Publishable Summary for 18HLT02 AeroTox
Measurements for mitigating adverse health effects from atmospheric particulate pollutants

Overview

Atmospheric particulate pollution has been linked to a broad spectrum of adverse health effects including respiratory problems, cardiovascular diseases, cancer and dementia. These effects depend not only on physical, but also on chemical properties of airborne particulate matter (PM) though to date it has proven difficult to disentangle the relative contribution of PM constituents to the reported population-level health effects. To address this issue this project used “tailored” reference aerosols, combined with high-resolution optical imaging of exposed cells and state-of-the-art cell analysis methods to study the cytotoxic effects of airborne PM in vitro. This was done in a systematic way to help inform which PM metrics are associated with the induction of toxic mechanisms so that they are linked to specific health effects. The project has developed new instrumentation, e.g. an automated oxidation flow reactor and a portable aerosol mixing chamber, and has shed light into the PM components that cause cytotoxic effects in *in vitro* studies.

Need

Airborne particles cause serious acute and chronic human health effects, associated with several hundred thousand premature deaths in the EU each year. For historical reasons, atmospheric particulate pollutants have been regulated for human health purposes by the mass concentration of discrete size fractions: PM_{10} and $PM_{2.5}$ (particles with diameter below 10 µm and 2.5 µm, respectively). However, PM mass concentration, fails to capture the chemical heterogeneity of airborne particulates and is uninformative concerning the toxicologically important contributions of ultrafine particles (<100 nm), which are of negligible mass. It has therefore been hypothesised that PM mass concentration, whilst useful, is not the most informative metric to characterise the potential of particles to cause the disparate detrimental health effects reported. The focus on mass also precludes the application of intelligent targeting of ‘health-relevant’ constituents. Therefore, there was a need to generate new data on the contribution of PM constituents to discrete toxicological relevant pathways. This can then be used to provide information on the causal link between the inhaled particles - and the down-stream health effects. Such information is vital if new metrics, such as particle size, number concentration and chemistry, are to be integrated into existing air quality guidelines.

The current literature evaluating the associations between air pollution and adverse health outcomes has been dominated by epidemiological studies, investigating the overall health effects of atmospheric air pollution, including particles, gases and mixtures. These studies, however, are limited in their capacity to distinguish independent effects of isolated aerosol components or properties on health. To disentangle the effects of the different aerosol properties on health, there is a need for well-defined reference aerosols generated in the laboratory. These aerosols should simulate the properties of real ambient aerosols whilst being stable and reproducible, with properties that can be "tailored" according to the experimental needs.

In vitro studies are essential for understanding the cause-effect relationship between airborne particles and cell/tissue damage. However, their value is fundamentally dependent on robust in vitro to in vivo correlation. To achieve this there is a need to go beyond the traditional cell-exposure techniques and simple biological models (e.g. 2D cultures). Novel methods for cell exposure that mimic the natural inhalation routes must be used and new biological models, such as lung organoids and lung scaffolds (3D multicellular structures), need to be developed to provide physiologically relevant models for measuring biological effects.

Cellular responses to pollutant stressors can be investigated with a combination of optical imaging techniques and biomedical assays. In both cases, quantification and integration of data from across multiple analytical platforms is challenging statistically and subject to measurement error and/or interpretive biases. For a meaningful integration of multiple endpoints to establish adverse outcome pathways (AOPs) in relation to

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specific PM properties and components, a metrology framework needs to be established to derive quantitative and reproducible response metrics.

Objectives

The overall goal of this project was to identify correlations between particle component/properties (metrics) with adverse outcome pathways that are associated with the induction of acute and chronic health effects within the European population. This was delivered through the development of a new method for studying in vitro cytotoxicity based on the use of "tailored" synthetic ambient aerosols combined with high-resolution optical imaging and state-of-the-art cell analysis methods. The specific objectives of the project were:

1. To develop a stable and reproducible laboratory-based source of well-controlled and chemically defined, synthetic reference aerosol mixtures that mimic real ambient aerosols at high concentrations (at around the limit values of the EU Air Quality Directive and up to a few mg/m^3). The aerosol properties should be tuneable and the source should be coupled to an oxidation flow reactor (OFR) to mimic atmospheric photochemical "ageing". To improve traceability for the physical and chemical characterisation of the synthetic aerosols using EU reference methods (target uncertainty in mass concentration 15 %, number concentration <15 %, analysis of major chemical components <15 %). Moreover, to quantify uncertainties for emerging techniques, such as Aerosol Mass Spectrometry (AMS) for chemical analysis and Brunauer–Emmett–Teller (BET) for surface area analysis and develop new approaches to ensure reproducibility and quality assurance.
2. To apply novel methods for cell exposure at the air-liquid interface (ALI) in order to mimic and quantify the effects of the in vivo aerosol inhalation routes. To study phenotypic effects using lung organoids. To compare these novel methods with the conventional cell-exposure techniques relying on submerged cell systems, where aerosol particles are collected in water with high-volume samplers.
3. To assess how the composition of the collected aerosols and their ageing impacts on their acellular and cellular oxidative characteristics, both in simple chemical models simulating human respiratory tract lining fluids (in health and disease) and in representative cell lines and lung tissue cultures maintained under near physiologic conditions. To evaluate adverse outcome pathways using proteomics and transcriptomics (high throughput sequencing), to examine known causal pathways, such as pro-/anti-inflammatory responses, cytotoxicity and genotoxicity, as well as novel 'component-specific' pathways. The project worked toward improved, validated protocols for harmonising/standardising cell analysis studies, as well as on the integration of multi-omic approaches for statistical analysis of complex data sets on a European level.
4. To push the frontiers of optical imaging and biological image analysis to quantify the effects of particle uptake on single cells and cell populations by using various types of optical microscopy including confocal, structured illumination, light sheet and fluorescence lifetime imaging.
5. To facilitate the take up of the technology and measurement infrastructure developed in the project by the measurement supply chain (accredited laboratories, instrumentation manufacturers), standards developing organisations (CEN, ISO) and end users (e.g. hospitals and health centres).

Progress beyond the state of the art

Whilst epidemiology has made major contributions to our understanding of the impacts of ambient pollutants on health, its capacity to discriminate independent effects in long-term studies is severely limited by the high correlation between pollutant types derived from modelling. This project has demonstrated a new method for studying health effects based on well-defined reference aerosols generated in the laboratory. Synthetic ambient aerosols of different complexity (from single to multiple component mixtures) have been generated in a systematic way and used for controlled exposure of biological models. This systematic approach enabled the correlation of the effects of isolated PM constituents with the in vitro toxicological response observed across biological models.

The in vitro to in vivo correlation has been improved by i) using methods for cell/tissue exposure to aerosols, which simulate the in vivo inhalation route and ii) developing and validating more physiologically relevant in

vitro and ex vivo models that mimic ongoing in vivo processes in the lung. The investigation of cellular responses to chemical stressors has been extended by examining not only panels of known markers in endpoints such as inflammation, oxidative stress and cytotoxicity but also using untargeted high-resolution imaging, proteomic and transcriptomic methods to examine the cellular response to synthetic aerosol particles.

To study how aerosol particles penetrate into cells and interact with subcellular compartments, novel fluorescent probes, imaging methods and image analysis tools have been developed. Finally, by building on experience developed during the preceding EMPIR 16ENV07 Aeromet project, this project has carried out an extensive comparison of the capacity of different imaging techniques to capture cellular responses to aerosol exposure, providing a benchmark for the development of future protocols and standards.

Results

Objective 1:

A new oxidation flow reactor, called Organic Coating Unit (OCU), has been developed by FHNW and validated by METAS. The new device (essentially a micro smog chamber) is portable, fully automated and therefore very user-friendly. It includes an automated precursor supply system, a built-in aerosol humidifier, touch screen for reading/displaying experimental parameters and automatic data-logging. Moreover, it enables inspection of UV-C lamp power and aerosol temperature during measurement. The device is versatile and can be readily combined with various soot generators, such as miniCAST and mini inverted burners. A series of combustion aerosols has been produced in a stable manner, ranging from black carbon aerosols (>95 % elemental carbon, EC, mass fraction) to aerosols containing mostly secondary organic matter (10 % EC and 90 % organic carbon, OC, mass fraction).

A portable aerosol mixing chamber was designed and built by METAS. The chamber is cylindrical with an adjustable height of 0.5-1 m and 5 cm internal diameter and is made of steel. Mixing is achieved by 3 turbulent air-jets located below the aerosol injection ports. The facility is modular, i.e. can be easily (dis)assembled and has a total weight of about 10 -20 kg depending on the exact height. The volume is <2 L, therefore more than 20 times smaller than the mixing chamber designed within the EMPIR 16ENV07 Aeromet project. The mixing chamber is equipped with three sampling outlets. The aerosol spatial homogeneity at the sampling zone was determined to be within 5 % in particle number concentration even for particles in the lower micrometre range. The mixing chamber enables the homogenisation of various aerosol components, such as soot, inorganic salt, dust and metal particles in order to generate ambient-like aerosols in the laboratory in a controlled way. The mass concentration and chemical composition of the aerosols can be tuned to simulate different urban, suburban and rural aerosols. This new facility can be applied in health-related studies, aerosol research and instrument calibration. Having these results, the project successfully achieved the objective.

Objectives 2 and 3:

LUND and NPL together with UBERN have performed an aerosol toxicity study using submerged systems with cultured A549 (human alveolar lung epithelial) cells, H441 (human alveolar lung epithelial cells), BEAS2B (human bronchial epithelial cells), THP-1 (human lung macrophages) and HFL1 (human lung fibroblasts) and the ex vivo model precision-cut lung slices (PCLS). As an intercomparison study between the different partners, cells were exposed to various aerosol components (uncoated fresh soot particles, copper oxide, ammonium sulphate and ammonium nitrate) in suspension with three different concentrations of each component for 5-72 hours. Exposures were also performed on H441 cells cultured in lung scaffolds (decellularised human lung tissue) and compared with H441 cultured on traditional plastic cell culture plates. Changes in cytotoxicity (LDH), metabolic activity (WST-1), generation of oxidative stress and immune responses were analysed. The results indicate that there are significant differences in toxicity of PM components and cellular responses that can be linked to adverse health effects. Cytotoxic responses were more pronounced in alveolar epithelial cells than fibroblasts and macrophages. Comparisons were also made between two commonly used metabolic assays: WST-1 and Presto Blue, which indicated that Presto Blue was less sensitive than WST-1 to detect metabolic alterations related to mitochondrial dysfunction. Overall, fresh uncoated soot particles and copper oxide concentration-dependently induced toxicity, reduced metabolic activity related to mitochondrial dysfunction, increased ROS and altered immune response. Increased ROS generation and mitochondrial dysfunctions are well known features in COPD pathology.

Three different studies with epithelial cells and the NACIVT (Nano Aerosol Deposition Chamber for In Vitro Toxicity) system have been performed. Two studies were performed with soot particles and one with ZnO (zinc oxide) particles. In the first case, the University of Bern (UBERN) and METAS performed a joint study with fresh (i.e. uncoated) and aged (i.e. coated) soot particles synthesised in the laboratory. Exposure of HBE (human bronchial epithelial) cells to aged soot particles led to a significant increase in LDH (Lactate Dehydrogenase) release. 102 inflammatory markers were analyzed and 35 of them were found altered. Our data suggest that secondary organic aerosol (SOA) coating might be important for induction of cell death and inflammatory pathways linking air pollution exposure to disease.

In the second study, conducted by LUND, the toxicological responses to ZnO nanoparticle (NP) exposure in the NACIVT chamber was compared with two different submerged exposure systems for three different doses. The primary site of respiratory NP deposition during inhalation exposure is in the distal airways, which were represented by A549 cells. The results showed that the medium dose (1.0 µg/cm2) used in the NACIVT system generated a significant increase in metabolic activity and release of the cytokines IL-8 and MCP-1 as compared to the unexposed controls, while no significant increases could be seen in the two submerged systems at this dose. This indicates that the NACIVT exposure system may be more sensitive than the two submerged exposure systems, because it utilizes a more physiologically realistic exposure model.

In the third study carried out by NPL with support from LUND, KCL/IC, METAS and FHNW four different respiratory cell types (A549, BEAS-2B, HFL1 and TT1) were exposed to uncoated and alpha-pinene coated soot particles (90 nm – 120 nm in diameter). After exposure, cells were analysed at three different time points (24 h, 48 h and 72 h) using a range of different analytical techniques including fluorescence and electron microscopy and assays for cytotoxicity (LDH) and metabolic activity (WST-1). The results showed significant differences in the magnitude of the responses of different cells to the same exposure conditions with TT1 cells (alveolar type 1 cells) being particularly sensitive. For the same particle concentration alpha-pinene coated soot was found to be significantly more cytotoxic.

LUND have performed toxicity studies with silver nanoparticles (AgNPs with the size 10 nm and 75 nm and two different concentrations 2 and 10 µg/mL) to compare responses in in vitro and ex vivo models. Results from in vitro cell cultures with HFL-1 and ex vivo lung tissue cultures with PCLS indicated that AgNPs at higher concentrations induced cytotoxic effects measured as increased LDH release and reduced metabolic activity in HFL-1 and PCLS after 48 h. These data indicate mitochondrial dysfunction which was visualized with TEM images. As a conclusion, the data indicate toxic effects of AgNP exposure on cell viability ex vivo and in vitro with altered procollagen and proinflammatory cytokine secretion in fibroblasts over time. Hence, careful characterizations are of importance and implicated that the exposure studies with this project's samples should include time points beyond 24 hours.

Moreover, LUND has worked on optimising the in vitro culture system with lung tissue scaffolds and successfully obtained novel in vitro models for exposure studies performed with this project's samples. Human lung fibroblasts and mast cells were cultured on cell culture plastic plates or decellularized human lung tissue (scaffolds) to create a more physiological milieu by providing an alveolar extracellular matrix (ECM). Culture in scaffolds altered the release of mediators compared to culture on plastic plates. These data indicate a modulatory role by the alveolar ECM microenvironment in the interplay between resident and inflammatory cells, highlighting the importance of advancing work with more complex culture systems such as the 3D lung scaffold to increase the in vivo relevance of cell physiology studies in vitro. LUND has also constructed a new prototype to mimic breathing movements *in vivo* when culturing and exposing cells. Obtained results with alveolar epithelial cells (H441) show that the cells cultured on human lung scaffolds with dynamic stretch mimic tidal breathing and induce increased surfactant production. Having these results, the project has largely achieved the objectives.

Objective 4:

NPL have developed experimental protocols for fluorescence microscopy analysis of different in vitro respiratory models. Extensive image datasets of a range of different cell types (A549, HFL1, BEAS-2B and TT1) exposed to different aerosol particles (uncoated soot particles, alpha-pinene coated soot particles, copper oxide, ammonium sulphate and ammonium nitrate) in submerged culture and at the air-liquid interface have

been captured using confocal laser scanning, structured illumination and light sheet fluorescence microscopy. Consistent with results from bulk assays for cell proliferation, metabolic activity and toxicity (WST-1, LDH release), qualitative analysis of reconstructed image data suggests exposure to some of the aerosol components impacts mitochondrial morphology. A computational workflow has been developed to quantify mitochondrial shape changes.

NPL have exposed BEAS-2B cells to fluorescently labelled silica particles synthesized by BAM. 3D super-resolution image data has been captured and analysed to assess the level of intracellular uptake and spatial localisation of the particles. NPL has captured image data of the particles in different pH buffers using confocal laser scanning and structured illumination microscopy. The results have been analysed to establish the capacity of these different fluorescent microscopy techniques to measure pH using the particles and assess the feasibility of using this data to identify the intracellular vesicle in which a particle is located. Due to delays because of the Covid-19 pandemic, the project could only partially achieve the objective.

Impact

The Kick Off meeting took place at the Federal Institute of Technology in Zurich (ETH Zürich), Switzerland, between 20 and 21 June 2019. Furthermore, a stakeholder committee was formed early in the project. The members' interests span the range of topics within the project. They are: Swiss Federal Laboratories for Materials Science and Technology (EMPA), University of Basel, University of Bern, University of Applied Sciences Northwestern Switzerland, Bundesanstalt für Materialforschung und -prüfung (BAM), Jing Ltd. (instrument manufacturer), NRC Canada and two representatives from the Swiss Federal Office for the Environment (Section "Traffic" and Section "Air Quality").

The project website has been created, at http://empir.npl.co.uk/aerotox/, and is regularly updated.

During the lifetime of the project, the consortium partners presented 18 oral or poster presentations at different European and national conferences. Eleven peer-reviewed papers and one Master's thesis have already been published, 1 more manuscript has been submitted and 2 are currently being drafted.

Several other dissemination activities including seminars, workshops, a Bachelor Thesis at Lund University and a Master's Thesis at METAS/EPFL have taken place. In particular, the NACIVT aerosol deposition chamber was exhibited at the ETH Conference on combustion-generated nanoparticles held in June 2019 at ETH Zurich, Switzerland. Consortium members have contributed directly to the EURAMET TC-MC meeting held at METAS in February 2020 and are also in close contact with regulatory agencies, such as the Swiss Federal Office for the Environment.

A successful stakeholder workshop was hosted by Lund University November 2022. The *In vitro respiratory toxicology: lung models, exposure methods and analytical techniques* workshop brought together leading researchers who are applying in vitro toxicological techniques to investigate the effects of exposure to air pollution on human health. The workshop was held at the Pufendorf Institute, Lund University, Sweden and was also available online.

Impact on industrial and other user communities

The novel oxidation flow reactor (OFR), which has been developed by FHNW and validated by METAS, is currently commercialised by FHNW and has already sold four units for use outside the framework of this project. Combined with a soot generator, such as a mini CAST or mini inverted burner, the OFR can produce a wide range of well-defined "fresh" and "aged" combustion aerosols, which can be applied in the field of toxicology, atmospheric sciences, calibration of common air-quality monitoring instrumentation (e.g. absorption photometers) and filter testing.

As an example, an international inter-comparison of commercial and prototype aerosol absorption instruments and BC absorption photometers took place at METAS in September 2020 with participation from national metrology institutes, academia and industrial partners. All instruments were exposed simultaneously to a series of "fresh" and "aged" (i.e. coated with secondary organic matter) soot aerosols. The goal of the inter-comparison was to characterise the performance of various BC monitors in a controlled and reproducible way,

help identify measuring artefacts, and provide feedback to our industrial partners on how to improve the design of the prototype absorption instruments.

Impact on the metrology and scientific communities

Our studies indicate that the use of ALI exposure on epithelial cells with the NACIVT chamber enables i) a more realistic particle dose and ii) a more accurate determination of the particle dose compared to submerged cell studies. Moreover, inter-comparison studies between cells cultured in 2D compared to 3D scaffolds and PCLS (which mimic the complex in vivo condition) indicate that the ECM microenvironment has an important role in modulating cellular responses involving cytokine and growth factor release. Changes in cell viability appear to be similar in vitro and ex vivo over time.

The new portable aerosol homogeniser developed in the project can be a useful tool in several areas, e.g. for laboratory studies using synthetic aerosols, instrument calibration and aerosol measurement at industrial and occupational settings. METAS has already sold one homogeniser along with custom-made isokinetic sampling units to a European institute for research and development purposes.

Impact on relevant standards

The project Consortium participated in international (ISO TC24/ SC4/ WG 9 & 12 and ISO TC172/ SC5/ WG 10) and national standardisation (BSI CPW/172) bodies where they provided input for the revision of relevant standards. The development of a portable aerosol mixing chamber has been within ISO TC24/ SC4/ WG 9 in view of the upcoming revision of the ISO standard 21501-1 on aerosol spectrometers (also known as optical particle sizers). Moreover, should new metrics, such as Black Carbon, particle number concentration etc., be found to correlate with adverse cellular responses consistent with the causal pathways associated with acute and chronic health effects, the calibration method developed by Kalbermatter et al. (see publication list below) could contribute to the development of entirely new standards.

Project partner NPL were also active in the light microscopy section committee of the Royal Microscopical Society (RMS) and a member of the QUAREP-LiMi (Quality Assessment and Reproducibility for Instruments & Images in Light Microscopy) consortium. For the former, NPL have contributed definitions to a new dictionary of light microscopy terms and for the latter they are particularly involved in working group 5, which is working to develop recommendations for the measurement of lateral and axial resolution in fluorescence microscopy. In ISO TC172/ SC5/ WG 3 NPL are working on a revision of ISO 10943:2020 – vocabulary for light microscopy.

Longer-term economic, social and environmental impacts

This project will support improvements to air quality data by reducing the measurement uncertainties for key air pollutants. This will be achieved through the newly developed instruments which can serve as transfer standards for the calibration of common air-quality monitoring systems and by extending the calibration and quality assurance procedures for emerging analytical techniques that are not yet adequately standardised.

Improving our understanding of the role of air pollutants in disease is essential in order to develop effective public health measures and reduce the exposure of the population to the most harmful components of ambient PM. The outputs from this project may also support a more targeted action on health-relevant components/properties, and promote the revision of air quality legislation, as well as extending the current legal framework to include metrics beyond PM mass concentration. Considering the enormous costs arising from hospitalisation and premature deaths, particularly in densely populated cities that constitute pollution hotspots, the protection of public health should also lead to substantial financial benefits.

List of publications

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This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

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Project start date and duration:	1 June 2019, 42 months	
Coordinator: Konstantina Vasilatou, METAS Tel: 0041583870382 E-mail: konstantina.vasilatou@metas.ch Project website address: http://empir.npl.co.uk/aerotox/		
Internal Funded Partners: 1. METAS, Switzerland 2. BAM, Germany 3. FMI, Finland 4. NPL, United Kingdom	External Funded Partners: 5. FHNW, Switzerland 6. KCL, United Kingdom (withdrawn from 7 July 2020) 7. LUND, Sweden 8. UBERN, Switzerland 9. IC, United Kingdom (joined from 7 July 2020)	Unfunded Partners: -
RMG: -		