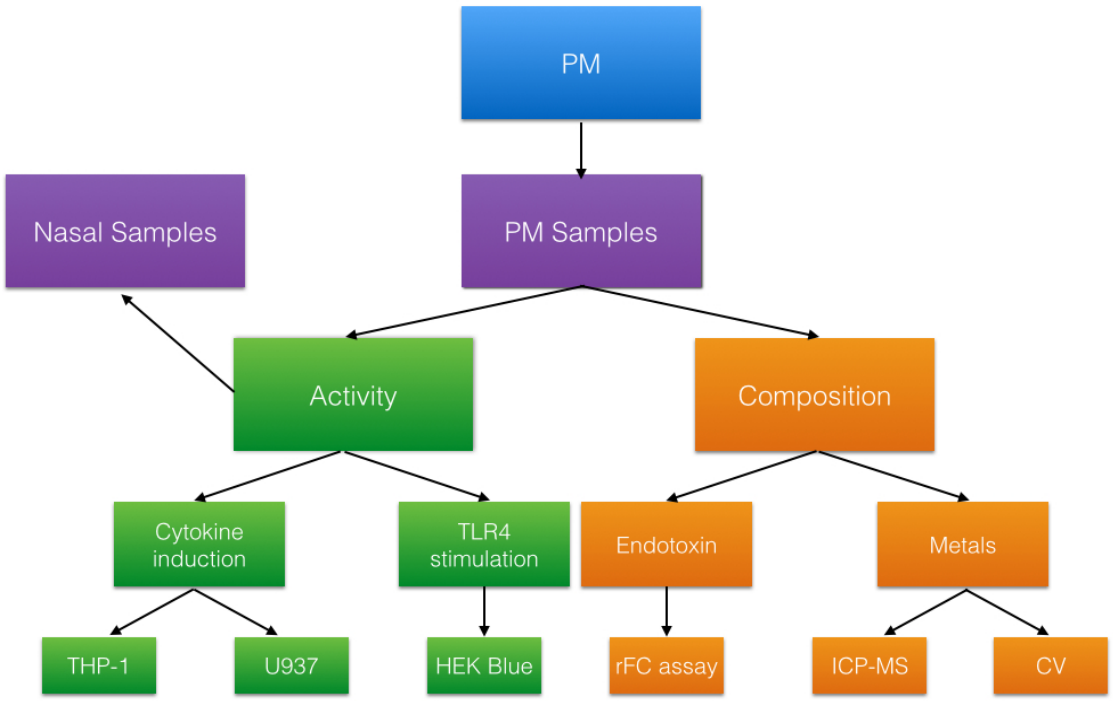

Figure 2.1: Overview of techniques used in the project

However, this project encompassed much more than the use of these experiments according to a certain protocol. For instance, the sampling and rFC assay protocols underwent several alterations, carefully considering the advantages and disadvantages of every implemented change. Furthermore, for the *in vitro* analysis of the pro-inflammatory capacity and endotoxin activity, several important skills had to be acquired even before starting the experiments. A good training and experience are indispensable when working with cell cultures, an experimental resource broadly applied in various research fields. The application of cell cultures does not restrict itself to the use of the cells in an experiment. It comprises the initiation, maintenance by growing and splitting the cells while keeping them free of contamination, and the preservation for long-term storage of a cell line. For each cell line, numerous variations exist in how these elements are carried out, and each lab has its own practices to avoid contaminations and ensure a safe working environment. During this project, it was possible to visit several labs and get accustomed with various working techniques, providing different views to the important aspects of cell culture. This knowledge proved to be vital when setting up the cell culture facility at the lab of Prof. Sarah Lebeer (ENdEMIC, Department of bioscience engineering, University of Antwerp). My encounters with various cell culture labs, together with my research for the purchase of the necessary equipment and products provided me with a deeper understanding of the technique. Furthermore, a good understanding of the possibilities and limits of certain techniques such as qRT-PCR was necessary in generating qualitative results. To this end, a training course was attended, provided by Life technologies™, highlighting the versatility of qRT-PCR, how to use the technique to its fullest potential and the essential prerequisites for publication, however often neglected (Bustin et al. 2009). Additionally, because of the multidisciplinary character of the project, several techniques mostly associated with chemical analysis were encountered and integrated with bio-molecular techniques. Furthermore, the experiments that were performed at or by different labs allowed a positive social interaction and collaboration with other researchers in different areas of expertise. Although picking up these competences do not directly lead to tangible data, this is in all probability the most important result of this project.

Chapter 3.
Sampling for airborne PM

3.1. Introduction

As was discussed in chapter 1, a range of sampling methods has been applied for the collection of PM and the airborne endotoxins it contains. In this project the Coriolis® μ air sampler, designed for the collection of bio-aerosols was used. To our knowledge the use of this air sampler has not yet been reported in literature for the collection of outdoor environments. Therefore, the sampling method needed significant optimization, which was one of the most important aims of this project.

3.2. Materials and methods

3.2.1. Use of the Coriolis® μ air sampler for outdoor PM collection

3.2.1.1. Sampling sites

At four sampling sites, each with a different character, PM was collected with the Coriolis® μ air sampler in order to obtain samples with a variable PM composition. The sites included Borgerhout (51° 12' 34.9" N, 4° 25' 54.3" E), the Antwerp Harbour (51° 16' 49.4" N, 4° 19' 50.0°E), Hoboken (51° 10' 12.7" N, 4° 20' 27.3" E) and Dessel (51° 14' 3.1" N, 5° 9' 48.3" E). The latter was only included on some sampling dates due to the travelling feasibilities and was intended to serve as a control for a less polluted area. Figure 3.1 indicates the sampling locations respectively on a map of Antwerp and surroundings.

Figure 3.1: Map of sampling locations. Location A: Harbour, location B: Borgerhout, location C: Hoboken, location D: Dessel. Reference: Google (2014)

3.2.1.2. Sampling preparations

Due to the ubiquitous nature of endotoxins together with their resistance to various treatments, care should be taken to avoid endotoxin contamination of the sampling materials. All used materials should be pyrogen-free and appropriate controls should be in place to test for contaminations. Glassware (borosilicate grade) used for sample collection, storage and endotoxin analysis, was dry heat sterilized for 3h at 250°C. The collection cones were dish washed, filled with 0.5M sodium hydroxide, incubated for 24h at room temperature, and rinsed with MilliQ three times (until the pH had stabilized). The parts of the sampling device suitable for sterilization were washed with Trigene, autoclaved and rinsed with MilliQ. It is important to note that these did not come into direct contact with the collection liquid, since autoclaving is not an efficient method for removing endotoxins (Sandle 2013).

3.2.1.3. Sample collection

Sample collection was performed using the Coriolis® µ air sampler (Bertin technologies) with compatible collection cones (Bertin technologies) previously sterilized and washed with MilliQ water, upon being filled with 15 mL of MilliQ water used as the collection liquid. Details on the collection conditions can be found in Table 3.1. When significant evaporation occurred during the first 10 minutes of sampling, the volume was topped up with MilliQ. Additional information such as the weather conditions (collected from the Royal Meteorological Institute website) and air quality was collected (website of Flemish environmental agency (VMM)) and can be found in annex.

Table 3.1: Sample collection dates and sampling details

Sampling day	Sampling sites (a)	Sampling time	Duplicate samples?	Air intake (L/min)	End volume of sample
25/09/13	3	10/30 min	Yes	250	15mL
02/10/13	3	20 min	Yes	250	15mL
25/10/13	3	20 min	No	250	20mL
06/11/13	4	20 min	No	250	20mL
13/11/13	4	20 min	No	250	20mL
02/12/13	3	20 min	No	300	20mL
17/12/13	3	20 min	No	300	20mL
13/03/14	3	20 min	No	250	20mL

(a) If sampling was performed at 3 sites, this includes Borgerhout, the harbour and Hoboken. In case a fourth sampling site was included, this includes Dessel.

3.2.1.4. Sample storage

After collection, cones containing the samples were transported on ice back to the lab.
Here, the cones were heated and vortexed for at least 1 minute, followed by transfer of the liquid sample to a 25 ml sterile glass vial. The empty cones were filled with 5 mL of MilliQ water, preheated to 37°C, vortexed, and the resulting liquid was added to the respective sample. These treatments were implemented to minimize losses of endotoxins against the polycarbonate wall of the sampling cone. After thorough mixing, the entire volume was aliquoted (by pouring) out into 5 mL vials (Filter Service) containing around 2 ml each. No pipettes or extra glassware was used to make the aliquots, in order to minimize losses of endotoxin. Samples were stored at -20°C.

3.2.2. Collection of indoor PM

3.2.2.1. PM collection with Coriolis® µ air sampler

Apart from the outdoor samplings, PM was also collected inside a music school, similarly as described in 3.1.1, using the Coriolis® µ air sampler. The thereby collected PM samples were included in subsequent assays.

3.2.2.2. PM collection with MicroDust Pro air sampler

In cooperation with the lab of Prof. Karolien De Wael (AXES research group, department chemistry, University of Antwerp), the MicroDust Pro (Casella) was used simultaneously with the Coriolis® µ to determine real-time PM concentrations in the music school. Airborne particulates were collected onto a glass microfiber filter (diameter 25mm, Macherey-Nagel), at a flow rate of 4L/min. Since the device could not measure PM_{10} and $PM_{2.5}$ simultaneously, PM_{10} concentrations were measured during the first indoor 30-min sampling, while the second sampling recorded $PM_{2.5}$ levels. Two outdoor reference samples were taken with the MicroDust Pro over 15 minutes each, first measuring $PM_{2.5}$ and afterwards PM_{10}.

3.3. Results

3.3.1. Selection of outdoor case studies for airborne sampling

In order to assess several techniques used later on in the project, samples with a highly variable composition were desirable. Three primary sites in and around Antwerp were chosen and regularly sampled, while a “greener” site (Dessel) was occasionally measured for comparison. The first sampling site, Plantin en Moretuslei in Borgerhout, is located in the city centre of

Antwerp and is regarded as a hotspot for PM in Flanders (Vlaamse milieumaatschappij 2010). This region has a high population and traffic density, which are expected to be the main influences for the local PM composition. The second location used for sampling was the Boudewijnsluis in the harbour of Antwerp. The industrial character of this site, with a high density in petrochemical companies, is expected to strongly affect the ambient PM composition, together with traffic of boats and trucks, typical for harbour regions. The third investigated region was Moretusberg, Hoboken, where samples were taken at Curiestraat, close to the Umicore plant. This mostly residential area with low traffic density in the suburbs of Antwerp has known severe air pollution in the past, more specifically of metals such as lead and arsenic, due to the vicinity of the Umicore plant. Today Umicore has made efforts to minimize the emission of metals (Meynen 2009). Later on in the project, a fourth location was added in order to obtain reference samples with minimal influence of human activity. For this purpose the site around the Sas4 tower in Dessel was chosen. The tower was placed at the crossing of two canals, in a nature reserve.

All sampling sites were chosen in the close vicinity of a VMM monitoring station that records ambient PM concentrations so that its data regarding the air quality could serve as background information for the collected samples.

3.3.2. Selection of indoor case studies for airborne sampling

As a case study, the air quality of a music school located in Borgerhout was investigated. At this school, several teachers experienced respiratory problems, including chronic sinusitis, and they expected the air quality to be a likely contribution factor. The walls of the classrooms were lined with an isolating material that produced a significant amount of dust. On top of this, classrooms were not well ventilated since the noise associated with opening the windows would disturb neighbours and passers-by. To estimate whether the air quality could indeed be a contributing factor for the health problems of the teachers, air samples were taken at the music school and included in the subsequent assays. Sampling was performed the 26th of February 2014 inside a classroom using the Coriolis® µ air sampler, with an air intake of 300L/min, for 30min and in duplicate, according to previous descriptions. A reference sample was also taken outside the classroom window, using the same settings. The simultaneous use of the MicroDust Pro (Casella) PM sampler to collect particles provided information concerning the PM concentrations inside and outside the music school.

3.3.3. Optimization of the Coriolis® µ air sampling protocol for the collection of bio-aerosols

While gaining more experience with the Coriolis® µ air sampler, the protocol for the preparation before and transport after sampling underwent various alterations over the course of this project. The changes to the sampling protocol are shown in table 3.2. A table with all sampling dates, including the sampling settings and environment conditions can be found in annex.

Table 3.2: Adjustments made to sampling protocol

Adjustment to	Details
Preparation: Storage of collection liquid	At first the collection liquid (MilliQ) was stored in glass bottles, sealed with aluminium foil, which were dry heat sterilized. Between the 2nd and 3th sampling, this was altered to avoid contact with aluminium, as this would interfere with ICP-MS data on the aluminium content of the samples. The heat sterilized bottles containing the MilliQ water used for washing the cones and as collection fluid, were sealed with lids, sterilized by a 24h 1M citric acid treatment. However, this treatment did not effectively destroy endotoxins as shown by contamination of the collection fluid and the samples (as tested with the rFC assay). Therefore, the bottles storing the collection liquid were again sealed with aluminium foil, dry heat sterilized, rinsed and filled with MilliQ as before.
Preparation: Cleaning of collection cones	After experiencing contamination of the samples, a 24h treatment with NaOH was added to the cleaning process of the cones. From then on, the cleaning procedure of the collection cones consisted of dishwashing, incubation with NaOH at room temperature, and rinsing with MilliQ three times (pH stabilization) before the cones were used for sampling.
Sampling: air intake	The first sample collections were performed with an air intake of 250L/min. However, the samples of December (and the music hall) were collected with an air intake of 300L/min, since airborne endotoxin concentrations were expected to be low.
Sample transport	During the first and second sampling, samples were transferred from the collection cone into a falcon tube or dry heat sterilized glass vial respectively, directly after sampling. These were then transported on ice to the lab for distribution in aliquots and stored at -20°C. From the third sampling on, samples were kept in the collection cone, transported on ice to the lab, heated, vortexed and transferred to a dry heat sterilized glass vial. To the empty cone, 5ml of preheated MilliQ (collection liquid) was added and vortexed to rinse out remaining endotoxin which may adhere to the cone wall. This was then added to the sample, vortexed and the solution was distributed in aliquots and stored at -20°C.

3.4. Discussion

3.4.1. Optimization of the Coriolis® µ air sampling protocol for the collection of bio-aerosols

A first step in this project was the optimization of sampling for PM, with respect of its components. The difficulty of sampling for endotoxins is evident, and this is also reflected in the lack of an internationally accepted protocol for the collection of airborne endotoxins. Most of these adjustments were aimed at two important factors influencing the measurement of the endotoxin concentration in the samples.

The first hurdle in the sampling process was avoiding contamination of endotoxins from sources other than the sampled air. This implied clearing endotoxins from the collection liquid and the sampling equipment such as the sampler itself, the collection cones and the used glassware. Due to the chemical stability of endotoxins, their elimination from various materials is a challenging task, which often requires harsh treatments. While dry heat sterilisation is a fairly easy and effective means of endotoxin destruction for borosilicate glass and certain aluminium caps, not all materials can withstand this. Alternatively, chemical breakdown of endotoxins by highly concentrated acid or bases can also be an effective method. However, in this project a 24-hour treatment of sampling cones with 1M citric acid did not cause a sufficient decrease in endotoxin concentration, which led to contamination. Subsequently, it was tested if treatment with 1M of NaOH for 1 hour at 37°C sufficed to reduce endotoxins to a satisfactory level (Sandle 2013). Although this was the case, the material (polycarbonate) suffered greatly from this treatment. Therefore, the concentration of NaOH was lowered to 0.5M and the incubation time increased to 24h at room temperature. This subsequently proved to be an acceptable method after testing various controls for the presence of endotoxins using the rFC assay.

A second important factor of the sampling optimization was the recovery of the collected endotoxin in the samples. The ability of endotoxin to adhere to various surfaces, and especially plastics, complicates the recovery of the collected endotoxin into the sample solution. To increase desorption from the polycarbonate wall of the sampling cone, the samples were heated and thoroughly vortexed before being aliquoted, together with an additional wash to dissolve remaining endotoxins. Additionally, whenever possible glassware was used instead of plastics. Regrettably, the efficiency of these alterations could not be studied experimentally.

It should be noted that some alterations made to the protocol could have a negative effect regarding the sampling of metals. Firstly, when working with metals in solutions, the use of glassware should be avoided, as this could lead to contamination of various elements, including

silicon, boron and sodium (Chen n.d.). Furthermore, metal ions have the tendency adhere to glass surfaces. Secondly, the used polycarbonate collection cones could also cause contamination of chlorine, bromine and aluminium (Chen n.d.). Finally, the use of aluminium foil to seal bottles prevented the analysis of aluminium content in the samples.

Chapter 4.
Characterization of PM composition: evaluation of endotoxin content

4.1. Introduction

A first step in analyzing the composition of the samples was the determination of the endotoxin content. For this purpose the PyroGene™ Recombinant Factor C (rFC) Assay (Lonza) was used. This assay and the Limulus Amoebocyt Lysate (LAL) assay from which it was derived were discussed earlier in chapter 1, including their principles and advantages.

4.2. Materials and methods

The determination of the endotoxin levels in the collected PM samples also underwent significant optimization over the course of this project. The basic protocol stated below includes all the adjustments made during this optimization. Before starting the assay, all samples and the LAL reagent water which was included in the kit, were heated to 37°C. Over the entire protocol, before using a standard or sample and after diluting it, the solution was vortexed for at least 1 minute, to avoid losses of endotoxin to the glass wall.

The first step in the protocol was resuspension of the *E. coli* O55:B5 endotoxin standard, included in the kit, in LAL reagent water according to the volume stated on the certificate of analysis to yield a 20 EU/mL solution. To assure proper resuspension of all the endotoxin in the vial, the standard solution was vortexed for 15 minutes as recommended. A volume of this solution was used to make dilutions for a standard curve, while the remainder was aliquoted and stored at -20°C in sterilized 5mL glass vials. Freeze/thaw cycles were avoided by using a new aliquot of the frozen stock for each assay. To make dilutions of the endotoxin standard, a volume of reagent water was first added to sterilized 1mL glass bottles, to which the appropriate volume of endotoxin standard was added. The used volumes, which can be found in table 4.1, were equalized when possible. Afterwards, the dilutions or spikes of the samples were prepared with LAL reagent water or the undiluted endotoxin standard respectively. The used volumes for spiking can also be found in table 4.1. Whenever spikes were used, the ‘normal’ samples were also diluted with the same volume of LAL reagent water to adjust for dilution of other components of the sample. Furthermore a ‘blank spike’ was included, as reference for the spike recovery and loss of endotoxin due to the use of plastic pipette tips.

Table 4.1: Dilution volumes used in rFC assay

Type	Solution	Volume of component 1	Volume of component 2
Standard	10 EU/mL	200µl of LAL reagent water	2x 100µl of 20 EU/mL standard
	1 EU/mL	360µl of LAL reagent water	40µl of 10 EU/mL standard
	0.1 EU/mL	360µl of LAL reagent water	40µl of 1 EU/mL standard
	0.02 EU/mL	320µl of LAL reagent water	2x 40µl of 0.1 EU/mL standard
Spikes	Sample	342µl of sample	18µl of LAL reagent water
	Spiked sample	342µl of sample	18µl of 20 EU/mL standard
	'Blank' spike	342µl of LAL reagent water	18µl of 20 EU/mL standard

When all samples and standards were prepared, 100 µl was added to a 96-well plate (Costar, Corning), which was then incubated for at least 10 min at 37°C. In the meantime, the Synergy™ MX microplate reader was set up according to settings in table 4.2. During the incubation time the reagent mix was prepared in a 5:4:1 ratio of fluorogenic substrate, rFC assay buffer and rFC enzyme, all included in the PyroGene™ Recombinant Factor C Assay kit, in an endotoxin-free reagent reservoir (Lonza). When reagents and the plate were ready, 100 µl of the reagent mix was added to each well with a multichannel pipette, after which the plate was shortly vortexed and placed in the microplate reader (BioTek). Fluorescence was measured at time 0 and after 1 hour.

Table 4.2: Microplate reader settings used for fluorescence measurement in rFC assay

Setting	Value
Time between reads (hh:mm:ss)	(01:00:00)
Excitation (nm)/ Bandpass	380/20
Emission (nm)/ Bandpass	440/20
Reads per well	10
Delay before reading (ms)	160
Optic position	Bottom
Sensitivity	64
Probe distance	4 mm

4.3. Results

4.3.1. Optimization of the rFC assay protocol

During optimization, several adjustments were made to the protocol, addressing various observed problems. Firstly, the recommendations of the manufacturer (Lonza) for the use of the rFC assay kit were vaguely written. To address this problem, previous optimization had been performed by Serena Moretti to adjust for unspecified details and to accommodate the use of a

Synergy™ Mx microplate reader (BioTek). During this project, optimization was primarily aimed at the use of air samples collected according to the protocol mentioned in section 2.2.

While first experiments showed problems with standard curves and variances between replicates of the same sample, most of these issues were minimized in the following experiments due to several improvements made to the protocol, which are summarized in table 4.3.

Table 4.3: Adjustments made in rFC assay over the course of the project

Adjustment to	Details
Standard curve concentrations	The ten concentrations used in the first experiment were replaced by 4 concentrations (0.01 EU/ml; 0.1 EU/ml; 1EU/ml; 10 EU/ml), which were tested in triplicate. Later on, the lowest concentration was replaced by 0.02 EU/ml.
Dilutions and spikes	All dilutions and spikes were made in small dry heat sterilized 1ml glass vials, in order to minimize the surface area that comes into contact with the diluted or spiked samples.
Spiking volumes	Spiking method was altered from the kit manufacturer's instructions in order to equalize total volumes of samples and spikes and compensate for endotoxin lost during pipetting.
Sample treatment	Before use, samples and standards were heated to 37°C, in order to facilitate de-adsorption of endotoxin from the vial wall.
Sample treatment	Before using a sample or standard and after making a new one, the solution was vortexed for at least one minute.
Loading 96-well plate	When adding samples to the 96-well plate used for analysis, the same pipette tip was used for all replicates of one sample or for all standards starting from the lowest concentrations.
Reagents and standard	Keep track of lot number and opening date of reagents and LPS standard.
Addition of reagents to plate	After adding the reagents, vortex plate shortly before putting it in the reader.
Setting of microplate reader	Turn off shake setting and increase amount of scans per well to 10.

4.3.2. Improvements to the standard curve

As mentioned above, some problems with the standard curve were encountered with the first experiments. The standard curve of the first experiment is shown in figure 4.1 as an example. This curve was obtained by using ten different concentrations of endotoxin, diluted from 3 standard solutions by adding a different volume of standard into the well and diluting it in the well itself. This is also illustrated by figure 4.1. The thereby obtained standard curve did not allow proper derivation of endotoxin values of the samples.

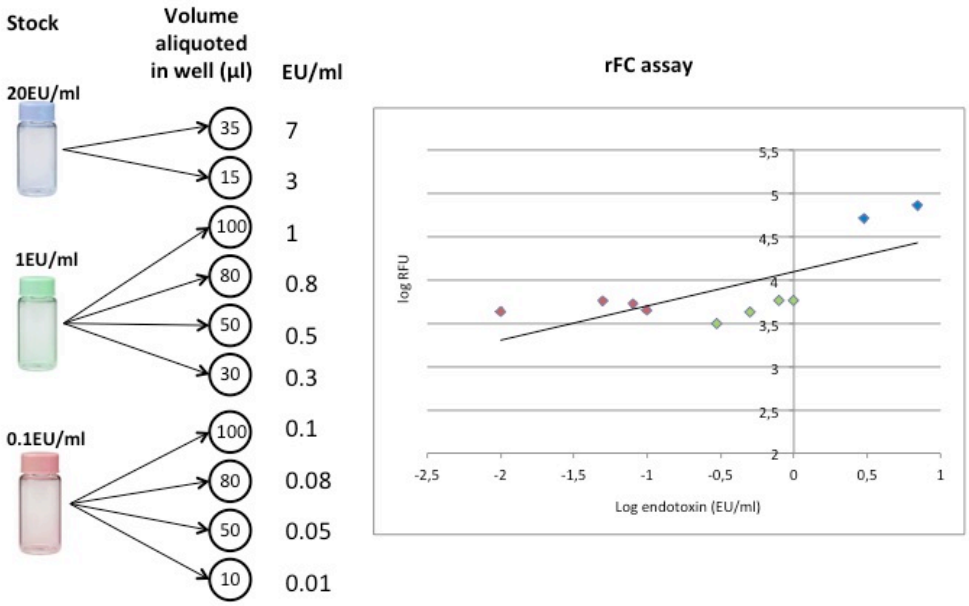

Figure 4.1: Set-up for standard curve in the first rFC experiment and the resultant standard curve on the 27th September. The concentrations used, diluted from the three stock concentrations are indicated on the figure.

Nonetheless, after improving the protocol, standard curves with a sufficiently high coefficient of determination (R^2) were obtained. These are represented in figure 4.2. Although most of these curves show a strong overlap, two curves deviate. It should be noted that all curves, except the curve from the 10th April, are derived from the same batch of endotoxin standard. For the standard curve of the 10th April, a new batch of endotoxin standard was freshly prepared and used before freezing.

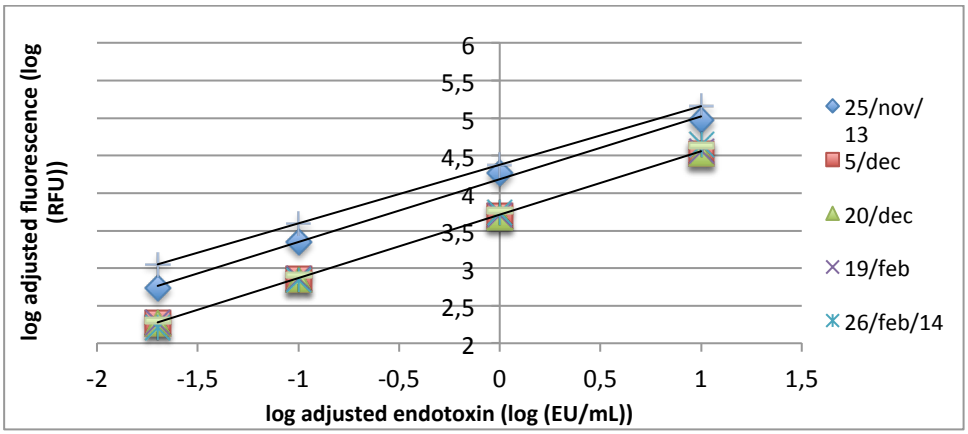

Figure 4.2: Graph showing standard curves obtained in 7 experiments. Data points are connected for the data sets of the 25th November, the 19th of February and the 18th March. The curves of the 5th and the 20th December, the 19th and 26th of February, and the 10th April overlap to a great extent and therefore only one line is represented.

4.3.3. Endotoxin concentrations of outdoor PM samples

Logically, the goal of optimization of the rFC assay protocol and the standard curve was to provide an accurate tool to determine the endotoxin concentrations in the collected air samples. The endotoxin concentrations of the samples were recalculated to the concentrations in the sampled air (as seen in figure 4.3.), and presented in figure 4.4. The resultant endotoxin concentrations are also plotted against the ambient PM_{10} concentrations during sampling in figure 4.5.

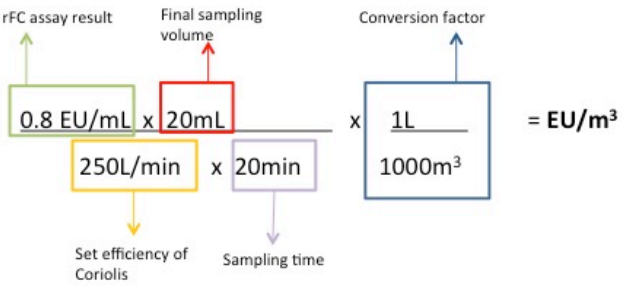

Figure 4.3: Calculation of airborne endotoxin concentration from the results of the rFC assay.

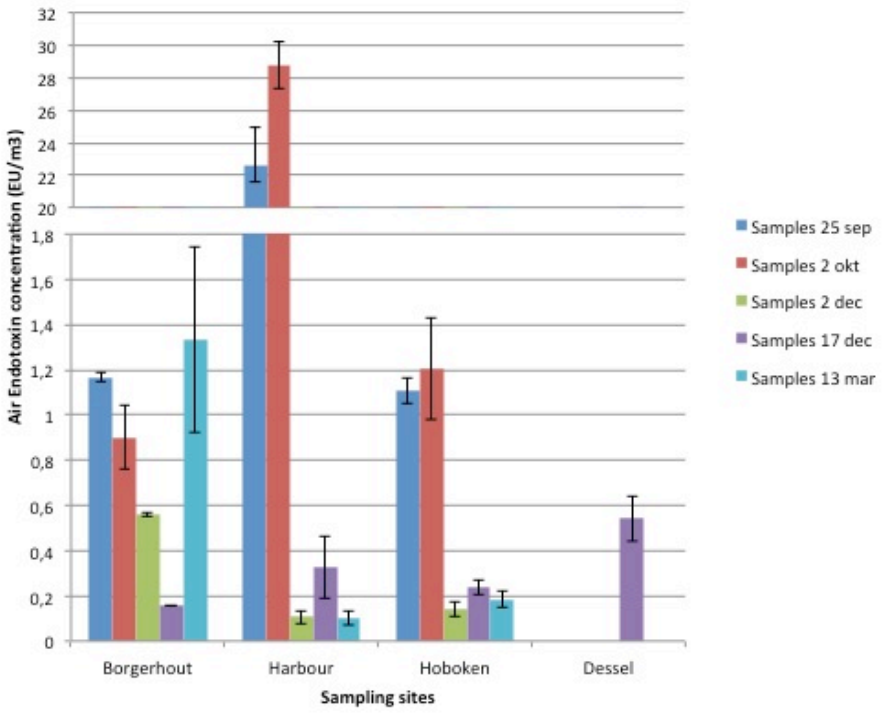

Figure 4.4. Concentrations of airborne endotoxin, as calculated from endotoxin levels in the collected samples, measured by the rFC assay. The error bars indicate a range of one standard deviation from the values.

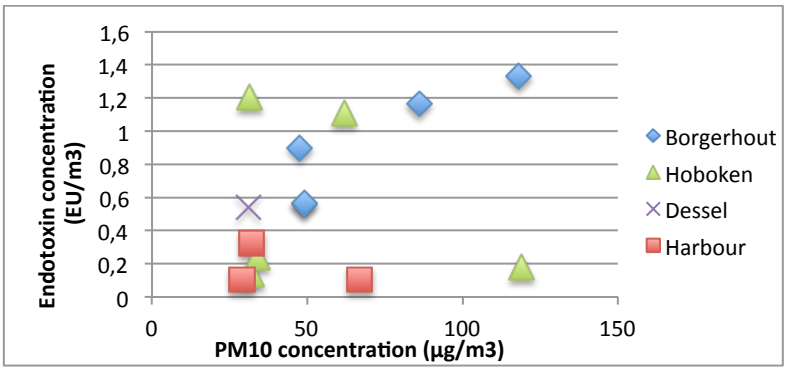

Figure 4.5: Variation of airborne endotoxin concentrations with ambient PM_{10} concentrations during collection of the PM sample.

Due to problems with the standard curves, the experiment was repeated for the samples of the 2nd October and the 13th March. While the results of the two assays were similar for the samples of the 13th March, this was not the case for the samples of the 2nd October. The results of the three experiments using these samples are presented in table 4.4.

Table 4.4: Results of three rFC assays performed on the samples collected on the 2nd October.

	4 October	**17 October**	**10 April**
Borgerhout repeat 1	23.2 ± 5.1	0.84 ± 0.19	ND
Borgerhout repeat 2	7.33 ± 2.0	0.41 ± 0.06	8.27 ± 2.2
Harbour repeat 1	358 ± 82	31.9 ± 0.19	ND
Harbour repeat 2	536 ± 34	42.9 ± 3.20	1.52 ± 0.13
Hoboken repeat 1	65.7 ± 7.6	2.50 ± 0.63	1.33 ± 0.19
Hoboken repeat 2	15.4 ± 1.8	0.50 ± 0.04	ND

4.3.4. Endotoxin concentrations of indoor PM sample

The samples obtained during sampling with the Coriolis® µ in the music school were also analysed on their endotoxin content. These results, together with the PM data recorded by the MicroDust Pro are represented in table 4.5.

Table 4.5: Airborne endotoxin concentrations calculated from sample concentrations, tested with the rFC assay.

Sample	**Airborne endotoxin concentration (EU/m^3) value ± SD** [(a)]	**PM_{10} concentration ($\mu g/m^3$) Mean (min; max)** [(b)]	**$PM_{2.5}$ concentration ($\mu g/m^3$) Mean (min; max)** [(b)]
Indoor sample repeat 1	0.09 ± 0.02	15 (7; 34)	13 (10; 20)
Indoor sample repeat 2	0.19 ± 0.02	15 (7; 34)	13 (10; 20)
Outdoor reference sample	1.43 ± 0.13	50 (42; 56)	38 (33; 44)

(a) SD: standard deviation (b) min: minimum; max: maximum

4.4. Discussion

4.4.1. Optimization of the rFC assay protocol

As was the case with the sampling protocol, the biggest issue to keep in mind during the rFC assay was the recovery of endotoxin from the samples, and in this case also from the endotoxin standards. The same measures, heating, vortexing and minimizing exposures to plastics were therefore also applied here to avoid losses of endotoxins due to adsorption.

4.4.2. Improvements to the standard curve

Analysis of the standard curve was an important part of every rFC assay and often it was used as criterion for the quality of all the measurements.
In the first rFC assay, the obtained results for the standard curve did not fit a linear model by far. The use of three stock endotoxin standards to prepare the 10 single concentrations is also reflected in the graph, with the highest volumes of one standard used producing a lower reading than expected for a linear increase, yielding a curved response, which is especially evident for the lower endotoxin concentrations. This suggested the importance of the pipetted volume, which was probably due to adsorption of endotoxin on the plastic pipette tip. Therefore, efforts were made to reduce the amount of pipetting steps, equalizing the used pipetting volumes and reusing pipette tips for the same solution. In addition, smaller glass bottles were used to prepare sample dilutions and spikes to minimize the surface area to which the samples were exposed since this could also cause losses of endotoxin due to adsorption.

Following these adaptions, the standard curves of later experiments showed high R^2 values, suggesting that this part of the assay was successfully optimized. However, on occasion an increased variation between the readings for the standard curve was observed, which could be traced back to deterioration of the reagents upon prolonged storage after opening. Therefore, it seems important to keep track of the lot number of the used standard and the reagents.
The most striking observation regarding these standard curves is the consistency of the obtained curves. Most of these curves overlap each other, which suggest a high reproducibility of the assay. The first curve that differs, the curve of 25th November, was obtained from a freshly prepared endotoxin standard, which was never frozen, unlike the other curves (with exception to the curve of the 10th April). As was also seen with the samples, storage at -20°C likely decreased the endotoxin concentration of the solution, which was supported by reports in literature (Douwes et al. 1995; Laitinen 1999; Milton et al. 1997). This could explain the higher

values measured for this standard, seeing as the gradient of the curve remained constant. From these experiments, it would seem that the endotoxin concentration was only influenced by the act of freezing and subsequent thawing rather than the duration of freezing, within the time limitations of the experiments. Another standard curve that deviated from the norm was the one of the 13th March. In this experiment, problems with the sensitivity settings were encountered, leading to overall higher readings for both the initial and second measurement. Furthermore, this standard curve was not completely parallel to the other curves. Due to the highest standard concentration (10 EU/ml) nearly attaining the upper limit of detection, the measurements of this standard might not be as accurate as the others, leading to a small decrease in the slope of the standard curve.

Finally, lot variations of endotoxin standards were tested. Curves from the 10th April and the 25th November were expected to be similar since they were both derived from freshly resuspended endotoxin lots. However, the more recent curve of the 10th April fell in the same range as (most of) the other curves, which had been frozen and thawed. This highlights again the importance of recording the lot number used for both the standards and the reagents. Nonetheless, if this reflects a lower reactivity of the endotoxin standard, the actual values of the samples would be underestimated, producing incomparable results between lots. On the other hand, this could also be due to a lower reactivity of the reagents, which would lower the obtained values for both the samples and the standards, which wouldn't necessarily invalidate the results. However, the cause might also lie with the microplate reader, seeing as problems with the sensitivity were observed before. This can be detected and solved by maintenance of the instrument.

4.4.3. Endotoxin concentrations in the samples

Over the course of this project several outdoor PM samples were analysed on their endotoxin content. Unfortunately, some samples, more specifically the samples of the 25th October, the 6th and 13th November 2013, were later excluded from further analysis as contamination had occurred in these samples. The obtained data will therefore not be considered here.

A first general observation on the endotoxin values of outdoor PM samples that can be made is the difference between the endotoxin concentrations in the autumn samples compared to the winter and early spring samples, with the latter yielding significantly lower results, especially in the harbour samples, and with exception of the spring Borgerhout sample. The Borgerhout sample of 13th March showed a strong increase in concentration as compared to the winter results. This observation was also supported by the similar concentration of the sample taken outside the window of the music school, which was taken 26th February, in similar weather

conditions. Additionally, literature supports this finding, finding low concentrations in winter months, and higher concentrations in spring and summer months (Carty et al. 2003; Longhin et al. 2013; Heinrich 2003)
However, the fact that this increase wasn't observed in the harbour or Hoboken could possibly indicate a difference in endotoxin sources. Furthermore, it might be entirely possible endotoxin concentrations rise later on in spring (a time span not possible within the time frame of this project).
Furthermore, the sample taken in Dessel during winter showed the highest endotoxin concentration of that sampling run, which was expected because of the 'green' character of the sampling site, hosting a high source of bacteria.

When looking at literature, the measured endotoxin concentrations in this project seem very acceptable. Overall, most results show the same order of magnitude as airborne endotoxin concentrations reported in literature, with exception of the two autumn samples taken at the harbour. Studies have shown lower concentrations (Nilsson et al. 2011; Menetrez et al. 2009), concentrations similar or lower (Morgenstern et al. 2005) and concentrations similar or higher (Traversi et al. 2011), than the airborne endotoxin concentrations measured in this project.

A second important observation regarding the endotoxin and PM concentrations is their apparent lack of correlation, as could be seen in figure 4.6. It seems the PM concentrations do not give an indication on the concentration of airborne endotoxin, which is most clearly demonstrated by the samples during the smog episode (13th March) and the samples of the autumn samples taken in the harbour. One the one hand, while the PM concentrations were abnormally high during the smog episode, especially in Borgerhout and Hoboken, their endotoxin concentrations were not elevated compared to other samplings (winter for Hoboken, music school for Borgerhout). One could argue that highly concentrated PM components present in the samples could cause inhibition of the rFC reaction. However, spikes were included in the assay to investigate this and although some inhibition was observed, this would not completely explain the observed results. On the other hand, exceptionally high endotoxin levels were observed in the harbour samples in autumn, although PM concentrations were comparable as in the other sampled locations. It should be noted that these results were not included in figure 4.6.

A last result of interest is the variance between the results of three assays, all determining the endotoxin concentration in the samples of 2nd October. While the first assay, which was performed before the samples were stored at -20°C, indicated very high concentrations, this was

no longer the case for later experiments, indicating a drop in endotoxin concentrations upon freezing. This phenomenon has previously been reported in literature with decreases in LAL reactivity of 20-25% (Douwes et al. 1995; Laitinen 1999)and of 86% (Milton et al. 1997) upon freezing at -20°C. On the other hand slight increases (Morgenstern et al. 2006) and no changes (Spaan et al. 2007) have also been reported.
Naturally one would think that if the endotoxin standards have been frozen it is best to apply this method likewise to the tested samples. However, this would imply that freeze/thawing results in a proportional loss for all samples, independent of the concentration of the endotoxins. An interesting experiment could consist of the determination of the endotoxin concentrations in fresh samples with a standard curve derived from a freshly prepared endotoxin standard, and repeating these measurements after the samples and the standard had been freeze/thawed.

Since some readings of the samples fell outside the range of the (unsatisfactory) standard curve in the first experiment, the experiment was repeated with dilution of the samples to a relatively large factor, based on the previous data. However, since freezing the samples had likely caused a drop in endotoxin concentrations, the samples were diluted much more than expected and results were subsequently lower than anticipated. Even when taking the dilution factor into account, observed concentrations were at least a tenfold lower than before. It is important to note here that at the time of these two assays, the assay procedure had not been fully optimised and therefore results could quite easily stray from the actual concentrations in the samples.
The poor quality of the results was also made evident by the analysis of the spikes. For various samples, spike recovery was higher or lower than the 'blank spike', which would normally indicate enhancement or inhibition. However, because of the strong dilution factors applied for some samples in this experiment, the effect of enhancing or inhibition should have been minimized. As even these strongly diluted samples showed enhancement, it might be possible another factor contributed more to the spike recovery than the presence of activating factors. No conclusions can therefore be made based on these spike recoveries.
A final conclusion in the analysis of the results of these two experiments concerns the large difference in endotoxin concentration in the sample repeats. Although these samples were taken directly after each other, they differ significantly in terms of their endotoxin content. This could possibly result from the increased susceptibility of short-term samples to bias due to temporal variation (Duchaine et al. 2001). However, the observed variation seems rather high to only result from this principle, but no other clear explanation can be provided.
A third and final rFC assay was performed on the 2nd October samples that were used in other

assays (B2.2, H2.2, HB2.1). This experiment showed a nice standard curve, providing a higher confidence for the results of the samples, but values again differed from the previously obtained results. In this last experiment, the used dilution factor lead to sample readings falling in the middle of the (higher quality) standard curve and samples spikes did not indicate activation of inhibition. Therefore, it is likely that these results can be used with a lot more confidence than the previously obtained data and differences between these likely result from suboptimal assay conditions in the first assays, together with the decline in endotoxin levels after storage at -20°C.

4.4.4. Music school

Simultaneously with the collection of the PM samples for analysis with the Coriolis® µ, the Microdust Pro recorded data on the PM_{10} and $PM_{2.5}$ concentrations. As expected, these results showed a higher concentration for PM_{10} than $PM_{2.5}$ and measured concentrations were higher outdoors than indoors. It is notable that the observed outdoor PM concentrations were at or above the EU and WHO guidelines, indicating the poor air quality on the sampling date.

The rFC assay, determining endotoxin content, was also performed on the music school samples. The obtained values for the indoor samples were relatively low, and remarkably lower than the samples taken outside the window. In fact, the observed endotoxin levels were similar and lower as the outdoor samples during winter. These results suggest that probably there is no significant source of endotoxins present in the empty classroom responsible for the observed health effects. Nevertheless, the minimal sampling size prevents totally excluding this possibility.

The endotoxin concentration of the sample taken outside the window corresponded very well to the relatively low endotoxin concentration of the Borgerhout sample of 13th March during the smog episode, although circumstances, including location and sampling height, were very different. This again suggests that factors contributing to the smog episode did not strongly influence endotoxin concentrations.

Chapter 5.
Characterization of PM composition: evaluation of transition metal content

5.1. Introduction

A next step in analysis of the sample composition was the determination of various metal concentrations in the samples by high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). This very reliable technology with excellent detection limits is based on the ionization of a sample by guiding it through an inductively coupled argon plasma discharge, and detection of these ions by mass spectrometry (Thomas 2001). A scheme of an ICP-MS instrument with the most important components indicated can be found in figure 5.1. Being a highly sophisticated technique, ICP-MS has been applied in the analysis of particulate matter samples numerous times, by various labs and government institutions (European Commission, 2001; Guastadisegni et al., 2010; Guo et al., 2012; Kroll et al., 2013; Manzano-Léon et al., 2013; Mesquita et al., 2014; Shang et al., 2013; Vlaamse milieumaatschappij, 2009).

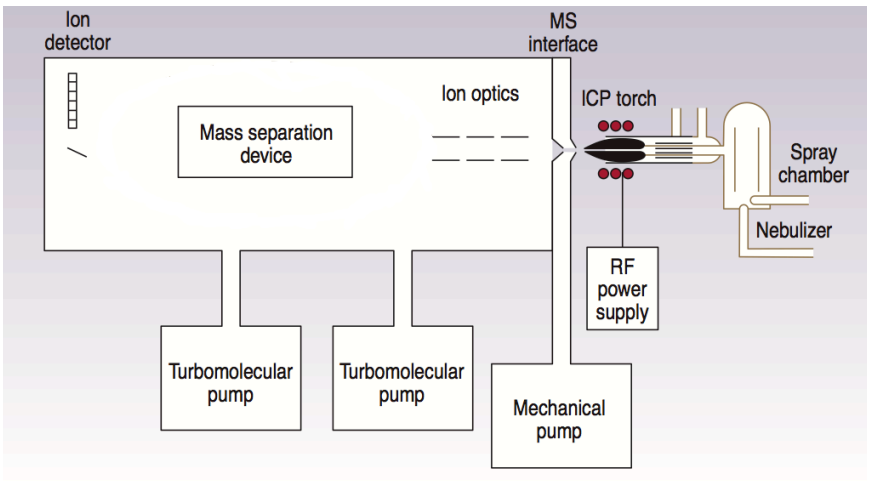

Figure 5.1: Elements of an ICP-MS instrument. First the sample is aerosolized in the nebulizer to form small sample droplets. The smallest of these droplets are then selected in the spray chamber and introduced into the ICP torch. Here an argon plasma discharge is generated and maintained by a radio-frequency (RF) power supply. This plasma discharge dries, vaporizes, atomizes and ionizes the sample to finally yield a beam of positively charged ions. These ions are then passed through the mass spectrometry (MS) interface, which adjusts for the difference in ambient pressure, to the ion optics, which focuses the ion beam. In the mass separation device, ions of a specific mass are filtered out which are subsequently detected by an ion detector. Reference: Thomas (2001).

Next to concentration, the redox state of the transition metals found in the PM samples is also an important factor for determining the provoked physiological response. However, this cannot be evaluated by ICP-MS. To provide additional information of the redox state of the samples, cyclic voltammetry was applied. In this technique a series of voltages is applied to a working electrode in an electrolyte solution containing specific analytes, such as redox active metals, while monitoring the electrical current flowing through the electrode. When the redox potential of an analyte present in the electrolyte solution is applied, electron transfer between the electrode and the analyte will occur resulting in a change in redox state of the analyte and a current flowing through the electrode, which is detected. This produces a voltammogram representing the observed current in function of the applied potential with peeks in current at potentials were electron transfer with an analyte has occurred. While the observed current of the peek gives an indication of the concentration of the analyte, the applied potential indicates which analyte is detected. Although this technique has been adopted in the research for environmental samples of water, sediment and soil (Buffle & Tercier-Waeber 2005), its application in PM research remains very limited (Baitimirova et al. 2011).

5.2. Materials and methods

5.2.1. Determination of PM transition metal concentration with ICP-MS

The ICP-MS technique was made available for this project by cooperation with the lab of Prof. Ronny Blust (SPHERE research group, University of Antwerp), where trained personnel assayed the samples. Before introduction of the sample in the instrument, the samples underwent acid destruction, to assure that all particulates were properly dissolved. The used instrument, a high performance high-resolution ICP-MS Element XR instrument (Thermo Scientific), employs double focusing magnetic-sector technology for mass separation and a secondary electron multiplier combined with a faraday detector for ion detection.

5.2.2. Determination of oxidative capacity of PM with cyclic voltammetry

In collaboration with Prof. Karolien De Wael (AXES research group, department chemistry, University of Antwerp), the PM samples were subjected through analysis by cyclic voltammetry. To this end, an Autolab potentiostad controlled by the NOVA 1.10 software package (Metrohm) was used for electrochemical measurements. A traditional three-electrode cell configuration was used, with a platinum electrode inlaid disk (with a diameter of 2.00 mm) functioning as the working electrode. A saturated calomel electrode (SCE) and graphite electrode were used as the

reference and the auxiliary electrode, respectively. The cell was placed in a Faraday cage to reduce electrical noise. All the experiments were conducted at room temperature (20 ± 1 °C).
Before starting the experiment the working electrode was cleaned by polishing with 0.05 µm alumina/water slurry on a polishing cloth to a mirror finish and sonication in an ethanol solution to eliminate traces of alumina. This cleaning is a crucial step, necessary to reduce the amount of adsorbed impurities on the electrode. The voltammetry was performed in 2ml of 0.4M KNO_3, as electrolyte buffer at a scan rate of 100mV/s. Before measurements were made in the first experiment N_2 gas was supplied to the solution, eliminating O_2 dissolved in the liquid, as this would produce noise in the measurements. Subsequently, a blank measurement was recorded, after which 1ml of the sample was added to the electrolyte buffer and a new measurement was taken. During the second measurement, a volume of a 0.01M Fe^{3+} solution was added to the electrolyte buffer to a final concentration similar as the Fe concentration measured in the sample by ICP-MS.

5.3. Results

5.3.1. Determination of PM transition metal concentration with ICP-MS

Transition metal concentrations of the samples were analysed by ICP-MS. A set of samples were chosen for this assay, more specifically the samples of the 2nd October 2013, the samples of the music hall and the samples of the 13th March 2014. The measured concentrations in the samples were calculated back to the airborne concentrations, similarly as in section 3.3.1.3. The calculated data is represented in table 5.1.

5.3.2. Determination of oxidative capacity of PM with cyclic voltammetry

Cyclic voltammetry was performed on the samples in hope to supply additional information on the oxidative state of some highly concentrated metals. While a preliminary test on one of the harbour samples of the 2nd October (not previously frozen) showed encouraging results, indicating the presence of redox-active species in sufficient concentrations to generate measurable signals. However, further analyses were unsuccessful and these results could not be reproduced. The data obtained in the first experiment can be found in figure 5.2.

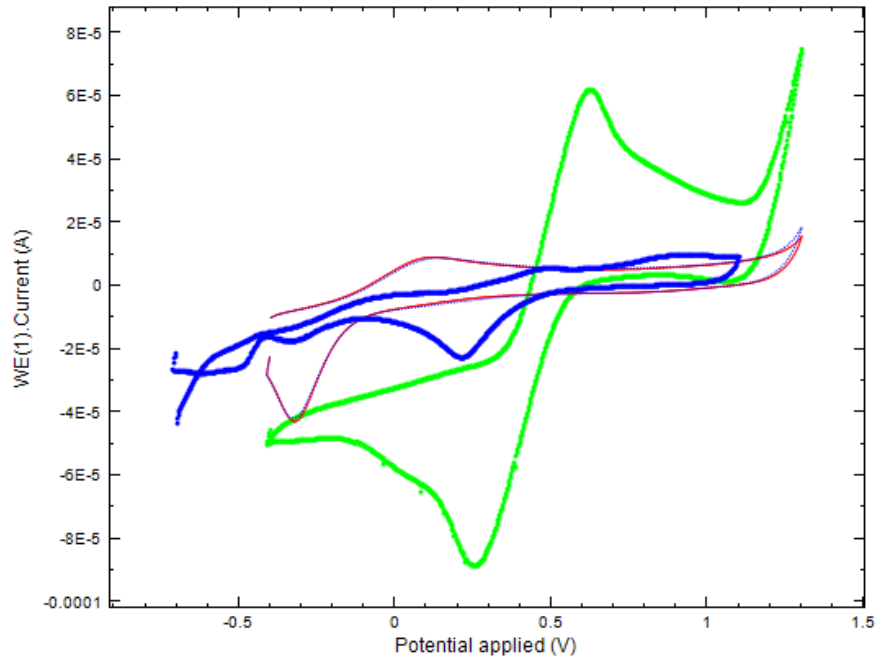

Figure 5.2: Voltammogram of main results of both experiments performed on the harbour sample of the 2nd October (2nd repeat). The red dotted line indicates the blank reading, which can almost not be distinguished from the blue dotted line, which represents the measurement after addition of the sample during the second experiment. The full blue line shows the measurement of the same sample during the first experiment. The green line indicates the measurement after addition of the Fe^{3+} solution. The indicated potentials are referred to the saturated calomel electrode. WE: working electrode

5.4. Discussion

5.4.1. Determination of PM transition metal concentration with ICP-MS

The large amount of ICP-MS data can be very elaborately discussed, but this might not be very relevant here. Therefore, some general observations will mainly be considered.

Firstly, when looking at the metal concentrations over all the samples, some metals are much more abundant than others. Iron and zinc are clearly the most abundant elements, complemented with intermediate concentrations of lead, manganese, copper and arsenic and fairly low concentrations of lithium, silver, cadmium, chromium and cobalt. A last measured element, nickel, showed very interesting results, with fairly low concentrations for the outdoor samples of the 2nd October, but high concentrations in the music hall and during the smog episode, which even surpassed the EU target value (with the exception of the Hoboken sample).

Table 5.1: Concentrations of transition metals in sampled air, calculated from sample concentrations analyzed by ICP-MS. Repeats refer to biological repeats as opposed to technical repeats.

Metal concentrations in sampled air (µg/m3)												
Sample	**Li**	**Ag**	**Cd**	**Pb**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Music hall (outdoor)	1.22	BMQL	BMQL	0.60	0.55	4.60	32.53	0.52	69.50	5.80	10.63	1.62
Music hall (indoor)	2.12	0.12	BMQL	1.22	0.85	1.62	13.58	0.58	38.92	11.37	11.45	2.83
Blanc 2 Oct	BMQL	BMQL	BMQL	BMQL	0.66	1.11	1.80	0.66	0.30	3.24	4.98	0.45
Borgerhout 2 Oct repeat 1	0.12	BMQL	BMQL	2.55	0.96	10.08	56.64	1.02	0.72	18.93	118.92	3.87
Borgerhout 2 Oct repeat 2	BMQL	BMQL	BMQL	0.87	0.78	6.75	27.57	1.05	0.66	12.72	22.71	2.19
Harbour 2 Oct repeat 1	BMQL	BMQL	0.24	48.00	2.01	101.25	586.47	2.37	3.30	44.07	193.80	5.91
Harbour 2 Oct repeat 2	BMQL	0.15	0.15	5.79	1.17	6.09	25.92	1.08	1.02	6.99	22.20	3.48
Hoboken 2 Oct repeat 1	BMQL	BMQL	0.24	60.30	2.91	49.29	654.45	1.56	2.04	47.31	138.84	4.41
Hoboken 2 Oct repeat 2	BMQL	0.33	BMQL	9.90	0.93	6.30	47.40	1.05	1.20	12.51	29.16	2.91
Borgerhout 13 Mar	4.28	0.08	0.80	34.28	2.20	31.44	298.60	1.16	35.36	75.92	304.72	ND
Harbour 13 Mar	5.96	0.04	0.48	3.28	3.84	6.68	41.48	0.36	20.76	19.52	70.96	ND
Hoboken 13 Mar	3.40	0.08	1.64	31.24	1.60	18.84	232.40	0.76	10.12	28.76	121.80	ND

Remark: BMQL: below minimum Quantitation level

ND: not determined

This is for the larger part in accordance with literature, seeing as fairly high levels of iron levels can be found, while lead, chromium, manganese, nickel en copper are also consistently present in PM (A. J. Ghio et al., 2012; Manalis et al., 2005; Schins et al., 2004). Some reports of the VMM have reported on metal concentrations in these areas. Comparing the obtained results against these, the findings are largely in the same order of magnitude but there are some differences. The samples of the 2nd October show lower transition metal concentrations in general, except for much higher concentrations of arsenic for the harbour and Borgerhout samples, and much lower concentrations for lead and chromium in the Borgerhout and Hoboken samples. These observations suggest that the measured metal concentrations are likely underestimated, resulting from the suboptimal sampling for metal collection. However, the lower concentrations observed in this project might also be caused by temporal variation, seeing as a comparison is made against an annual time-average.

Comparison of the reported data with the samples of the 13th March, during the smog episode, paints a different picture. These smog samples show generally higher concentrations for the Borgerhout samples, and while some elements were increased, others decreased in the harbour and the Hoboken sample. These results suggest that the smog episode contributed significantly to airborne transition metal contents.

On the other hand, comparing the tested samples between each other can also provide important information. Firstly, some trace levels of metals were found in the analysed blank but these remained below an acceptable level, so it was assumed no important contamination occurred. Regarding the other samples of the 2nd October, the second sampling repeats consistently showed lower metal concentrations than the first repeats, for which no clear explanation could be found. Although analysis of the blank did not indicate contamination, these observations might suggest otherwise. However, it would be surprising that consistently the first repeat suffered from contamination, while collection cones were randomly selected at the sampling site.

Comparing the sampling locations for the samples of 2nd October amongst each other, it is clear that the highest concentrations can be found in Hoboken and harbour samples.

This trend was not confirmed by the samples of 13th March, taken during the smog episode, seeing as in this case the highest concentrations were found for the Borgerhout and Hoboken samples. While concentrations of the tested elements all increased as compared to the 2nd October samples in the Borgerhout sample, this was not seen for the other two sites. Interestingly when comparing between the two sampling dates per element and region, similar conclusions can be made for both Hoboken and the harbour samples. For both regions an overall increase in lithium, cadmium and nickel, an overall decrease in cobalt

concentrations and similar silver concentrations were observed. On the other hand, comparing the samples of 13th March with the two repeats of the 2nd October, lead, manganese, iron, copper and zinc showed a decrease in concentration compared to the first sampling repeat and an increase in concentration compared to the second repeat, for both regions. An exception was chromium, which was elevated compared to both repeats in the harbour sample, although the Hoboken sample showed a lower concentration than the first repeat but higher concentration than the second repeat of the 2nd October samples.

Metal analysis by ICP-MS, was performed on two of the music school samples, more specifically one of the two indoor samples and the outdoor sample. The results thereof did not indicate an overall higher metal concentration for the outdoor or indoor sample. In fact, manganese, iron and nickel were elevated in the outdoor sample as compared to the indoor sample, while it was the other way around for lithium, silver, lead, chromium, copper and arsenic. Levels of cobalt, zinc and cadmium were similar for the two samples. It should also be noted that these observed differences were relatively small. This was expected seeing as the arrangement of the sampler while collecting the outdoor sample couldn't exclude interference of the indoor air.

5.4.2. Determination of oxidative capacity of PM with cyclic voltammetry

The first experiment performed with this technique showed some promising results, which indicated the presence of redox-active metals in the samples. The observed signals were suggested to result from iron, and possibly lead in the samples, based on the applied potential resulting in a peek. Later on, the results of the ICP-MS analysis confirmed a high concentration of iron present in the samples. However, to determine the origin of the peeks more accurately, it was decided to repeat the experiment after ICP-MS analysis of the samples, which would allow a more targeted approach. Sadly, the following experiments did not show similar results and no noteworthy peeks could be observed compared to the blank. This might suggest that the used storage conditions did not allow for the samples to be analysed after a long period of storage, although this seems an insufficient explanation for the observed effects.

Chapter 6.
In vitro analysis of biological activity of PM: TLR4 stimulation

6.1. Introduction

Due to the large structural variability of LPS between various microbial species and the subsequent difference in potential to activate the TLR 4-pathway, quantification of LPS does not necessarily correspond to the biological activity of LPS in a certain sample. Therefore, a sample's LPS activity can possibly be a better indicator in investigating the importance of endotoxin in PM toxicity.

To provide information concerning the ability of the samples to trigger TLR4 pathway activation, the HEK-Blue™ hTLR4 cell line was used. To our knowledge, these cells haven't been applied in PM research beforehand. Previously, phosphorylation of IRAK1, TAK1 (transforming growth factor β- activated kinase) and IκBα (NFκB inhibitor α), proteins involved in the downstream TLR4 pathway, was used to confirm TLR4 activation in cells (Farina et al., 2012). Other studies investigated the involvement of TLR4 in the response to PM mixtures by comparing responses of cells transfected with TLR4 and accessory proteins and cells not showing TLR4 expression (Becker et al. 2002; Shoenfelt et al. 2009). However, the HEK-Blue™ hTLR4 cell line provides an alternative, easy and rapid way to monitor TLR4 activation.

This TLR4 reporter cell line was obtained by transfection of the HEK293 cell line, derived from embryonic kidney cells. Genes coding for hTLR4, co-receptors MD-2 and CD14, and a secreted embryonic alkaline phosphatase (SEAP) reporter gene were co-transfected, rendering reporter cells sensitive for TLR4 stimulation. Activation of TLR4 leads to NF-κB activation, which allows transcription from the IL-12 p40 minimal promoter fused to five NF-κB and activator protein 1 binding sites, controlling expression of the SEAP reporter gene. In this way, SEAP will be produced and secreted in the cell medium upon TLR4 activation. Measuring phosphatase activity of the cell medium will then give an indication of production of the secreted SEAP reporter, and thus TLR4 activation. An overview of the assay principle is given in figure 6.1.

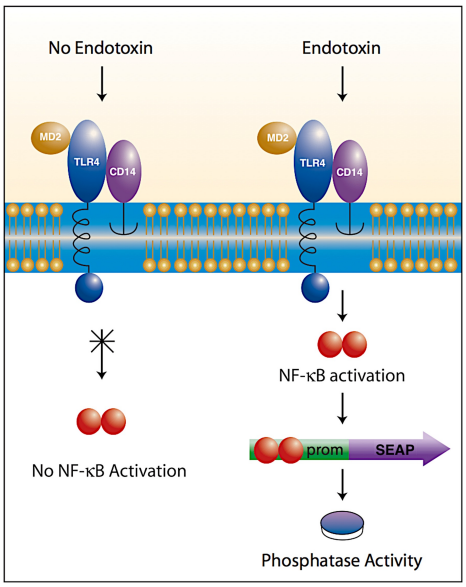

Figure 6.1: HEK-Blue hTLR4 assay principle

6.2. Materials and methods

To evaluate the ability of the air samples to stimulate TLR4 and the associated pathway, the HEK-Blue™ h-TLR4 cell line was used (Invitrogen), which was kindly donated by the lab of Prof. Rudy Beyaert (VIB inflammation research Centre).
Marijke Segers first maintained this cell line at the lab of Prof. Jos Vanderleyden (S&P research group, CMPG, K.U. Leuven), where the experiments were also performed. Afterwards, this cell line was transported and maintained at the lab of Prof. Ronny Blust (SPHERE research group, department biology, University of Antwerp).

A first experiment using the HEK-Blue™ hTLR4 cells was performed to provide proof-of-principle for production of SEAP under influence of TLR4 stimulation. Cells were grown in DMEM medium containing 4.5g/l glucose (Gibco), 10% fetal bovine serum (Perbio Science) 100µg/ml Normocin™ (Invitrogen), 1X HEK-Blue™ Selection (Invitrogen), 2mM L-glutamine (Gibco) at 37°C, 5%CO_2 and >95% humidity.
The day prior to an experiment, cells were detached by 0,05%Trypsin/ 1X EDTA treatment (Gibco). Following detachment, trypsin was deactivated by adding medium containing FBS. The cell concentration of this suspension was estimated with the trypan-blue exclusion method, using a Neubauer Improved hemocytometer. In the meantime, 11ml of the cell suspension was centrifuged at 1200 rpm for 10 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in complete DMEM growth medium to yield a density of 0,3x 10^6 cells/ml. The cells were seeded in a 12-well plate by adding 1ml of the cell suspension to each tested well. Cells were grown overnight at 37°C, 5%CO_2.
The following day, the medium was replaced by fresh DMEM medium without FCS or

antibiotics. A LPS standard, isolated and purified from *E. coli* K12 (LPS-EK, Invitrogen), was resuspended, diluted and added to the cells to yield final concentrations of 0 EU/ml, 150 EU/mL, 250 EU/ml, 500 EU/ml, 1000 EU/ml and 5000EU/ml. The cells were incubated with the standards/samples for 24h at 37°C, 5%CO_2, allowing the cells to react to the components of the standards/samples and start production of SEAP. After 24h the SEAP levels were determined by conversion of *para*-nitrophenylphosphate (pNPP) to *para*-nitrophenol, a yellow coloured product. Due to the rapid degradation of pNPP, the pNPP solution was prepared right before the read-out of each experiment and was protected from light. For 1ml of substrate solution, 1.5mg of pNPP was added to 150µl 10X substrate buffer, containing 100mM Tris-HCl, 100mM NaCl and 5mM $MgCl_2$, at pH 9.5. This was subsequently diluted to 1ml with distilled H_2O. The SEAP secreted by the cells was then quantified by taking 50µl of supernatant of each well (in duplicate), transferring it to a 96-well plate and adding 100µl of the substrate solution. After 20 minutes of incubation shielded from light, absorbance was measured at 405nm.

A second experiment, using the samples of the 2nd October, used largely the same protocol, except for the volume of sample used. For the Borgerhout and Hoboken sample, 100µl of sample was added to 900µl of fresh medium, while for the Harbour sample, 10µl of sample was added to 990µl of fresh medium. LPS isolated from *E.Coli* O55:B5, which was provided in the rFC assay kit (Lonza), was used as positive control at a concentration of 1EU/ml. As a negative control, 1000µl of medium was added to the cells. Unfortunately, due to problems culturing these types of cells, experiments could not be continued or repeated.

6.3. Results

To test the ability of the cell line to respond to a TLR4 stimulus, several endotoxin standards of known concentration were tested. This experiment showed significant elevations of the measured absorbance compared to the negative control, as can be seen in figure 6.2. Subsequently, air samples (2nd October) were tested, together with a positive and negative control. The results of this experiment can be found in figure 6.3. The responses to the samples are slightly elevated compared to the negative control, but remain much lower than the positive control.

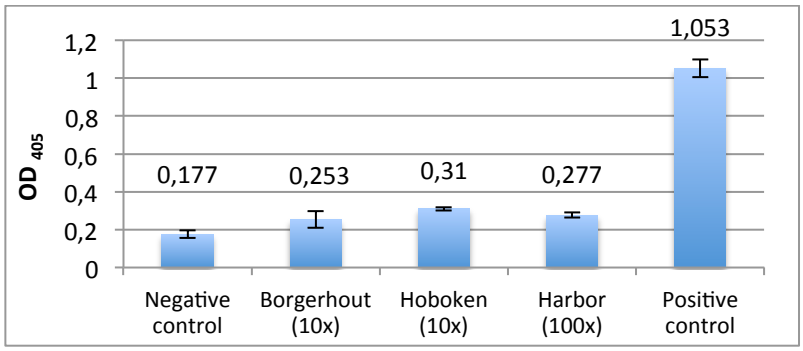

Figure 6.2: Absorbance measured at the end of the first experiment using the HEK-Blue™ TLR4 cells. In this experiment several endotoxin standards were incubated with the cells for 24h to provide proof-of-concept for the assay principle. The error bars indicate a range of one standard deviation from the values.

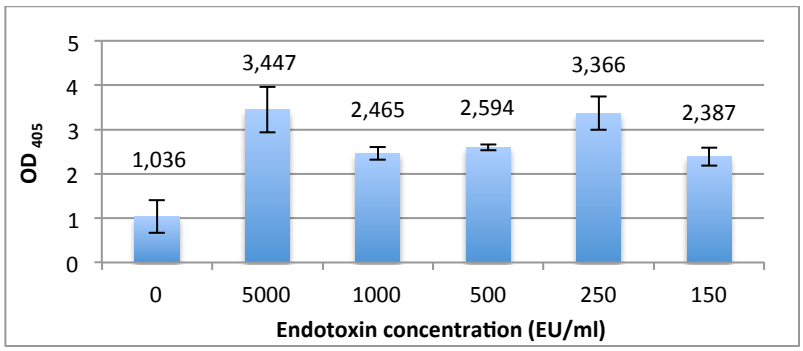

Figure 6.3: Absorbance measured at the end of the second experiment using the HEK-Blue™ TLR4 cells. In this experiment three air samples were incubated with the cells for 24h in 10-fold or 100-fold dilutions. A positive control (1 EU/ml) and a negative control were included in the assay. The error bars indicate a range of one standard deviation from the values.

6.4. Discussion

A first experiment with the HEK-Blue™ hTLR4 cell line was designed to test the ability of the cells to respond to a TLR4 stimulus, LPS, and the possibility to quantify this response by means of spectrophotometry. This experiment gave positive results, with readings of the LPS stimulated cells well above readings of the negative control. However, the readings did not show an increase in absorption proportional to the increase in concentration. It was presumed this was the result of the relatively high endotoxin concentrations used, which could cause saturation of the reaction, or saturation of SEAP production by the cell.

A second experiment tested the effect of the samples of the 2nd October 2013. In this experiment a strong increase in absorbance was again observed for the positive control (1EU/ml), as compared to the negative control, but this response did not attain the level of the previous experiment. Seeing as the signal of the positive control was caused by a much lower concentration compared to the first experiment, this supports the assumption that the previously used LPS concentrations were relatively high, leading to saturation.

Furthermore, this experiment also showed a slight increase in absorbance for the samples compared to the negative control, giving a first suggestion for the applicability of this assay with PM samples. The obtained values were still much lower than that of the positive control, which coincides with the lower concentrations of endotoxin concentrations in the samples. Additionally, the presence of inhibiting factors in the samples could also decrease their readings. Matching the data with a standard curve could possibly help to clarify this.

Regarding the differences between the samples, the lower value of the harbour sample compared to the Hoboken sample cannot be explained by concentration differences alone. Although the harbour sample was diluted more, the final tested endotoxin concentration should still have been higher. This might suggest the presence of activating factors contributing to the response, which would have been diluted out more in the harbour sample. A possible activating factor could be nickel, cobalt and palladium, which were recently shown to also activate the TLR4 pathway (Rachmawati et al. 2013) and the first two were found to be present in the samples as shown by ICP-MS analysis. However, no real conclusions concerning this issue can be made on this preliminary data alone, without any follow up experiments. Seeing as the samples were able to elicit a measurable response, this type of experiment could be extended in the future to include a standard curve of LPS concentrations and the use of LPS inhibitors (and possibly metal inhibitors) and/or spikes. On top of expanding the tested conditions, the use of the same dilution factors seems advisory, avoiding extra variance between the samples. Furthermore, the parental cell line, HEK-Blue™ Null 2 cells, which do not express TLR4 and lipopolysaccharide binding protein (LBP) could also serve as an improved negative control.
Although efforts were made to include some extra experiments with this cell line, several problems encountered in the culture of these cells did not allow this.

Chapter 7. *In vitro* analysis of biological activity of PM: pro-inflammatory capacity

7.1. Introduction

When evaluating the pro-inflammatory capacity of PM samples, finding a good model system that correctly represents the biological responses of a cell type or tissue is an essential factor in sample analysis. In this project, the use of the THP-1 cell line (ATCC(R) TIB-202(TM)), a human monocytic cell line, was examined first. The cell line is derived from an acute monocytic leukaemia of a one year old infant and can be differentiated to a macrophage-like state by treatment with certain phorbol esters (ATCC 2014; Daigneault et al. 2010; Maess et al. 2010). THP-1 cells have previously been used in literature to estimate pro-inflammatory responses of human macrophages to PM samples (McConnell et al. 2013; Longhin et al. 2013; Manzano-Léon et al. 2013; Don Porto Carero et al. 2001)

Later on another human cell line, U937, derived from a histolytic lymphoma of a 37 year old male was included in the project. These cells show various similarities with the THP-1 cells including their monocytic morphology and the ability to differentiate into a macrophage-like state upon stimulation with, amongst others, phorbol esters (ATCC 2013; Baek et al. 2009). These cells have also been used to estimate the pro-inflammatory character of PM (Bastonini et al. 2011; Kang 2010; Vogel et al. 2005; Vogel et al. 2012), however in a lesser extent than THP-1 cells.

7.2. Materials and methods

7.2.1. Evaluation of the pro-inflammatory capacity of PM with the THP-1 monocyte/macrophage cell line

7.2.1.1. Exposure of the cells to the PM samples

The THP-1 cell line was maintained in culture at the Lab of Prof. Jos Vanderleyden (S&P research group, CMPG, K.U. Leuven) by Cynthia Vargas Garcia and Ilke De Boeck. Experiments using this cell line were also performed at this lab. The basic steps of the protocol are schematically shown in figure 2.6.

THP-1 cells were cultured in complete RMPI-1640 medium (Gibco), supplemented with 10% heat inactivated FBS (Perbio Science), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 1X non-essential amino-acids (Gibco), 100µM β-mercapto-ethanol (Sigma-Aldrich), 100U/ml penicillin and 100µg/ml streptomycin (Gibco).

Before an experiment, cell concentration was estimated by trypan-blue exclusion, using a Neubauer Improved hemocytometer. The volume of cell suspension necessary for the assay was centrifuged at 1800 rpm for 5 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in complete RMPI-1640 growth medium, to which a final concentration of 10ng/mL of phorbol-12-myristate-13-acetate (PMA; Sigma Aldrich) was added. The PMA cause the THP-1 monocytes to differentiate and become adherent. Cells were seeded in a 12-well plate at a density of 10^6 cells per well and incubated overnight at 37°C, 5% CO_2.

The next day, cells were first washed with 1mL of RPMI-1640 medium (Gibco) without FBS or antibiotics. Subsequently, 1ml of diluted sample was added to each well. Samples were dissolved in RMPI medium without FBS or antibiotics with a minimum dilution of the samples of 1:10, to assure cells were not deprived of nutrients. Each sample was tested in triplicate, while positive and negative controls were tested in duplicate. LPS isolated from *E.coli* O55:B5, provided as part of the rFC assay kit (Lonza) was used as a positive control at a concentration of 1EU/ml. The cells were incubated with the samples for 2h at 37°C, 5%CO_2. After incubation, cells were washed three times with phosphate buffered saline (PBS), free of calcium and magnesium ions (Gibco), and finally resuspended in 200µl of PBS after cell scraping.

7.2.1.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the cell suspensions obtained in 7.2.1., using the High Pure RNA isolation kit (Roche), according to the manufacturer's instructions. The RNA yield was estimated using a Nanodrop spectrophotometer (Thermo Scientific). If very low RNA yields or purity were apparent from these readings, the RNA sample was not included in following steps. RNA concentrations were equilibrated by diluting the samples with qPCR-grade water (Invitrogen) to the RNA concentration of the sample with the lowest RNA yield that was included in cDNA synthesis (between 200-500ng). The Superscript™ III Reverse Transcriptase kit (Invitrogen) was used for first-strand cDNA synthesis, according to manufacturer's instructions using an oligodT_{20} primer (Integrated DNA technologies; IDT). The other components that were not included in the kit, the 10mM dNTP mix and RNaseOUT™, were purchased from Invitrogen. The obtained cDNA was diluted five fold to a total volume of 100µl by adding qPCR grade water (Invitrogen) and stored at -20°C.

7.2.1.3. Analysis of cytokine expressions with qRT-PCR

As a final step in the evaluation of the pro-inflammatory capacity of the PM samples with the use of the THP-1 cell line, cytokine expression was measured by quantitative real time PCR (qRT-PCR), using the StepOnePlus real time qPCR system (Applied Biosystems®). For

every tested gene, a mastermix was prepared according to table 7.1. Sequences of primers and probes can be found in table 7.2. These sequences were obtained from the CMPG (K.U.Leuven), which had previously been optimized for sequence and concentration, and used extensively in this lab. Mastermix (20µl) and cDNA (5µl) were added per well to a MicroAmp® Fast Optical 96-Well Reaction Plate. Each cDNA sample was tested in duplicate for each gene. A StepOnePlus qRT-PCR machine was used to perform the assay, using the pre-set 'standard run' settings of the StepOnePlus software. Data was analysed using the $2^{-\Delta\Delta Ct}$ method, as described by Livak & Schmittgen (2001).

7.2.2. Evaluation of the pro-inflammatory capacity of PM with the U937 alveolar macrophage cell line

7.2.2.1. Exposure of the cells to the PM samples

Due to unexpected results with the THP-1 cell line, an alternate cell line was included in the project, the U937 (ATCC (R) CRL-1593.2 (TM)) cell line. The experiments performed on this cell type were very similar to those performed on the THP-1 cells, but were slightly adapted according to the use of this cell line in the lab of Prof. Ingrid De Meester (Medical biochemistry research group, department pharmaceutical sciences, University of Antwerp).

The day prior to an experiment, cells of a confluent T_{75} flask were centrifuged, the supernatant was discarded and cells were resuspended in fresh RMPI-1640 medium (Gibco), supplemented with 100U/ml penicillin, 100µg/ml streptomycin (Gibco) and 100ng/ml PMA (Sigma Aldrich) to a concentration of 1.10^6 cells/ml. Cells were seeded in a 12-well plate by adding 1ml of cell suspension to each well, and allowed to differentiate and adhere overnight at 37°C, 5%CO_2.

The following day, the cells were washed with RPMI-1640 medium (Gibco), supplemented with Penicillin/Streptomycin (Gibco), and 1 ml of a ten-fold dilution of samples in the RPMI-1640 medium was added to the cells. The positive control, LPS isolated from *E.coli* O55:B5, supplied as part of rFC assay kit (Lonza), of 10EU/ml was diluted accordingly, while the negative control consisted of undiluted RMPI. Samples were tested in triplicate and controls in duplicate. After 2h incubation at 37°C, 5%CO_2, cells were washed twice with PBS (free of calcium and magnesium ions) and resuspended in 200µl PBS.

7.2.2.2. RNA isolation and cDNA synthesis

Proceeding from the cell suspension obtained in 7.2.4, cells were scraped off the surface of the wells and 400µl of lysis buffer of the High Pure RNA isolation kit (Roche) was added prior to vortexing. The 12-well plates containing the cell lysates were then transported on ice to the lab of Prof. Sarah Lebeer (ENdEMIC research group, department Bio-engineering,

University of Antwerp). Here, the RNA isolation protocol was resumed, according to manufacturer's instructions directly after transportation. In the following steps, measurements of RNA concentration and cDNA synthesis were performed identical as described in 7.2.1.2 and therefore will not be repeated here.

7.2.2.3. Analysis of cytokine expression with qRT-PCR

The final step, qRT-PCR, differed from the THP-1 protocol, primarily in final reaction volume, which was adjusted from 25 µl to 20 µl, without altering the reagent concentrations, as shown in table 7.1. The same primer and probe sequences, listed in table 2.5, were used. To a MicroAmp® Fast Optical 96-Well Reaction Plate (Invitrogen), 15µl of the mastermix was added in each tested well. Afterwards, 5µl of cDNA was added. Each cDNA sample was tested in duplicate for each gene. Again a StepOnePlus qRT-PCR instrument was used to perform the assay. The used instrument settings can be found in annex. As previously mentioned, data was analyzed using the $2^{\Delta\Delta Ct}$ method.

Table 7.1: Composition of qRT-PCR MasterMixes

Component	Concentration	Added volume per well (THP-1 cells)	Added volume per well (U937 cells and nasal RNA)	Final concentration in well
TaqMan Universal PCR Master Mix [(a)]	2X	12,5µl	10µl	1X
Forward primer [(b)]	30µM	0,25µl	0,20µl	300nM
Reverse primer [(b)]	30µM	0,25µl	0,20µl	300nM
TaqMan Probe [(b)]	5µM	1,00µl	0,80µl	200nM
PCR grade Water [(a)]	/	6,00µl	3,80µl	/
Total		20µl	15µl	

[(a)] Purchased from Invitrogen

[(b)] Purchased from IDT

Table 7.2: Used primer and probe sequences

Gene	Oligo	Sequence (5' – 3')
PPIA	Forward primer	CGC GTC TCC TTT GAG CTG TT
	Reverse primer	CTG ACA CAT AAA CCC TGG AAT AAT TC
	Taqman probe	CAG ACA AGG TCC CAA AGA CAG CAG AAA ATT T
TNFα	Forward primer	TCT TCT CGA ACC CCG AGT GA
	Reverse primer	CCT CTG ATG GCA CCA CCA G
	Taqman probe	TAG CCC ATG TTG TAG CAA ACC CTC AAG CT
IL1β	Forward primer	CTG ATG GCC CTA AAC AGA TGA AG
	Reverse primer	GGT CGG AGA TTC GTA GCA GCT GGA T
	Taqman probe	TTC CAG GAC CTG GAC CTC TGC CCT C
IL6	Forward primer	GGT ACA TCC TCG ACG GCA TCT
	Reverse primer	GTG CCT CTT TGC TGC TTT CAC
	Taqman probe	TGT TAC TCT TGT TAC ATG TCT CCT TTC TCA GGG CT
IL8	Forward primer	TGG CAG CCT TCC TGA TTT CT
	Reverse primer	TTA GCA CTC CTT GGC AAA ACT G
	Taqman probe	CAG CTC TGT GTG AAG GT

Remark: all Taqman probes used FAM as reporter dye and TAMRA as quencher dye

7.3. Results

7.3.1. Evaluation of the pro-inflammatory capacity of PM with the THP-1 monocyte/macrophage cell line

Following the partial characterization of the sample contents, their pro-inflammatory capacity was estimated using monocyte/macrophage cell lines, such as the THP-1 cell line. Exposing this cell line to the samples, and measuring expression of pro-inflammatory genes, such as TNFα, IL1β, IL6 and IL8, provided a measure of the ability of the samples to trigger an inflammatory response. In total, three experiments were performed on these cells using the samples of the 2nd October, and the 2nd and 17th December, each accompanied by positive and negative controls. The results of these experiments, in terms of fold change in gene expression, can be found in figures 7.1, 7.2, and 7.3 The main observation that can be made regarding these results is the down-regulation observed with all tested interleukins, and in most experiments also with TNFα, even with the positive controls. Only the experiment with the samples of the 2nd October showed relatively strong up-regulation in TNFα gene expression. Furthermore, almost all samples showed an undesirable high variability between the three biological repeats.-

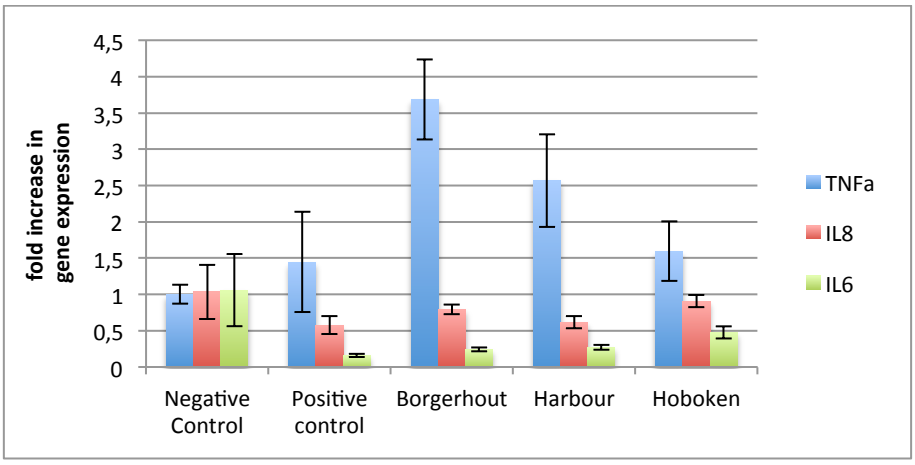

Figure 7.1: Changes in gene expression of TNFα, IL8 and IL6 of THP-1 cells exposed to the positive control (1 EU/ml) or the samples of the 2nd October, compared to the negative control, as measured by qRT-PCR. The error bars indicate a range of one standard deviation from the values.

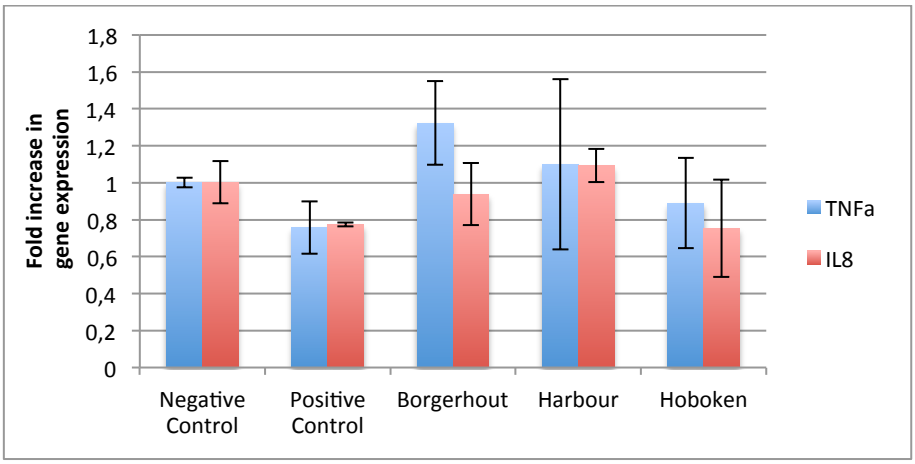

Figure 7.2: Changes in gene expression of TNFα and IL8 of THP-1 cells exposed to the positive control (1 EU/ml) or the samples of the 2nd December, compared to the negative control, as measured by qRT-PCR. The error bars indicate a range of one standard deviation from the values.

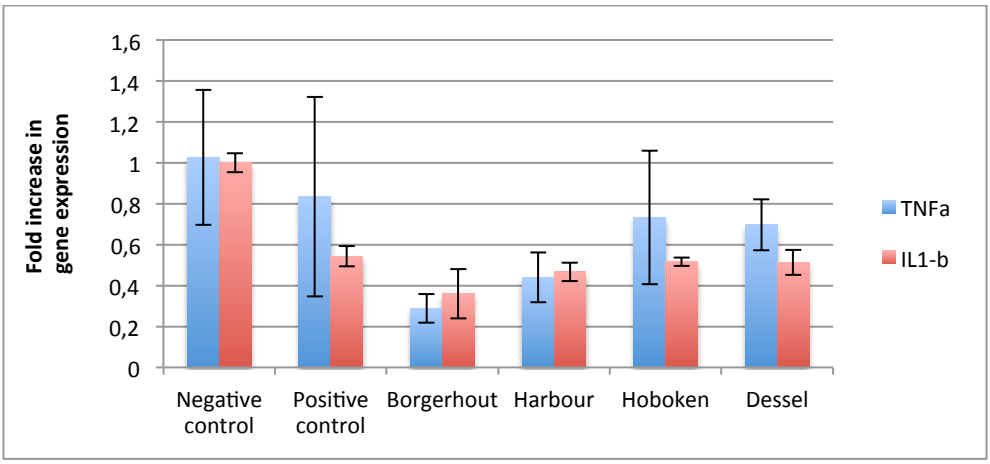

Figure 7.3: Changes in gene expression of TNFα and IL1β of THP-1 cells exposed to the positive control (1EU/ml) or the samples of the 17th December, compared to the negative control, as measured by qRT-PCR. The error bars indicate a range of one standard deviation from the values.

7.3.2. Evaluation of the pro-inflammatory capacity of PM with the U937 alveolar macrophage cell line

A second cell line, U937, was also used to investigate the pro-inflammatory potential of the collected samples. A similar principle was applied in the use of these cells as with the THP-1 cells. Again, the gene expression levels of an inflammatory response related gene, namely TNFα, was assessed by qRT-PCR after exposure of the cells to the samples. Unfortunately, only one experiment could be performed with this cell type. In this experiment, the samples of the 13th March were tested, together with a positive and negative control. The obtained gene expression data is represented in figure 7.4. In this assay, a down-regulation of TNFα gene expression levels was observed compared to the negative control.

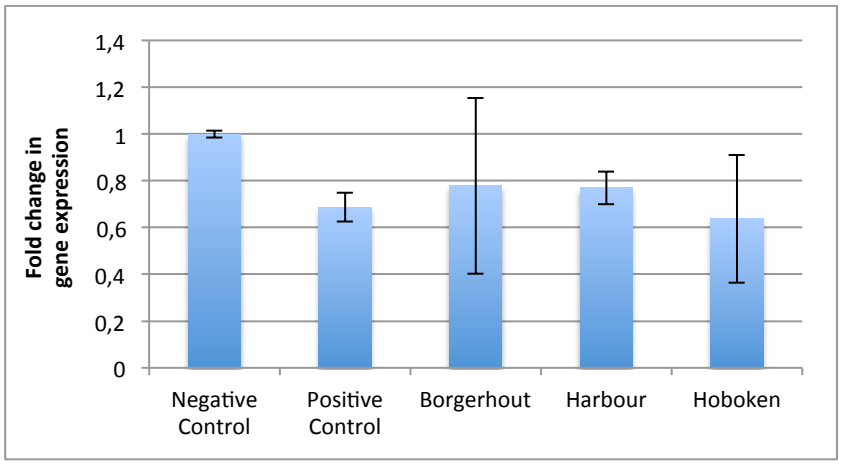

Figure 7.4: Changes in gene expression of TNFα of U937 cells exposed to the positive control (1 EU/ml) or the samples of the 13th March, compared to the negative control, as measured by qRT-PCR. The error bars indicate a range of one standard deviation from the values.

7.4. Discussion

7.4.1. Evaluation of the pro-inflammatory capacity of PM with the THP-1 monocyte/macrophage cell line

A first experiment evaluating the ability of tested PM samples to trigger expression of pro-inflammatory cytokines used the samples of the 2nd October and showed an up-regulation of TNFα and down-regulation of IL6 and IL8. This down regulation was not expected as several other studies had shown increased production of these three cytokines, as was indicated in table 1.3 in section 1.3.3.4. It should be noted that these studies measured cytokine production at protein level, and not at mRNA level as was performed in this project.

Regarding the TNFα responses, the highest value was found for the Borgerhout sample, while this sample had lower endotoxin concentrations than both other samples, lower metal concentrations than the Hoboken sample and showed the weakest TLR4 stimulation, taking

the dilution factors into account. This could suggest another component in the sample, which contributed to the increase in TNFα expression. On the other hand, the Hoboken sample, which showed the strongest TLR4 stimulation and contained the highest amount of metals, showed the weakest TNFα response. However, the harbour sample was diluted a factor of 10 more than the other samples, so possibly this sample still contains the strongest pro-inflammatory potential. A second observation of the TNFα expression data is the relatively high variances between biological repeats for both the samples and the positive control. This could potentially give a false impression of the obtained results and therefore caution should be taken when making conclusions.

The data on the interleukins gives a very different view on the samples. All three samples and even the positive control showed diminished expression of both IL6 and IL8, with the strongest down regulation for IL6. This seemed an unlikely result, since both the samples and definitely the positive control are expected to elicit a pro-inflammatory response.

On the other hand, the negative control, to which the other data is compared in the $2^{-\Delta\Delta Ct}$ method, shows an abnormally high variance between biological repeats, especially for IL6. This will, in all likelihood, negatively influence the results of the other tested conditions.

Two extra experiments were performed using the samples of the 2nd and the 17th December, to test whether the same response was observed. The samples of the 2nd December showed a slight up regulation of TNFα in the Borgerhout sample and an even smaller increase in both TNFα and IL8 gene expression was elicited by the harbour sample. All other samples of both the 2nd and the 17th December showed down regulation of TNFα mRNA levels, with the latter giving the strongest decrease. The samples of the 17th December were tested for expression of IL1β as an alternative target, and all samples induced a down-regulation. High variances between biological repeats were again a common observation in both experiments.

These winter samples all had relatively low endotoxin contents, and combined with the dilution factor necessary to provide the cells with enough nutrients and thus medium, a lower response as compared to the previous experiment was expected. On top of this, it has been shown that PM collected in winter showed a decreased pro-inflammatory potential (Farina et al. 2013). Nonetheless, a resultant down regulation was not foreseen. Additionally, the positive controls also showed diminished expression of the tested cytokines, which normally should not be observed. The unexpected results and high variances between biological repeats suggested a problem within the assay itself.

Firstly, the use of the THP-1 cell line might not accurately represent responses elicited by human macrophages in their natural environment. The THP-1 cells are known to give highly variable results with limited reproducibility and sometimes do not respond well to endotoxin challenge (verbal communication).

Secondly, the observed problems could also be rooted in the set-up of the assay. For instance, the time frame between stimulation of the cells with PMA to differentiate, and the initiation of the experiment might be too short. If the PMA over stimulates the cells and they haven't fully recovered before starting the experiment, this might influence gene expression. Furthermore, the used culture conditions could be a source of constant exposure to endotoxins desensitising the cells for this stimulus, since some components added to the culture media, such as fetal bovine serum, are known to contain endotoxins as high as 50 EU/ml (Ryan 2008; Scientific 2012; Gibco 2007). Additionally, an alternative explanation could be an inappropriate incubation time of the cells with the samples. If, within this time frame, a strong initial increase in mRNA levels of pro-inflammatory cytokines is followed by a compensatory decline, it is possible that the latter is observed in the experiments performed here. This could possibly be demonstrated with by larger experiment including different incubation times.

Finally, if the expression of the endogenous control is affected by exposure to the samples, this would invalidate the use of the $2^{-\Delta\Delta Ct}$ method. As can be seen in the unprocessed results of the qPCR (can be found in annex), some variance is present in the detected levels of PPIA throughout the cDNA samples. Therefore, the use of an alternate, better endogenous control or using two could strengthen the results of future experiments.

7.4.2. Evaluation of the pro-inflammatory capacity of PM with the U937 alveolar macrophage cell line

An additional macrophage cell line, U937, was included in the project to investigate the potential of the samples to trigger cytokine expression and possibly complement the data obtained in the THP-1 cell experiment.

Unfortunately due to time constraints, only one experiment could be performed with these cells, which measured the TNFα response upon exposure to the samples of 13th March. Again the samples and positive control induced a down regulation in TNFα expression, with high variances between biological repeats for Borgerhout and Hoboken samples. These results suggested that the assay problems did not originate from the use of a suboptimal model system for this type of assay. In all likelihood, the problems observed with these results, as well as with the results of the experiments using the THP-1 cell line, originate from the previously mentioned issues with the assay set-up or a combination thereof. This seems the most likely option, regarding the vast amount of evidence supporting production of pro-inflammatory cytokines by macrophages upon PM exposure.

Chapter 8.
Nasal respiratory epithelium as model for inflammatory responses to air pollution

8.1. Introduction

The use of *in vitro* models to evaluate biological responses have some important advantages, including the price, rapid regeneration of cells and reproducibility of experiments, which contribute to the frequent application of this useful tool in PM research. However, despite these advantages, the use of these models also has some significant limitations. Most importantly, they do not necessarily represent the human responses accurately. Responses of isolated immortalized cell lines might differ from responses of cells of the same cell type in their natural environment. On the one hand, immortalized cells might react differently than native cells, for instance because of differential receptor expressions. On the other hand, interactions between various cell types within the exposed tissue, like macrophages and respiratory epithelial cells, can also influence the provoked response (Genc et al. 2012). Seeing as PM research always investigates the co-exposure of several pollutants, this could possibly be an important issue. Although these *in vitro* models are a valuable tool, they cannot replace exposure studies on human subjects. However, these often include invasive techniques such as induced sputum collection (Baines et al. 2011; Behbod et al. 2013). The nasal respiratory epithelium could possibly provide a non-invasive alternative to evaluate biological responses to air pollution. Sampling of the upper airway epithelium is an easy and relatively non-invasive method which may be applied to young children (Giovannini-Chami et al. 2012), and is therefore preferred above sampling of bronchial epithelium. Furthermore, it was suggested by McDougall et al. (2008) that nasal epithelial cells could be used as a surrogate for studying lower airway inflammation. Supporting this, some studies investigating nasal and bronchial gene expression found similar patterns for both cell types (Giovannini-Chami et al. 2012; Zhang et al. 2010)Therefore, some first steps were made in this project to investigate if and how nasal epithelium samples could be used for this purpose. If feasible, collection of nasal epithelial cells could later be applied in a project of the ENdEMIC research group of the University of Antwerp (Department of bioscience engineering), on the involvement of TLR receptors in the human response to air pollution. The proposed experimental set-up of one of the work packages includes collection of nasal samples before and after a walk in an environment with highly polluted air for transcriptome analysis, while simultaneously collecting PM concentration data and PM samples for later analysis on endotoxin content.

8.2. Materials and methods

8.2.1. Sampling of nasal respiratory epithelium and RNA isolation

To investigate the applicability of the use of nasal epithelial cells in this type of research, nasal epithelial cells were sampled from healthy volunteers by brushing the inferior turbinates, under guidance of Dr. Kristine De Sager and after the approval of the ethical committee. Nasal samples were collected with a FLOQSwab™, included in an eSwab™ package (Copan). This swab has a flocked tip, consisting of nylon fibres, enhancing the amount of specimen collected in a thin adsorbent layer and allows rapid elution of the specimen (Copan n.d.). The flocked swab tip is shown in figure 8.1

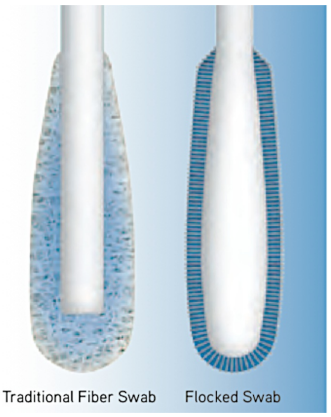

Figure 8.1: Comparison of traditional fibre swab and flocked swab. Reference: Copan (n.d.)

After the cells were collected onto the swab, different treatments, as listed in table 8.1, were tested to investigate treatments suitable for a satisfactory RNA yield with ease of use. The RNeasy Mini kit (Qiagen) was used, according to the manufacturer's instructions, to extract RNA larger than 200 nucleotides from the nasal samples. This provides an enrichment of mRNA in the isolated RNA, compared to traditional extraction of total RNA. The suggested DNase treatment was included in the protocol, using the RNase-Free DNase Set (Qiagen) according to manufacturer's instructions. RNA was eluted in 30µl of RNase free water, included in the kit, and stored in aliquots at -80°C until proceeding with cDNA synthesis. The RNA concentrations were estimated using a Nanodrop Spectrophotometer. Beforehand, the samples were diluted 10-fold in final concentration of 10mM tris-hydroxymethyl-aminomethane (TRIS) buffer (pH 7.5) and then used for nanodrop measurement as recommended by QIAgen. The A_{260}/A_{280} ratio is influenced considerably by pH, therefore measuring in unbuffered water may reduce sensitivity to protein contamination. Samples with a sufficient yield were equated to the same RNA concentration and converted to cDNA with the Superscript® III Reverse transcriptase kit (Invitrogen) according to manufacturer's instructions for first strand cDNA synthesis, as described in 7.2.2.2. cDNA samples were stored at -20°C for future analysis.

Table 8.1: Tested treatments of nasal samples for RNA isolation

Sample	Swab placed in	Storage	Remarks
Experiment 1			
Sample 1	Amies medium	-80°C, several days	RNA isolated from swab tip [(a)]
Sample 2		Swab medium	RNA isolated from medium [(a)]
Sample 3		Both	RNA isolated from both [(a)]
Sample 4	Lysis buffer	Proceeded directly to RNA isolation	Small swab tip
Sample 5		Proceeded directly to RNA isolation	Normal swab tip
Experiment 2			
Sample 1-3	Lysis buffer	Stored on ice for 30 minutes	Performed on 3 test subjects [(a) (b)]
Sample 4-6	Lysis buffer	Proceeded directly to RNA isolation	Performed on 3 test subjects [(a) (b)]

(a) Swab with normal size tip was used for the isolation of the sample

(b) Sampling was performed on the same three test subjects, using one nostril in the first isolation and the other in the second isolation

8.2.2. Evaluation of primer efficiency

In a first assay, primer efficiency was tested, using a 5 different cDNA concentrations of one cDNA sample, added in the q-PCR reaction mix, falling in the range suggested by the manufacturer for gene expression analysis. The amounts of added cDNA included 50ng, 25ng, 12.5 ng, 6.25ng, and 1 ng.

An easy method to evaluate primer efficiency is to plot the obtained C_T values against the used amounts of cDNA, of which the slope should lie between -3.58 and -3.10. The primer efficiency can be calculated from the slope by using the following equation:

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1$$

Furthermore, when data is analyzed by the $2^{-\Delta\Delta Ct}$ method, it is important that the primer efficiencies for the tested gene and the endogenous control are as identical as possible. In the previous graph, this would represent two perfectly parallel lines connecting the data of the two genes. Additionally, if primer efficiencies are comparable, subtracting the C_T genes of the one gene from the other, and plotting this against the logarithmic values of the added cDNA amount, should yield a horizontal plot with a slope smaller than 0.1. (Life technologies 2012; Qiagen 2010).

8.3. Results

8.3.1. Sampling of nasal respiratory epithelium and RNA isolation

The possibility of estimating responses of human subjects to air pollution by analyzing gene expression in nasal samples was investigated. To this end, RNA of cells collected by brushing the inferior turbinates was isolated, using different swab tip sizes and storage possibilities. RNA yields obtained by the different methods can be found in table 8.2. RNA samples with sufficiently high yields were converted to cDNA for storage and later evaluation of gene expression of TNFα and PPIA, as endogenous control, with qPCR.

Table 8.2: RNA yields and integrity of nasal samplings with various treatments

Sample	Method	RNA concentration (ng/µl)	OD_{260}/OD_{280}	OD_{260}/OD_{230}
Experiment 1: 27th March				
1	Medium (defrosted)	2	-0.4	0.05
2	Medium and normal swab (defrosted)	6.7	3.46	0.31
3	Normal swab (defrosted)	5.7	1.67	0.07
4	Swab with small tip (fresh)	31.8	2.33	0.71
5	Normal swab (fresh)	118.7	2.25	1.27
Experiment 2: 17th April				
1	New swab (normal tip), kept on ice, in lysis buffer for 30 minutes	45.78 (volunteer x)	2.09	0.67
2		23.17 (volunteer y)	2.00	0.32
3		90.39 (volunteer z)	2.09	1.72
4	Normal swab (fresh)	156.50 (volunteer x)	2.09	0.90
5		68.74 (volunteer y)	1.98	0.88
6		87.29 (volunteer z)	2.03	1.54

8.3.2. Evaluation of primer efficiency

The primer efficiency of a qRT-PCR reaction is an important parameter in the assay performance. Ideally, primer efficiencies reach 100% but efficiencies ranging from 90% to 110% percent are regarded as acceptable. When primer efficiencies fall outside this range, this can skew results leading to invalid conclusions, and affect assay sensitivity. Possible causes for a low efficiency can be suboptimal reagent conditions, too large difference between primer melting points, enzyme quality, suboptimal thermocycling conditions and amplicon variables such as length, secondary structure and GC content. An efficiency above 110% most likely results from the presence of PCR inhibitors, poor RNA or DNA quality, high template concentration or carryover from nucleic acid purification (Life technologies, 2012; Qiagen, 2010).

The efficiency of the primers was evaluated by performing qPCR on 5 different cDNA concentrations of one sample, more specifically the sample with the highest RNA yield

isolated the 27th March. The results of this assay are plotted in figure 8.2. The experiment showed a primer efficiency of 87% for PPIA and 99% for TNFα. To compare the efficiencies of the primer and probe set of the two genes, the difference in their C_T values is plotted against the logarithmic transformation of the used cDNA amount, as shown in figure 8.3. The slope of this curve should not exceed 0.1, which was not the case. This is also reflected in the graph, showing the C_T values of both genes with the logarithmic transformation of the used cDNA amount. The linear model fits the data nicely, and the curves lie parallel.

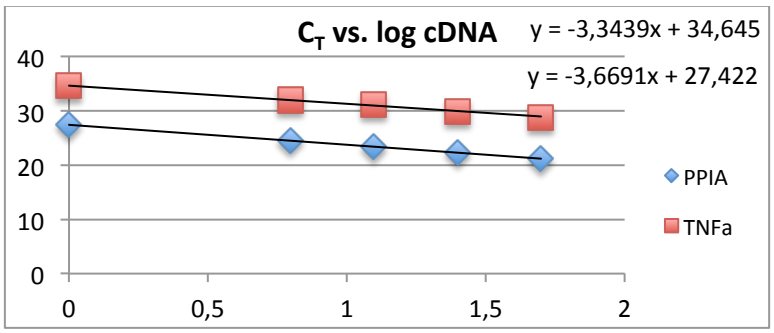

Figure 8.2: Plot of measured C_T values against the logarithmic transformation of the added amount of cDNA in the reaction volumes, in the primer efficiency assay. When acceptable efficiencies were obtained, the slope of these plots should lie between -3.58 and -.3.1 (Life technologies, 2012).

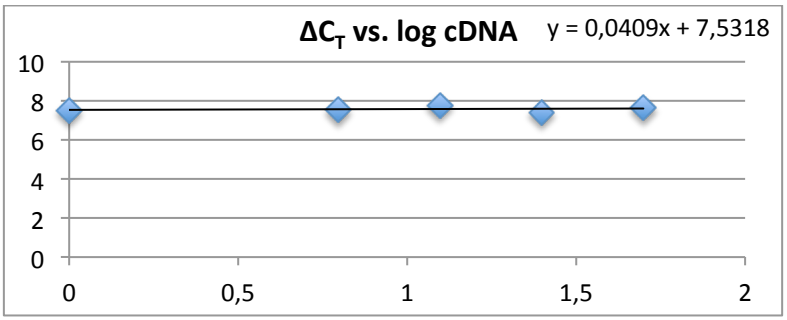

Figure 8.3: Plot of the difference in measured CT values between PPIA and TNFα against the logarithmic transformation of the added amount of cDNA in the reaction volumes, in the primer efficiency assay.

8.4. Discussion

8.4.1. Sampling of nasal respiratory epithelium and RNA isolation

A first goal of the work with the nasal samples was to determinate if RNA could be obtained in a sufficient yield, and integrity, using the different applied methods with variations in used sample tip and storage conditions. Although not all methods were as successful, it was possible to isolate RNA in a relatively high concentration with minimal protein contamination. The highest RNA yields were obtained when RNA isolation was performed directly after collection. However, in most experiments this will not be possible, seeing as collection of the nasal swabs and RNA isolation will likely not be executed in the same space at the same time, requiring storage of the samples. Freezing the samples in the supplied medium

(eSwab, Copan) at -80°C instantly after collection did not adequately preserve the RNA, leading to very low RNA yields. Therefore, this preservation method was not suited for this application. Nonetheless, storage of the swab tips in lysis buffer (from RNA isolation kit), on ice for a limited time (30 minutes) before proceeding to RNA isolation showed to be an acceptable storage method. Although RNA seems to drop slightly during storage, the obtained RNA concentrations remained sufficiently high.

Besides the storage treatments, the influence of the tip size of the used swab on the RNA yield was investigated by using two different swabs with either a normal sized swab tip or a smaller one. Although a much higher RNA concentration was obtained with the normal tip, the RNA concentration obtained from the smaller tip was acceptable for further analysis. Since the discomfort during sampling is reduced with the use of the smaller tip, these might be better suited when collecting samples from children. On the other hand, since both the storage and the use of swabs with the smaller tip size both reduce the amount of isolated RNA, it should be tested whether RNA concentrations aren't diminished too strongly when these are used together.

8.4.2. Evaluation of primer efficiency

As an important factor in the quality of a qRT-PCR analysis, the primer efficiencies for the two tested genes, PPIA and TNFα were evaluated. The slopes of the plots of the C_T-values against the logarithmic transformations of the added cDNA show that although the efficiency for TNFα (99%) was in the optimal range, this was not the case for PPIA, which showed a lower efficiency (87%). This was not expected, seeing as optimization of these primers had previously been performed at the CMPG (K.U. Leuven). However, some important differences exist between the experiments for which they were optimized and the one described here. Firstly, while here RNA was extracted from freshly isolated human tissues, in the reference experiment RNA was extracted from cells grown in culture. Furthermore, a different kit for RNA isolation was used, and qRT-PCR was performed in an alternate reaction volume.

As mentioned in section 8.3.2, decreased primer efficiency might be due to various factors, including suboptimal primer concentrations. This can be easily confirmed and solved by designing an experiment using various primer concentrations. However, if this problem persists, it might be advisory to use an alternate endogenous control.

Furthermore, it was tested if the two primer efficiencies were comparable, as this would influence results analysed by the $2^{-\Delta\Delta Ct}$ method. This could be analysed with the plot in figure 8.3. Seeing as the slope of the curve is lower than 0.1, the primer efficiencies are comparable, allowing for analysis by the $2^{-\Delta\Delta Ct}$ method

Chapter 9.
Conclusion

The main purpose of this project was the evaluation and optimization of several new or altered methods for the investigation of the pro-inflammatory capacity of PM and the relative importance of endotoxins and transition metals therein. To this end, several techniques, represented in figure 9.1, have been applied, with various successes and future recommendations.

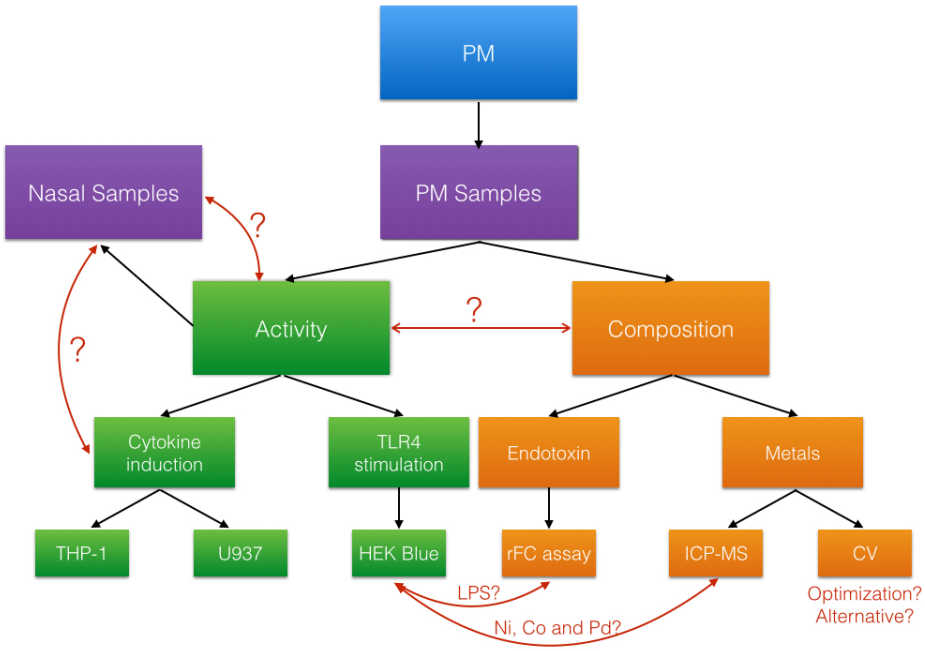

Figure 9.1: Diagram of performed experiments and possible research questions.

Firstly, extensive optimization was performed for two methods used here, more specifically sampling of airborne PM and endotoxin quantification with the rFC assay. A first and crucial step in the project was the sampling of PM with the Coriolis® μ, so as to optimize maximal recovery for endotoxins. In the future, it might be recommended to design an experiment investigating the influence of all the implemented steps on the final endotoxin recovery. Nevertheless, since procedures recommended for collection of transition metals and endotoxins often contradict (e.g. use of plastics vs. use of glassware), this may have coincided with a reduced efficiency for sampling of transition metals, although to what extend is unknown.

Secondly, the rFC assay was successfully optimized to accurately represent endotoxin levels in the collected samples and to reduce variability, as shown by the obtained standard curves. The use of spikes was integrated in the assay protocol to check for inhibition or

enhancement by the other PM components, as their influence on the rFC assay has not previously been thoroughly characterised. Since the experiments performed in this project did suggest the presence of interfering factors, it might be worthwhile to investigate this issue further.

Two other techniques providing information on the composition of the PM samples were ICP-MS, to determine transition metal concentrations, and CV, to determine the redox state of some of these metals. ICP-MS was performed by another lab by experts and had already been optimized for this purpose. On the other hand, the use of cyclic voltammetry was not as successful and therefore could not easily be integrated with the other assays. In order to adopt this technique in PM research in the future, in all likelihood it will need some significant optimization, including testing the effect of storage conditions, which might have been an issue in this project.

After analysis of the sample composition, the biological activity of the samples was investigated, including the ability of the samples to stimulate TLR4. The use of the HEK-Blue hTLR4 cell line for this purpose showed promise, although the culture of these cells restricted their further use. Therefore, if culture conditions would be optimized, this cell line might be a useful tool to investigate TLR4 activation in the future.

To determine whether the tested components indeed have a strong influence over the pro-inflammatory capacity of the sample, a good model is necessary to estimate these effects. While often used in literature, the application of monocyte/macrophage cell lines to evaluate the induction of pro-inflammatory cytokines by the samples did not produce results in accordance with other studies. As this might be due to the different level at which responses were measured (protein vs. mRNA) and assay conditions such as incubation time and sample concentration, protocol optimization might eliminate these problems in the future.

A last investigated technique was the collection of nasal epithelial samples to estimate biological responses to PM. Encouragingly, some positive first results were obtained here, supporting the continuation of method development. In the future, it should be confirmed that the sample storage in lysis buffer doesn't affect RNA integrity, the baseline expression of the tested cytokines is high enough to measure, and whether it is possible to observe significant changes in gene expression of the tested cytokines. Furthermore, to improve the quality of the assay, although often neglected in literature, primer efficiencies should be optimized and the use of an alternate endogenous control or a combination thereof should be considered.

Evaluation of these techniques, illustrated in figure 9.1, has generated some research questions, which could be investigated when methods are optimized

- Can optimization of cyclic voltammetry be performed and is there an alternative method to determine the oxidative capacity of the PM sample?
- Does TLR4 stimulation correlate with the endotoxin concentrations in the tested sample?
- Does the transition metal component of the sample contribute to the TLR4 response, and to what extent?
- Can nasal samples be used to evaluate biological responses to PM? And do these responses relate to the observations made with the used *in vitro* models?
- Lastly, can a link be found between the biological activity of a sample and it's composition, including possible synergistic effects?

In the future, answers to these questions could possibly contribute in providing insights in the importance of endotoxins and/or metals in the pro-inflammatory capacity of PM.

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Addendum

Annex 1: Sources and effects of airborne metals

Table 1: Common airborne metals, their origin and the health effect they might cause

Metal	Origin	Health effects
Arsenic (As)	Metallurgical industry, combustion of fuels, pesticides, tobacco smoke (EEA 2013a; Manalis et al. 2005)	Cardiovascular effects, neuropathy, gangrene of extremities, cancer of skin and lungs (EEA 2013a; Manalis et al. 2005)
Cadmium (Cd)	Soil dust and fires, non-ferrous metal production, stationary fossil fuel combustion, waste incineration, iron and steel production, cement production and road transport (EEA 2013a; Manalis et al. 2005)	Impaired kidney function, increased risk of osteoporosis, increased risk of lung cancer (EEA 2013a; Manalis et al. 2005)
Chromium (Cr)	Metallurgical industry and oil refineries (Manalis et al. 2005)	Toxic and carcinogenic effects of bronchial tree (Manalis et al. 2005)
Copper (Cu)	Metal production and other industrial processes. Also wear of vehicle's brake linings (Manalis et al. 2005)	Respiratory irritancy (Manalis et al. 2005)
Iron (Fe)	Suspension of mineral dust, metal industry and wear of vehicles (Vlaamse milieumaatschappij 2010)	Inducer of oxidative stress (Valko et al. 2005)
Mercury (Hg)	Combustion of coal and other fossil fuels. Metal production, cement production, waste disposal, cremation and gold production. (EEA 2013a)	Damage to liver, kidneys, digestive and respiratory systems. Also brain and neurological damage and growth impairments.(EEA 2013a)
Manganese (Mn)	Suspension of crustal particles, industrial activities, and generic engine wear (Manalis et al. 2005; Schauer et al. 2006)	Neurotoxic impairments (Manalis et al. 2005)
Nickel (Ni)	Wind-blown dust, vegetation. Combustion of oil, nickel mining and primary production, waste incineration and sewage sludge, steel manufacturing, electroplating, coal combustion and road transport (EEA 2013a; Manalis et al. 2005)	Cancer of the lung, nose, larynx or prostate. Furthermore; allergic skin reactions, disruption of endocrine regulation, and damage to the respiratory tract and the immune system. (EEA 2013a; Manalis et al. 2005)
Lead (Pb)	Soil dust and sea spray, forest fires. Fossil fuel combustion, waste incineration and production of non-ferrous metals, iron, steel and cement. Previously leaded gasoline. (EEA 2013a; Manalis et al. 2005)	Neurotoxic effects: serious brain damage and impairment of neurodevelopment in children. Furthermore: damage of kidneys, liver, brain and nerves. (EEA 2013a; Manalis et al. 2005)
Vanadium (V)	Combustion of crude or residual oil (Manalis et al. 2005)	Health effects of the respiratory tract (Manalis et al. 2005)

Annex 2: Photo's of sampling sites

Borgerhout:


Harbour:


Hoboken:


Dessel:

Use of MicroDust Pro in music school:

Annex 3: Background information on sampling conditions

Table 2: Sampling date and details (continued)

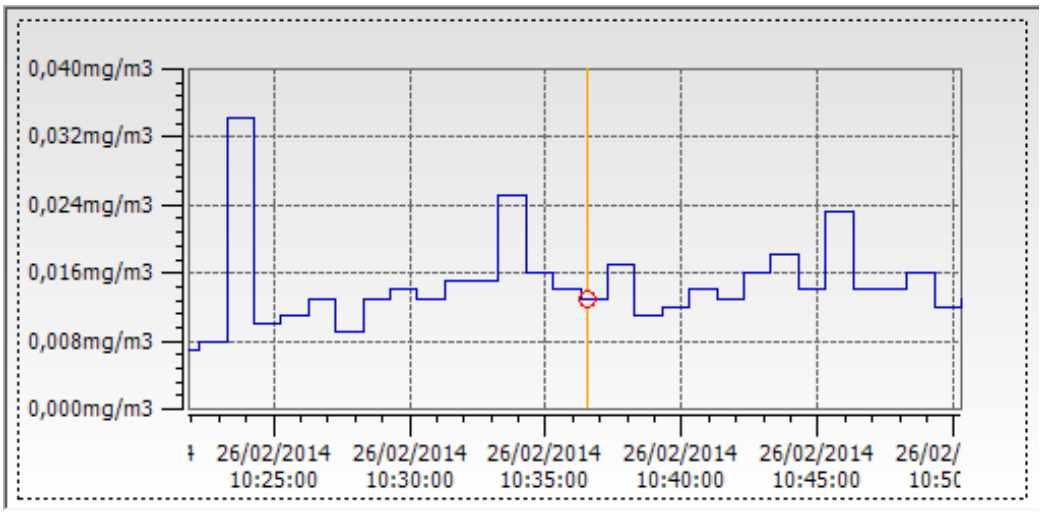
	Sampling site	Name	Sampling time (min)	Air intake (L/min)	End volume (ml)	ambient PM10 conc. (µg/m3)	Ambient PM2.5 conc. (µg/m3)	Black Carbon conc. (µg/m3)	Temperature (°C)	Humidity (%)	Remarks
25 September 2013	Borgerhout	B1.1	10	250	15	86	64	10,0	17,4	85	Misty Misty
		B1.2	30	250	15	86	64	10,0	17,4	85	
	Harbour	H1.1	10	250	15	72	/	9,3	17,4	85	
		H1.2	30	250	15	72	/	9,3	17,4	85	
	Hoboken	Hb1.1	10	250	15	62	/	/	17,4	85	Sunny, construction works further down the road
		Hb1.2	30	250	15	62	/	/	17,4	85	
2 October 2013	Borgerhout	B2.1	20	250	15	40	25	5,2	7,5	83	Some clouds, light to moderate wind
		B2.2	20	250	15	55	25	4,7	7,5		
	Harbour	H2.1	20	250	15	42	/	2,9	16,1	64	
		H2.2.	20	250	15	37	/	2,8	16,1		
	Hoboken	Hb2.1	20	250	15	35	/	/	19,8	54	
		Hb2.2.	20	250	15	28	/	/	19,8		
25 October 2013	Borgerhout	B3	20	250	20	34	10	5,2	14,1	85	Rain before sampling Cloudy, moderate to strong wind
	Harbour	H3	20	250	20	/	/	/	16,4	81	
	Hoboken	Hb3	20	250	20	25	/	/	18,8	77	
6 November 2013	Borgerhout	B4	22,5	250	20	47,5	22	5,5	9,0	86	Light rain to heavier rain, light wind
	Harbour	H4	20	250	20	17	/	1,5	9,0	86	
	Hoboken	Hb4	20	250	20	12	/	/	10,7	91	
	Dessel	D4	20	250	20	7		1,6	10,7	91	
13 November 2013	Borgerhout	B5	20	250	20	65	20,5	12,06	3	94	Clear sky, light wind

Table 2: Sampling date and details (continued)

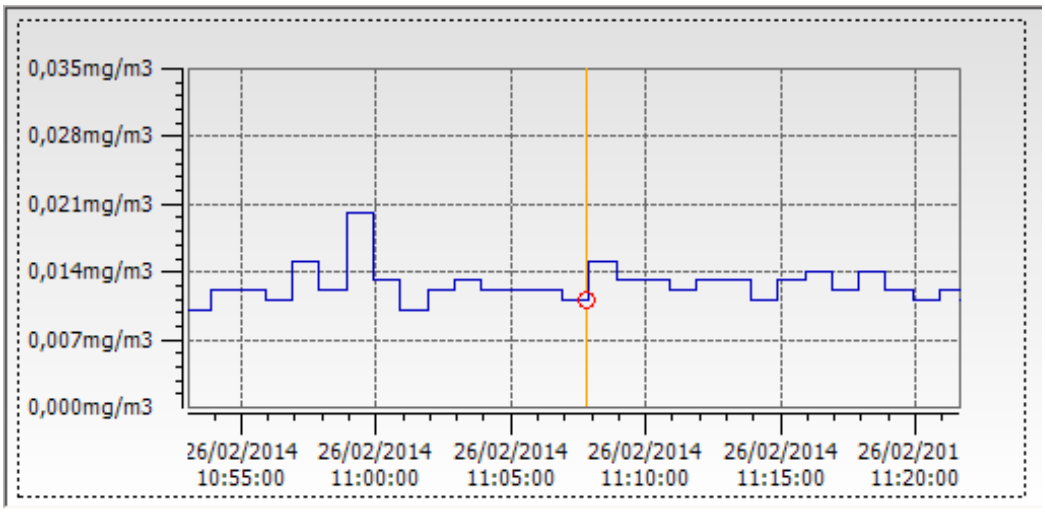
	Sampling site	Name	Sampling time (min)	Air intake (L/min)	End volume (ml)	ambient PM10 conc. (µg/m3)	Ambient PM2.5 conc. (µg/m3)	Black Carbon conc. (µg/m3)	Temp. (°C)	Humidity (%)	Remarks
	Harbor	H5	20	250	20	42	/	6,87	5	94	Clear sky, light wind
	Hoboken	Hb5	20	250	20	36	/	/	7,1	95	
	Dessel	D5	20	250	20	13	/	0,84	10,7	66	
2 December 2013	Borgerhout	B6	20	300	20	49	14	3,2	6,0	86	Clear sky, light wind
	Harbour	H6	20	300	20	29	/	0,2	7,4	76	
	Hoboken	Hb6	20	300	20	32	/	/	8,9	65	
17 December 2013	Borgerhout	B7	20	300	20	49	25	6,7	7,7	72	Cloudy, no rain Light wind
	Harbour	H7	20	300	20	?	/	3,8	7,7	72	
	Hoboken	Hb7	20	300	20	34	/		7,7	75	
	Dessel	D7	20	300	20	31	/	3,1	8,0	77	
13 March 2014	Borgerhout	B8	20	250	20	118	86	5,81	18	42	Smog episode Sunny, no wind
	Harbour	H8	20	250	20	67	/	4,25	18	42	
	Hoboken	Hb8	20	250	20	119	/	/	18	42	

Annex 4: Real-time data recorded with MicroDust Pro

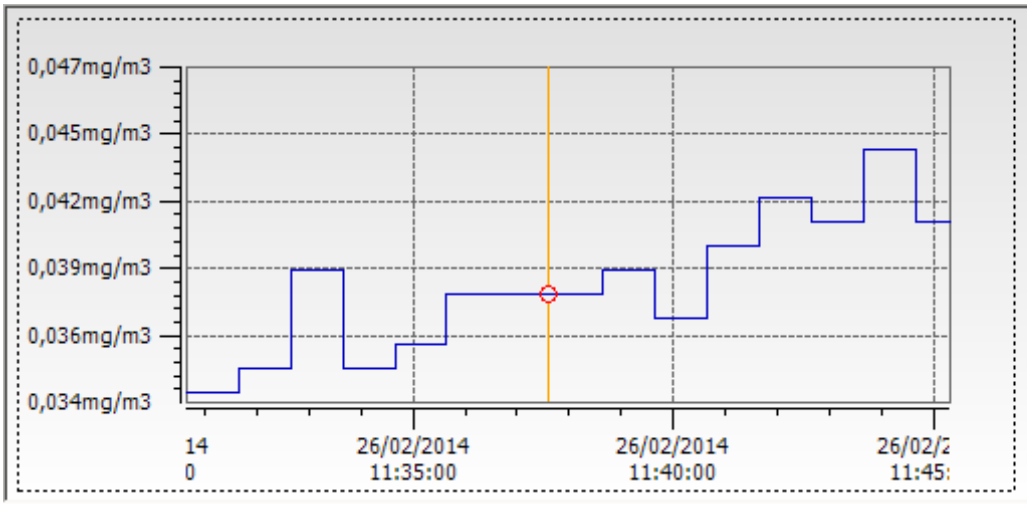
Real time PM10 concentrations of the music hall



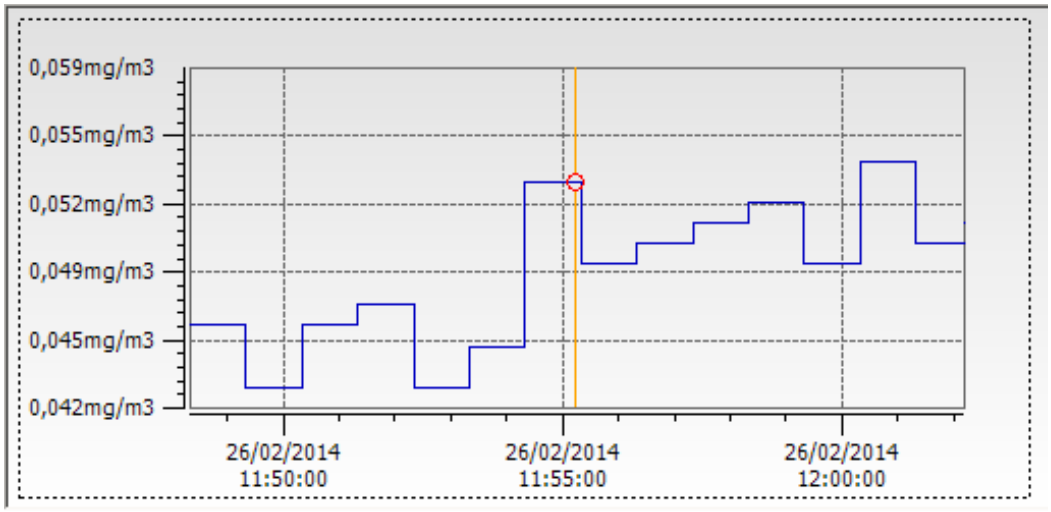
Real time PM2.5 concentrations of the music hall



Real time PM2.5 concentrations outside of the music hall



Real time PM10 concentrations outside of the music hall



Annex 5: Unprocessed qRT-PCR data

Table 3: Unprocessed qRT-PCR data

Sample			2 October 2013					2 December 2013			17 December 2013			13 March 2014	
Date qRT-PCR			4 December 2013			28 February 2014		13 December 2013			19 March 2014			17 April 2014	
Cell line			THP-1											U937	
Condition	BR	TR													
Gene			PPIA	TNFα	IL8	PPIA	IL6	PPIA	TNFα	IL8	PPIA	TNFα	IL1β	PPIA	TNFα
Negative Control	1	1	20,014	25,616	18,856	22,102	27,281	20,935	24,958	19,937	21,279	27,464	30,541	20,875	26,718
	1	2	19,918	25,719	19,091	ND	28,178	20,902	25,051	20,243	21,230	27,765	30,666	20,952	26,790
	2	1	20,550	26,500	18,720	21,391	28,117	20,714	24,933	20,132	21,004	28,243	30,749	20,617	26,459
	2	2	20,614	26,521	18,487	21,408	27,929	20,814	24,875	20,206	21,350	28,166	30,493	20,883	26,517
Positive control	1	1	19,948	24,788	19,505	22,364	29,958	20,763	25,079	20,316	20,474	26,626	29,447	20,351	26,452
	1	2	19,962	24,907	19,532	21,953	ND	20,668	25,022	20,402	21,281	26,459	29,521	20,068	26,634
	2	1	21,408	27,265	20,526	22,294	30,976	20,848	25,451	20,523	21,088	27,878	29,369	20,356	26,738
	2	2	21,434	26,792	20,409	21,566	30,722	20,781	25,615	20,455	20,749	27,826	29,311	20,026	26,907
Borgerhout	1	1	20,648	24,533	19,533	20,767	29,326	20,338	23,830	19,438	21,950	26,849	28,415	21,566	27,378
	1	2	20,491	23,941	19,451	20,746	28,816	20,819	23,797	19,573	20,889	26,461	28,922	21,514	27,452
	2	1	20,878	24,619	19,661	21,685	29,126	20,740	24,578	20,178	20,560	25,282	28,975	20,158	26,308
	2	2	20,997	24,427	19,626	21,198	29,761	20,875	24,799	20,199	20,831	25,761	28,983	20,148	26,465
	3	1	20,914	25,039	19,805	20,862	29,349	20,383	24,244	19,928	20,648	25,172	28,773	19,420	25,804
	3	2	20,975	24,540	19,981	21,441	29,390	20,447	24,258	20,233	20,590	25,172	28,561	19,590	25,958
Harbour	1	1	20,663	24,937	19,562	21,855	29,488	20,736	23,997	19,711	20,936	26,347	29,163	19,612	25,743
	1	2	20,354	25,019	19,559	21,246	29,411	20,437	24,005	19,536	20,990	26,651	29,227	19,798	25,762
	2	1	20,124	24,916	20,270	21,579	29,567	20,820	25,155	20,009	20,837	25,769	29,026	19,516	25,872
	2	2	21,501	25,267	20,097	21,357	29,650	20,872	24,981	20,060	20,859	25,978	29,067	19,648	25,849
	3	1	21,468	26,465	20,790	22,162	ND	20,730	25,288	20,012	21,347	26,793	29,894	19,116	25,820
	3	2	21,796	26,146	20,695	28,816	ND	20,701	25,200	19,951	21,499	27,751	29,847	19,823	25,835

Table 3: Unprocessed qRT-PCR data (continued)

Sample			2 October 2013					2 December 2013			17 December 2013			13 March 2014	
Date qPCR			4 December 2013			28 February 2014		13 December 2013			19 March 2014			17 April 2014	
Cell line			THP-1											U937	
Condition	BR	TR													
Gene			PPIA	TNFα	IL8	PPIA	IL6	PPIA	TNFα	IL8	PPIA	TNFα	IL1β	PPIA	TNFα
Hoboken	1	1	21,313	26,279	19,751	21,748	28,758	20,819	24,766	20,144	20,077	26,138	28,827	19,546	25,742
	1	2	21,163	26,494	19,842	21,265	28,947	20,918	24,741	20,018	20,787	26,128	29,007	19,895	25,975
	2	1	20,951	25,598	19,677	21,164	28,211	20,678	25,157	20,746	ND	ND	ND	19,921	25,918
	2	2	20,903	25,739	19,624	21,095	27,886	20,717	25,228	20,842	ND	ND	ND	19,373	25,980
	3	1	20,255	25,618	19,194	20,470	27,738	20,690	25,223	20,637	20,195	26,963	28,330	19,077	26,804
	3	2	20,741	26,082	19,213	20,250	27,672	20,826	25,421	20,782	20,233	26,746	28,897	19,111	27,587
Dessel	1	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	21,918	27,568	30,043	N/A	N/A
	1	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	21,038	27,344	29,522	N/A	N/A
	2	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	ND	ND	ND	N/A	N/A
	2	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	ND	ND	ND	N/A	N/A
	3	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	20,620	26,780	28,878	N/A	N/A
	3	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	20,329	26,854	29,169	N/A	N/A

ND: not determined

N/A: not applicable

Summary in layman terms

Particulate matter (PM) is the general term for tiny liquid or solid particles (with the largest particles about 5-7 times smaller than the diameter of a human hair) floating in the air, which can be inhaled and deposited in the lungs. The PM mixture is composed of a high number of different molecules, coming from different sources. Because of this, the PM composition can be very different from time to time and place to place.

When these particles are inhaled they can cause various health problems, including illnesses of the lung such as asthma, but also problems outside the lungs. This is because like the oxygen we breathe, the smallest particles can enter the bloodstream and distribute themselves across the body. It has been found in research that through this mechanism PM can hurt the heart and bloodstream, for instance by contributing to atherosclerosis, but also the brain, by contributing to the development of Alzheimer's and Parkinson's disease. It has also been seen that PM levels don't need to be very high to damage our health. Scientists have demonstrated that the current levels of PM in Europe and Belgium drastically shorten the lives of their inhabitants. Additionally, governments and environmental agencies often focus on the levels of PM in terms of the combined weight of all the particles in a certain volume of air. However, this might not be the best way to estimate how harmful the polluted air is, seeing as some particles might be more dangerous than others. Therefore, it might be better to also look at the levels of particular harmful components in PM. Two PM components that have been suggested as especially unhealthy are endotoxins, derived from bacteria, and metals. To provide clear evidence that these components are in fact important for the toxic character of PM and how they cause their health effects, good methods to study this have to be developed first. This would include techniques to measure the amount of endotoxins and metals in PM samples, and on the other hand techniques to investigate how the body responds to these samples. The latter can be studied in different ways. First of all, the response of different types of cells grown in the lab to the PM samples can be tested or the response of human volunteers to polluted air can be analyzed by taking a small amount of cells from inside the nose and testing these on effects of PM exposure.

This project attempted to develop methods for these purposes. As a result, some techniques were optimized and future work necessary for the other techniques was suggested.