Journal of Aerosol Science 96 (2016) 38–55

Contents lists available at ScienceDirect

Journal of Aerosol Science

journal homepage: www.elsevier.com/locate/jaerosci

Toxicity testing of combustion aerosols at the air–liquid interface with a self-contained and easy-to-use exposure system

Sonja Mülhopt [a,i,*,1], Marco Dilger [a,b,i,1], Silvia Diabaté [b,i], Christoph Schlager [a,i], Tobias Krebs [c,i], Ralf Zimmermann [d,h,i], Jeroen Buters [e,i], Sebastian Oeder [e,g,i], Thomas Wäscher [f], Carsten Weiss [b,i], Hanns-Rudolf Paur [a,i]

[a] Karlsruhe Institute of Technology, Institute for Technical Chemistry, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[b] Karlsruhe Institute of Technology, Institute of Toxicology and Genetics, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[c] Vitrocell Systems GmbH, Fabrik Sonntag 3, 79183 Waldkirch, Germany
[d] University of Rostock, Institute of Chemistry, Dr.-Lorenz-Weg 1, 18051 Rostock, Germany
[e] Center of Allergy & Environment (ZAUM), Technische Universität and Helmholtz Zentrum München, Biedersteiner Str. 29, 80802 München, Germany
[f] Ingenieurbüro für Energie- und Verfahrenstechnik, Von-Dalheim-Str. 2, 69231 Rauenberg, Germany
[g] Kühne Foundation, Christine Kühne Center for Allergy Research and Education (CK- CARE), München, Germany
[h] Cooperation Group "Comprehensive Molecular Analytics" – CMA, Helmholtz Zentrum München, 85764 Oberschleißheim, Germany
[i] HICE – Helmholtz Virtual Institute of Complex Molecular Systems in Environmental Health – Aerosols and Health, Germany[2]

ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form
24 February 2016
Accepted 26 February 2016
Available online 9 March 2016

Keywords:
Air–liquid interface exposure
Nanoparticle
Lung cell culture
In-vitro
Ship diesel emission
Wood combustion

ABSTRACT

In vitro toxicity testing of airborne particles usually takes place in multi-well plates, where the cells are exposed to a suspension of particles in cell culture medium. Due to the artefacts caused by particle collection and preparation of suspensions, the air–liquid interface (ALI) exposure is challenging this conventional exposure technique to become the method of choice. The ALI technique allows for direct sampling of an aerosol and exposure of cell cultures to airborne particles. At the same time, it reflects the physiological conditions in the lung to a greater extent. So far, the available ALI systems have mostly been laboratory set-ups of the single components. Here, we present a mobile and complete system providing all process technology required for cell exposure experiments at dynamic aerosol sources. The system is controlled by a human machine interface (HMI) with standard routines for experiments and internal testing to assure reproducibility. It also provides documentation of the exposure experiment regarding process parameters and measured doses. The performance of this system is evaluated using fluorescein-sodium dosimetry, which is also used to determine the factor of dose enhancement by optional electrostatic deposition. The application of the system is shown for two different technical aerosol sources: wood smoke particles emitted by a household log wood stove

* Corresponding author at: Karlsruhe Institute of Technology, Institute for Technical Chemistry, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany. Tel.: +49 721 608 23807; fax: +49 721 608 24303.
E-mail addresses: Muelhopt@kit.edu (S. Mülhopt), marco.dilger@kit.edu (M. Dilger), silvia.diabate@kit.edu (S. Diabaté), christoph.schlager@kit.edu (C. Schlager), t.krebs@vitrocell.com (T. Krebs), ralf.zimmermann@uni-rostock.de (R. Zimmermann), buters@lrz.tu-muenchen.de (J. Buters), sebastian.oeder@lrz.tum.de (S. Oeder), IB-waescher@t-online.de (T. Wäscher), carsten.weiss@kit.edu (C. Weiss), Paur@kit.edu (H.-R. Paur).
[1] Both authors contributed equally to this work.
[2] www.hice-vi.eu.

http://dx.doi.org/10.1016/j.jaerosci.2016.02.005
0021-8502/

and emissions from a ship diesel engine. After exposure of lung cells, cytotoxicity and gene regulation on a genome-wide scale were analysed.

Nomenclature

A	surface area of cell culture [cm²]
$c_{m,SMPS}$	aerosol mass concentration calculated from SMPS measurement [mg/cm³]
N_i	number concentration in channel i of the SMPS measurement [1/cm³]
d_i	particle diameter in channel i of the SMPS measurement [nm]
ρ_P	particle density [g/cm³]
f	deposition efficiency, deposited particle fraction [%]
RID	relevant in vitro dose (Cohen, Teeguarden & Demokritou, 2014)
$RID_{m,FSD}$	deposited particle mass measured by fluorescence spectroscopy [μg/cm²]
$RID_{SMPS,diff}$	diffusional deposited dose calculated from SMPS data [μg/cm²]
$RID_{TEM,diff}$	diffusional deposited dose calculated from TEM data [μg/cm²]
$RID_{SMPS,HV}$	electrostatic deposited dose calculated from SMPS data [μg/cm²]
$RID_{TEM,HV}$	electrostatic deposited dose calculated from TEM data [μg/cm²]
$t_{exposure}$	duration of exposure [h]
$V_{exposure}$	aerosol flow rate [l/h]

1. Introduction

1.1. Toxicity testing of submicron particles

During the first half of the 20th century, several episodes of extreme air pollution in European and US cities demonstrated that airborne particulate matter adversely affects human health (Dockery & Pope, 1994). Since then, many epidemiological studies have consistently linked air pollution to higher morbidity and mortality (Anderson, Thundiyil & Stolbach, 2012; Dockery, 2009). *In vivo* and *in vitro* data available on the toxicity of aerosols from specific sources generally support the epidemiological findings and give important insights into molecular mechanisms and the effects of specific physical and chemical properties of aerosol components, as was summarised by recent reviews (Kelly & Fussell, 2012; Nemmar, Holme, Rosas, Schwarze & Alfaro-Moreno, 2013; Schwarze et al., 2006).

Toxicity of airborne particles following inhalation can be studied either by *in-vitro* or by *in-vivo* experiments. The advantages and limitations of both test methods have been discussed in detail elsewhere (Maier et al., 2008; Sayes, Reed & Warheit, 2007). *In-vitro tests* are conducted with organ-specific, often human, test cells. The deposition of originally airborne particles onto test cells is carried out either from the liquid phase (submerged exposure) or from the gas phase at the air–liquid interface (ALI). Classical submerged testing of particles allows for straightforward analyses of a large number of different particles, concentrations, and time points within a short period in particular when high-throughput methods are applied (Nel et al., 2013). However, this test method has several limitations with respect to particles and cells:

(1) It is not representative of the conditions in the lung, because the cells are covered by a few millimetres of culture medium. This changes the oxygen partial pressure in comparison to the lung surface, where the layer of lung-lining fluid covering the cells is extremely thin (Blank, Rothen-Rutishauser, Schurch & Gehr, 2006).
(2) For submerged exposure of particles, which are components of complex aerosols, the particles must be separated from the gas phase by filtration. Collection of the solid particles, however, may change their agglomeration state and their chemical composition. Semi-volatile compounds in the filtered gas may adsorb to the deposited particles or be removed partly (Subramanian, Khlystov, Cabada & Robinson, 2004).
(3) The particle properties will be changed by dispersion in cell culture medium, which contains a large number of biomolecules, including serum proteins. Proteins are known to adsorb to the particles, form a corona, and may prevent adverse effects to the cells (Monopoli, Wan, Bombelli, Mahon & Dawson, 2013; Panas et al., 2013).
(4) In submerged exposure the dose cannot be determined correctly because of several reasons: as the agglomeration state is unknown, settling velocity is not defined; particles may also dissolve partially in the culture medium (Teeguarden, Hinderliter, Orr, Thrall & Pounds, 2007). For submerged exposure the particle dose is often delivered as a bolus. During inhalation of aerosols, by contrast, the particles are deposited linearly over a defined period. This may have an effect on the quality and intensity of the biological effects.

To overcome these problems, the ALI exposure technique applies the aerosol directly to the cell cultures (Paur et al., 2011). This particularly holds for the toxicity testing of unmodified complex gas-particle mixtures, such as combustion aerosols which contain thousands of substances in both the gaseous and the solid fraction. The ALI technique allows for a direct dynamic delivery of the aerosol to the test cells which are covered with a very thin liquid film like in the lung (Aufderheide, 2005; Bakand & Hayes, 2010). This direct exposure to airborne substances over a defined period is therefore more physiological. Further advantages are the facts that the dose can be determined more precisely and the gaseous phase of an aerosol can be examined separately by removing the particulate phase with filters. In sum, the ALI exposure technique using a direct sampling system represents a convenient method combining aerosol sampling and exposure in one step while avoiding the disadvantages of filter sampling, chemical extraction, purification, suspension and undefined dose (Fig. 1).

1.2. *Performance of ALI studies in comparison with classical approaches*

Cigarette smoke aerosol has adverse effects on human health and was studied intensively during the early development of the ALI exposure technique. With surprisingly high consistency, researchers found a dose-dependent toxicity when exposing cultured cells to diluted cigarette smoke (Aufderheide, Ritter, Knebel & Scherer, 2001; Fukano, Ogura, Eguchi, Shibagaki & Suzuki, 2004; Li et al., 2013; Weber, Hebestreit, Conroy & Rodrigo, 2013). In contrast to the classical submerged exposure, use of the ALI exposure technique allowed for the investigation of the volatile cigarette smoke constituents. Indeed, the gas phase of the cigarette smoke partly contributes to cigarette smoke toxicity, as was shown by the removal of the particulate phase with filters or testing of denuded smoke by the use of charcoal filters (Fukano et al., 2004; Fukano, Yoshimura & Yoshida, 2006; Nara, Fukano, Nishino & Aufderheide, 2013; Okuwa et al., 2010).

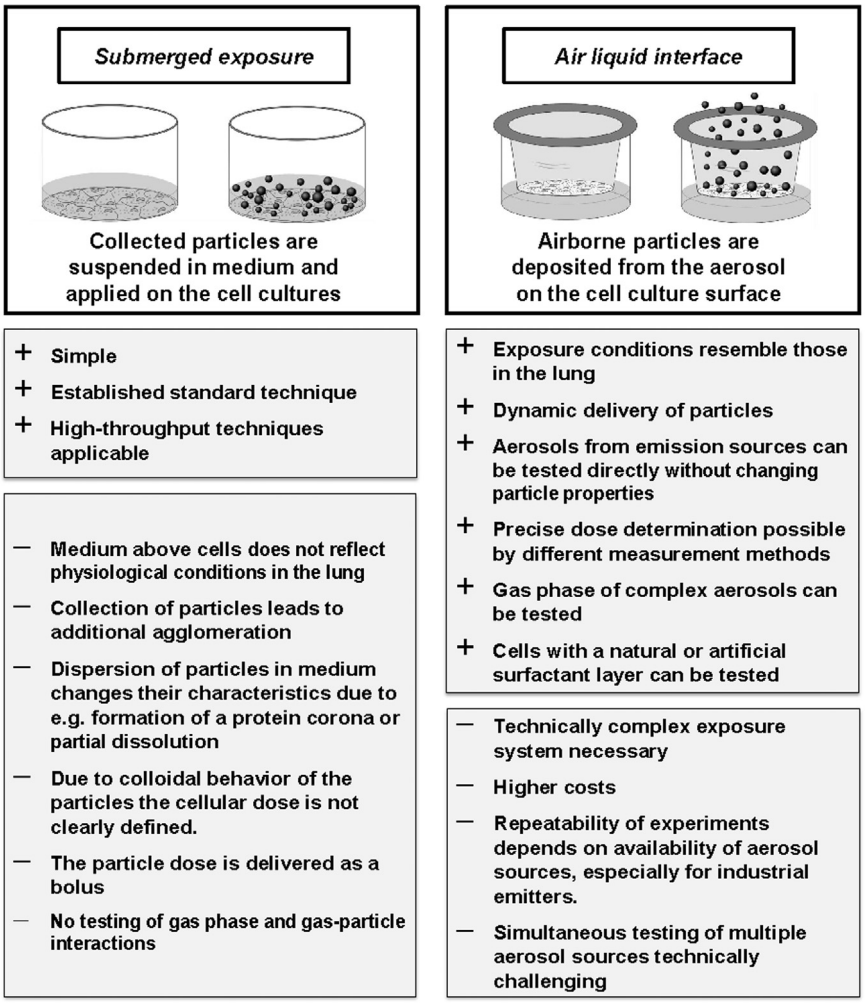

Fig. 1. Comparison of the cell culture exposure methods with respect to the influences of particle properties and the quality of dose determination. Left: submerged exposure reflecting the state of the art, right: air–liquid interface exposure (short: ALI).

Emissions from diesel and gasoline engines have also been analysed frequently using the ALI exposure technique. Several groups observed acute cytotoxicity of diesel or gasoline combustion aerosols tested under ALI conditions (Abe, Takizawa, Sugawara & Kudoh, 2000; Joeng, Hayes & Bakand, 2013; Knebel, Ritter & Aufderheide, 2002; Kooter et al., 2013; Müller et al., 2010; Seagrave et al., 2007; Tsukue, Okumura, Ito, Sugiyama & Nakajima, 2010). In comparison to similar particle doses in submerged exposure, a much higher toxicity was reported when the ALI technique was used (Cooney & Hickey, 2011; Lichtveld et al., 2012). ALI exposure was further used to examine the contribution of volatile compounds to the observed toxicity. The toxicity of engine exhausts was not altered when particles were removed, suggesting that the toxicity is attributable to the gas phase (Holder, Lucas, Goth-Goldstein & Koshland, 2008; Knebel et al., 2002). However, Seagrave et al. report a lack of acute toxicity of the particle-free exhaust (Seagrave et al., 2007). Induction of inflammatory processes might be attributable to the particulate phase, which was indicated by a loss or reduction of inflammatory response when the particles were removed (Abe et al., 2000; Holder et al., 2008; Steiner et al., 2013).

Wood smoke particles show adverse biological effects in submerged studies, as was summarised by Kocbach Bølling et al. (Kocbach Bølling et al., 2009). Although wood combustion is an important source of human particulate matter (PM) exposure (Naeher et al., 2007), its toxic effects have been poorly addressed by ALI exposure studies so far. Hawley and Volckens compared the emissions of wood stoves used for cooking and found a rapid induction of inflammatory and antioxidative genes by the emissions produced by a traditional stove, but not by modern stoves (Hawley & Volckens, 2013). Künzi et al. exposed cells covered with a thin liquid layer to aged beech combustion aerosol. Under these conditions, however, no changes in the investigated biological endpoints in comparison to freshly emitted aerosol or control cells remaining in the incubator were detected (Künzi et al., 2013).

Apart from combustion-derived aerosols, the ALI exposure technique can also be used to investigate aerosols generated from manufactured nanomaterials (MNM). Also here, the ALI exposure technique on several occasions was found to provide important insights that could not have been achieved by submerged exposure. Holder et al., for instance, reported a much higher toxicity of nickel oxide particles exposed at the ALI when compared to similar doses in submerged exposure (Holder & Marr, 2013). Similar results exist for zinc oxide nanoparticles (Lenz et al., 2013; Raemy et al., 2012). In striking contrast to many submerged studies (AshaRani, Low Kah Mun, Hande & Valiyaveettil, 2009), neither toxicity nor changes in inflammatory or anti-oxidative gene or protein expression have been reported so far for aerosolised Ag NPs (Herzog et al., 2013; Holder & Marr, 2013). Reduced toxicity in lung cells at the ALI compared to submerged conditions has also been observed after exposure to silica nanoparticles (Panas et al., 2014).

Comparisons between ALI exposure and *in vivo* data are scarce, but look promising. A genome-wide gene expression study of *in vitro* cell cultures exposed to cigarette smoke under ALI conditions revealed an enrichment of gene signatures and marker proteins associated with human smoking behaviour (Mathis et al., 2013). A pre-validation study performed by the German Federal Institute of Occupational Safety (BAuA) to assess the reliability of an ALI exposure model tested four gases (NO_2, SO_2, ozone, formaldehyde) and derived EC50 values. Comparison with LC50 values for rodents published in literature revealed a close quantitative relationship between *in vitro* cytotoxicity and *in vivo* lethality (Linsel et al., 2011).

1.3. Air–liquid interface exposure systems

The air–liquid interface exposure method is used in various equipment solutions in which the cells are cultivated on porous membrane inserts and subsequently placed in exposure devices. The design of the exposure devices determines the size of the inserts and has to be matched with appropriate tubing dimensions and flow rates. For a sophisticated system, other equipment components, such as aerosol generators, humidification systems, and heating devices, have to be integrated. For the deposition of particles on the cell culture surfaces, different deposition principles are used. The continuous flow principle with deposition by diffusion was reported by Aufderheide et al., Tippe et al., and Bitterle et al. (Aufderheide, Halter, Möhle & Hochrainer, 2013; Aufderheide, Scheffler, Möhle, Halter & Hochrainer, 2011; Bitterle et al., 2006; Kim, Peters, O'Shaughnessy, Adamcakova-Dodd & Thorne, 2013; Tippe, Heinzmann & Roth, 2002). Broßell et al. used thermophoretic forces to deposit the particles on the lower side of the membrane inserts on which the cells have been grown upside down (Broßell et al., 2013). Humidification was incorporated in the systems described by Tippe et al. and Bitterle et al. and allowed for high flow rates of up to 250 ml/min over 47 mm membrane inserts without cell death.

For toxicity testing of aerosols with low toxicity, an increased particle deposition in the continuous flow systems was desired. Savi et al. were the first to charge particles and integrate an electrical field (Künzi et al., 2013; Savi et al., 2008) to increase the efficiency of particle deposition to 15–30% in comparison to 1.5–2% achieved by diffusion-controlled systems before (Mülhopt, Paur, Diabaté & Krug, 2008; Tippe et al., 2002). The systems by de Bruijne et al. (de Bruijne et al., 2009), by Stevens, Zahardis, MacPherson, Mossman & Petrucci (2008) and Aufderheide et al. (2013) are working with electrical fields as well.

For quantification of deposited particle mass, an online dose monitoring using the quartz crystal microbalance (QCM) technique was developed by Mülhopt et al. and integrated into the exposure chamber usually containing the membrane inserts with cell cultures (Mülhopt, Diabaté, Krebs, Weiss & Paur, 2009). For validation of the deposition efficiency, fluorescein sodium nanoparticles are deposited on the Transwell membrane and quantified spectrophotometrically.

In contrast to continuous flow feeds, the Cloud system developed by Lenz et al. (2014) uses one-shot exposures of aerosolised liquid and particle suspensions. It is especially suitable for liquid aerosols and reaches a high deposition efficiency by single droplet sedimentation within a relatively short period of time.

Some of the systems described are commercially available, e.g. Cultex Laboratories offers a diffusional exposure and electrostatic deposition system. Vitrocell Systems also offers diffusional exposure systems as well as the Cloud technology, both of which might be equipped with the QCM online monitoring technique.

Here, we report the development of an advanced and reproducibly operating ALI exposure system with integrated Vitrocell exposure modules. It was set up as a further development with significant improvements of the first generation of the Karlsruhe Exposure System described in detail before (Comouth et al., 2013; Mülhopt et al., 2008; Panas et al., 2014; Paur, Mülhopt, Weiss & Diabaté, 2008). The major improvements include the implementation of internal controls, with cells being exposed to clean humidified air only, as well as an automated controlling of the exposure parameters. Furthermore, the choice of materials for the exposure module was optimised with regard to biocompatibility. Part of the development was performed within the framework of the Helmholtz Virtual Institute for Complex Molecular Systems in Environmental Health – Aerosols and Health (HICE). In HICE a comprehensive analysis of the physical and chemical properties of the combustion aerosols is combined with a comprehensive acquisition of the molecular biological effects of the emissions in human lung cell cultures during field campaigns at various test facilities. The biological effects are monitored using a multi-omics approach by vertical integration of transcriptomics, metabolomics, and proteomics. Due to the high amount of cell material required for the multi-omics approach, the system was scaled-up to 18 exposure positions of the 6-well format. The ALI system was further developed as an integrated system at any location.

2. Material and methods

2.1. ALI exposure system – description of technology

2.1.1. Main components

The system can be divided into several main components (Fig. 2): the test aerosol enters the system by passing a size-selective inlet to exclude the particle size fraction above 2.5 μm, since large particles can cause artefacts in the dose determination and biological effects. This inlet also mimics the function of the upper respiratory system, where larger particles are deposited and, hence, do not reach the alveoli. The sampling flow rate of the exposure system is 1 m³/h, driven by a vacuum pump at the end of the line. The flow rate for each exposure unit is measured and controlled by a mass flow controller (MFC) between the off-gas filter and the pump and additionally monitored by the pressure drop caused by the size-selective inlet. In the main reactor the aerosol is conditioned to 85% relative humidity and 37 °C. Humidification is performed by controlled steam injection. The stabilized aerosol is sampled for distribution to the VITROCELL® modules as well as for external measurements, such as gravimetric filter sampling or mobility spectrometry.

2.1.2. Exposure chambers

The system comprises three VITROCELL 6/6 CF Stainless® (VITROCELL SYSTEMS GmbH, Waldkirch, Germany) modules for the exposure of six inserts of the 6-well format. Each of these 18 exposure chambers is supplied with separate aerosol feeds taken by metallic sampling probes from the conditioning reactor. Here, the aerosol is passed over the cells growing on transferable Transwell® inserts.

For each of the 18 exposure positions, the flow rate of 100 ml/min is adjusted by a MFC in the off-gas of the chamber, which is protected by a filter. The exposure positions are equipped with an electrode below the membrane insert that induces an electrical field, if desired, to increase the deposition efficiency. Without an electrical field, the deposition is controlled by diffusion only and therefore is significantly lower. However the electrical mobility of a charged particle depends on the charge number and the particle size. So in consequence the deposited fraction of the particles may show another size distribution which warrants further investigation. Every electrode is connected to a separate high-voltage supply to establish voltages in the range of 400–1500 V in the polarity of choice.

For exposure of cells to clean air, the inlets of the lower module can be supplied with a separate gas stream which consists of synthetic or HEPA-filtered ambient air humidified to 85% r.h. at 37 °C. The humidification is controlled by passing the air over a water reservoir with software-controlled heating. All exposure parameters, such as temperature, pressure losses, flow rates, and the voltage of the electrodes, are controlled and protocolled by a Lab View data acquisition system. Additionally, there is the possibility to change one freely selectable cell exposure position against a quartz crystal microbalance (QCM) sensor. With this online dose determination system, the particle dose per area can be monitored, as was described previously (Mülhopt et al., 2009).

2.1.3. Leak testing

An essential criterion for the successful execution of exposure experiments is the leak tightness of the modules. If there is leakage between the sampling probe and the modules, ambient air, which is not humidified, is sucked in. Even low volumes of non-humidified air readily compromise cell vitality.

For this reason, a mandatory detection procedure was implemented which checks the tightness of the modules before and after each exposure. After closing the module, the valves for aerosol supply are closed and the nominal value of the

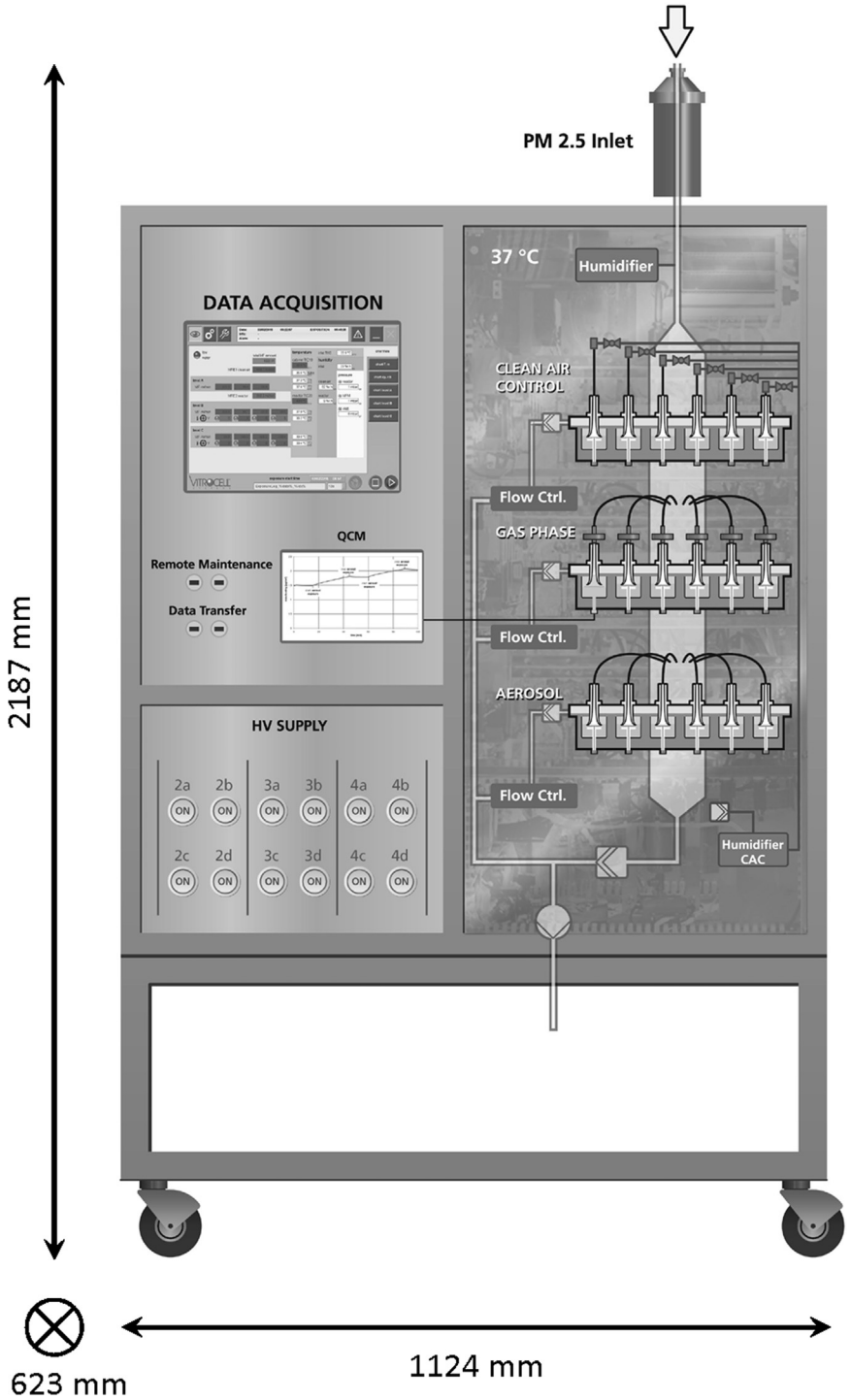

Fig. 2. Flow chart of the ALI exposure system: the left part displays the data acquisition and the control units for high-voltage supply and the flow controllers. The right part shows the 18 exposure chambers, which are thermostatted to 37 °C. In the set-up shown, the upper 6 aerosol feeding tubes are equipped with particle filters to test the gas phase only. The middle 6 exposure chambers are flushed with complete aerosol and the lower 6 exposure chambers with filtered room air for the clean air controls (CAC).

MFC's is set to 100 ml/min. If the modules are tight, the flow will decrease from 100 ml/min to 0 ± 3 ml/min. In case of a detected flow of higher than 3 ml/min, the system has to be checked for leaks. It has been ensured that the inherent pressure drop of a few seconds duration has no effect on the viability of the cells.

2.1.4. Exposure conditions

For testing a complex aerosol, several exposure conditions were studied in addition to the complete diluted "*aerosol*". The "*gas phase" is* filtered aerosol. Differences between "*aerosol*" and "*gas phase"* samples will provide information on the contribution of the particulate fraction of the aerosol. In order to consider the effects of the ALI procedure alone, cells are exposed to humidified filtered air "*clean air controls*". Negative controls, called *"incubator control",* are cell cultures without medium on top which remain in an incubator at 37 °C without CO_2 supply.

2.1.5. Determination of the deposition efficiency

The procedure used for the validation of the ALI exposure system is based on the detection of fluorescent particles on the Transwell® membranes of each individual position. For this purpose, a fluorescein sodium aerosol is generated and introduced into the aerosol reactor AEOLA (Mülhopt et al., 2008). In this reactor the aerosol is homogeneously distributed and reproducible sampling is ensured. The ALI exposure system is fed with the test aerosol from the AEOLA reactor and distributed to the VITROCELL® modules, which are equipped with clean Transwell® inserts. The membranes of the inserts have contact with deionised water from below. After exposure to fluorescein sodium aerosol, the membranes are cut out and rinsed with deionised water. This solution, as well as the water below the membrane, are analysed for fluorescence intensity in an Aminco Bowman Series 2 fluorescence spectrometer (Polytec, Waldbronn, Germany). The mass of deposited fluorescein sodium is calculated by linear regression from fluorescein sodium standards.

The test aerosol is characterised regarding number size distribution by SMPS by an additional sampling line from the conditioning reactor within the ALI exposure system as described in 2.1.1. SMPS data were corrected for the particle losses within sampling tubes from exposure system to SMPS following Soderholm et al (Soderholm, 1979). Using a particle density ρ_P of 1.49 g/cm³, the particle mass concentration $c_{m,SMPS}$ in the aerosol is calculated according Eq. 1 and corrected with respect to gravimetric measurements to the total mass concentration of $c_m = 14.4$ mg/m³. The relationship between the deposited particle mass on Transwell® membranes $c_{m,FSD}$, measured by spectroscopy and equivalent to $RID_{m,FSD}$, and the mass concentration in the aerosol is defined as the deposition efficiency f (Eq. 2). This deposition efficiency was also used to estimate the deposited dose from wood exhaust, as its size distribution was comparable to the fluorescein sodium aerosol in the submicron region and there was no coarser fraction above 1 μm. To calculate the particle mass concentration in the wood exhaust, a particle density ρ_P of 2.70 g/cm³ was used (Lanzerstorfer, 2015).

$$c_{m,SMPS} = \Sigma_i(N_i * (d_i^3/6 * \pi * \rho_P)) \quad (1)$$

$$f = RID_{m,FSD}/c_m \quad (2)$$

To verify the dose calculations of the wood smoke aerosol with a second method, image analysis of particles deposited on TEM grids on a Transwell® membrane was performed. TEM grids (plano GmbH, Wetzlar Germany), 3.05 mm in diameter, 200 mesh and carbon-coated, were exposed with the same aerosol as cell cultures, but without any liquid beneath the membrane, as this would interfere with TEM analysis. For dosimetry with electrostatic deposition, the electrode under the transwell membrane was repositioned to achieve the same electric field strength as in cell exposure experiments. From each grid, 10 images were taken with the transmission electron microscope EM 109 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at a magnification of 3000. These images were evaluated regarding particle load and particle size using the software ImageJ (Abràmoff, Magalhães & Ram, 2004).The particle number per area was determined in 1/cm² by creating a binary picture from the TEM micrograph and applying watershed segmentation, followed by particle analysis. The particle mass per area was calculated from the deposited particle number using the same particle density as in the SMPS data evaluation.

2.2. Cell culture

The human lung alveolar epithelial cell line A549 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultivated in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin (all from Life Technologies, Darmstadt). The BEAS-2B cell line derived from normal human bronchial epithelium was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA, CCL-185™) and cultivated in Bronchial Epithelial Growth Medium (BEGM, Lonza Inc., Walkersville, MD) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin instead of provided gentamycine. For BEAS-2B, culture plates were pre-coated with 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen Type 1, and 0.01 mg/ml BSA to improve cell adherence. All cultures were maintained at 37 °C in a 5% CO_2 atmosphere, when not otherwise stated. Cells were passaged every 2–3 days before reaching confluence.

2.2.1. Preparing cells for ALI exposure

Cells were seeded on transferable 24 mm Transwell® inserts with a 0.4 μm pore polyester membrane (Corning, Tewksbury, MA, USA) 24 h before exposure at a density of 4E+05 (A549) or 5E+05 (BEAS-2B) cells/ml/insert (corresponding to a cell density of 8.6E+04 or 1.1E+05 cells/cm² growth area, respectively) with 1.5 ml cell culture medium provided beneath the insert membrane. For cell exposure, the cell culture medium on the apical side was removed and medium underneath the insert membrane was changed to RPMI 1640 medium without FBS, supplemented with 25 mM

HEPES for A549 cells (Life Technologies, Darmstadt, Germany) or bronchial epithelial basal medium (BEBM, Lonza Inc., Walkersville, MD) supplemented with 10 mM HEPES for BEAS-2B cells. Both HEPES media were additionally supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. 7.6 ml HEPES medium were used per exposure position in order to achieve proper contact of the medium with the insert membrane. Cells were then exposed under ALI conditions for the specified time with an aerosol flow rate of 100 ml/min at each position.

2.2.2. Testing of material compatibility with cell culture

A549 cells were seeded in 24 well plates at a density of 8.7E+04 cells/cm² growth area. The isolator cups of the exposure modules were assembled as used in the ALI exposure system, i.e. cups made of polyoxymethylene (POM) or polypropylene (PP) with electrode, filled with 7.6 ml of RPMI-FBS medium, and placed in the incubator at 5% CO_2 and 37 °C. For negative controls, medium was put in standard cell culture conical flasks (PP) and also incubated. After a 24 h contact period, the medium was removed from the cups and aliquots of 600 μL of conditioned medium were applied into each well of the 24-well plate.

2.2.3. Toxicity test (LDH release)

A549 cells were seeded 24 h before treatment according to the respective experiment. After treatment, medium from the supernatant or from the compartment under the membrane (ALI exposure experiments) was collected and an aliquot of 100 μl was used for quantification of released lactate dehydrogenase (LDH), an indicator of plasma membrane integrity. An LDH detection kit was used in accordance with the manufacturer's instructions (Roche, Mannheim, Germany) with slight modifications: the dye solution was diluted 1:1 (v/v) with PBS to slow down the fast reaction time caused by elevated LDH values due to the high cell densities used for ALI exposure experiments. The absorbance of the reaction mix was measured at 490 nm with a microplate reader (Molecular Devices, Ismaning, Germany). Cell-free medium kept at the same CO_2 concentrations as the tested cells was used to generate blank values, which were subsequently subtracted from all samples. Cells kept under untreated conditions were lysed with 0.1–1% Triton-X 100 (Roth, Karlsruhe, Germany) for 30 min prior to the end of the exposure period to generate samples with the highest LDH release achievable, and the measured values were set to 100% toxicity.

2.2.4. Viability test (AlamarBlue®)

A549 cells were seeded 24 h before treatment according to the respective experiment. After treatment, AlamarBlue® reagent (AbD Serotec, Düsseldorf, Germany) diluted 1:10 (v/v) with RPMI 1640 without FBS was added to the cells and incubated at 37 °C and 5% CO_2. Before the maximum turnover was reached by control cells, the supernatant was transferred to 96-well plates and fluorescence was quantified with a microplate reader (Bio-Tek FL600, MWG-Biotech AG, Ebersberg, Germany) at 580 nm excitation and 620 nm emission. The fluorescence intensities of the samples were normalised to the untreated controls, which were set to 100%.

2.2.5. Whole-genome expression analysis

Directly after exposure in the ALI system, BEAS-2B cells were lysed using APL buffer of AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany). Total RNA was extracted with provided columns according to the manufacturer's protocol. RNA was spiked (One-Color RNA Spike-in Kit, Agilent, Waldbronn, Germany), reversely transcribed into cDNA using T7 promoter primers, and Cy3-labelled with Cy3-coupled CTP in a T7 RNA polymerase transcription reaction (Low Input Quick Amp Labeling Kit, one-color, Agilent, Waldbronn, Germany). Generated labelled cRNA was purified on RNeasy mini spin columns (Qiagen, Hilden, Germany), quantified spectrophotometrically by UV–vis (NanoDrop ND-1000 UV–vis, Thermo Fisher Scientific, Waltham, MA, USA), and analysed fluorospectrometrically for the calculation of labelling efficiency. Purified labelled cRNA was then fragmented and hybridised on Human Gene Expression Microarrays (Sure Print G3 Human Gene Expression Microarray 8 × 60K, Agilent, Waldbronn, Germany). After 17 h at 65 °C in a hybridisation oven, microarray slides were washed (Gene Expression Wash Buffer Kit, Agilent, Waldbronn, Germany) and scanned (Agilent C microarray scanner, Agilent, Waldbronn, Germany). Data were extracted using Feature Extraction software (Agilent, Waldbronn, Germany) and analysed with GeneSpring software (Agilent, Waldbronn, Germany). Significantly, at least 3-fold, regulated genes (compared to the clean air control group) were used for further analysis. Variance in whole-genome expression due to aerosol treatment was determined by principle component analysis.

2.2.6. Statistics

Results are reported as mean+standard deviation (StdDev) of multiple independent experiments except when otherwise indicated in the statistical analysis of biological results except for principle component analysis (GeneSpring software, Agilent, Waldbronn, Germany) was performed using R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). P-values were calculated using an analysis of variance (ANOVA), followed by a post-hoc Tukey test for pairwise statistical comparison. Values of $p < 0.05$ were considered statistically significant and annotated as indicated in the figure legends.

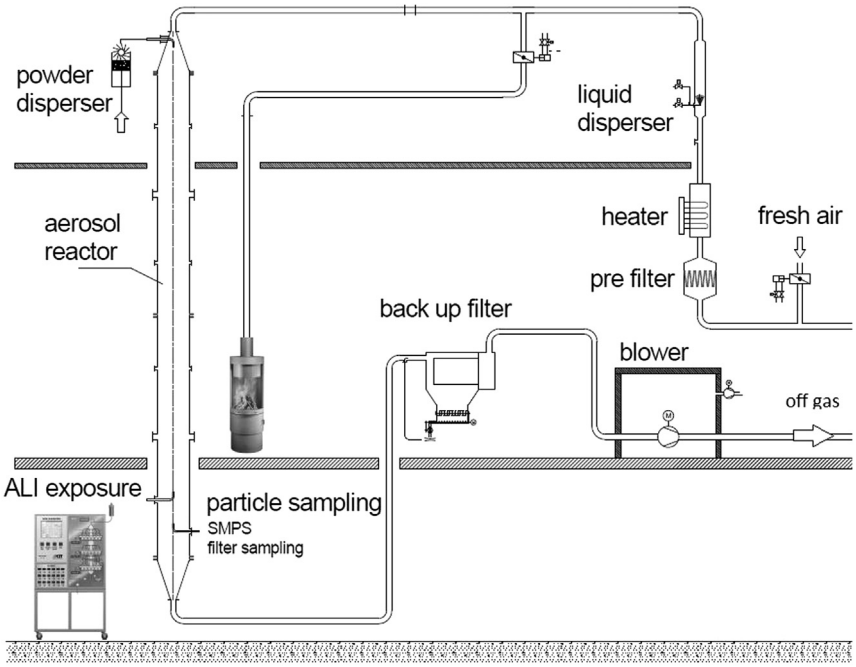

Fig. 3. Flow chart of exposure experiments at AEOLA with the different aerosol sources: the liquid disperser used to spray the fluorescein sodium solution, a dry powder disperser, and the wood stove.

2.3. Application examples

The ALI system was applied at typical combustion sources. The ALI system was installed at the exhaust of a ship diesel engine which was chosen as a representative technical process and at biomass burners as a model of typical household heaters. Both systems are relevant emitters contributing to the ultrafine particle air pollution (Corbett, 2003; Naeher et al., 2007). At the ship diesel engine (one cylinder, four stroke cycle, max. power output: 80 kW) the off-gases were sampled and diluted as described by Oeder et al. (2015) the comprehensive aerosol characterisation is published by Reda et al., (2014) and Sippula et al. (2014).

For the biomass combustion experiments, a 8 kW log wood stove (type "Toronto", Hase Kaminofenbau GmbH, Germany) was installed at the aerosol reactor AEOLA (Mülhopt et al., 2008) (Fig. 3). The stove was fired with beech logs of 1.2 kg each and a humidity of less than 15% according to DIN EN ISO 17225-5. The AEOLA reactor was operated with a flow rate of 250 m³/h, resulting in a nominal dilution of the off-gas stream of the wood stove of 25 m³/h by the factor of 10. The diluted aerosol was allowed to stabilise in the 6 m long AEOLA reactor and downstream, after a residence time of 2.1 s, the samples were taken and directed to the ALI exposure system. The aerosol was characterised by a Scanning Mobility Particle Sizer SMPS (Model 3934C-3 TSI Inc., Minnesota, USA) after sampling from the conditioning reactor of the exposure system. The SMPS was operated at a flow rate of 0.3 l/min and determined the particle number and size distribution in a range of 14.1–763.5 nm. Measurements were repeated every 5 min to monitor the changes in the aerosol which were dependent on the burning phase of the log wood burner, as the logs were applied once or twice an hour.

3. Results and discussion

3.1. Physical validation by fluorescein sodium dosimetry

To test the reproducible and homogeneous deposition of particles in the automated exposure system, fluorescein sodium dosimetry was applied. As shown in Fig. 4, a mean deposition of 0.29 ± 0.0375 μg/(h cm²) was obtained without electrical field. Referring these values to the exposed particle mass concentration $c_m = 14.4$ mg/m³ leads to a deposition efficiency of $f = 1.5\%$ corresponding to earlier data (Mülhopt et al., 2008), the numerical simulation by Comouth et al. (2013), and similar systems (Bitterle et al., 2006; Tippe et al., 2002). Applying an electrical field at -1000 V, the mean deposition was increased by a factor of nearly 9 to 2.49 ± 0.19 μg/(h cm²) with a very low deviation of 5–8% between the positions within a single experiment. This deviation rose to 8–13% when comparing all independent experiments, due to the daily differences of the aerosol source. As shown for example in the studies of Savi et al. and de Bruijne et al., the deposition efficiency can be further increased by additional charging of the particles (de Bruijne et al., 2009; Künzi et al., 2013; Savi et al., 2008).

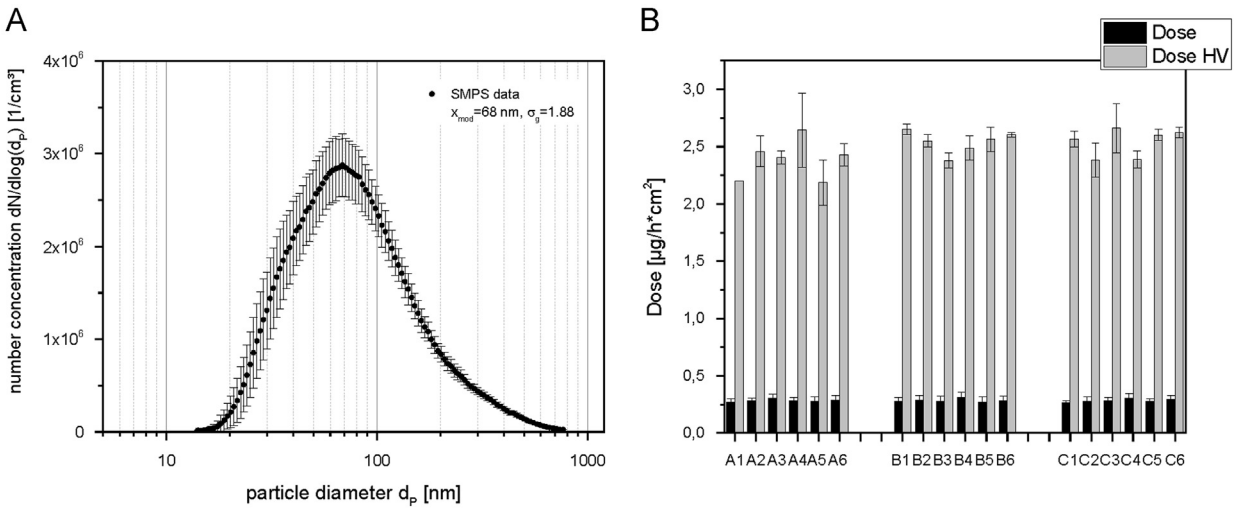

Fig. 4. Dose determination in all 18 modules of the HICE exposure system. A: particle number size distribution of fluorescein sodium aerosol in the reactor of the exposure system determined by SMPS, mean ± StdDev of 12 measurements shown in each channel B: fluorescein sodium mass deposited on the membrane surface per hour with (grey columns) and without (dark columns) electrical field (HV) caused by a high voltage of −1000 V. The values are means ± StdDev of three independent experiments with three technical replicates.

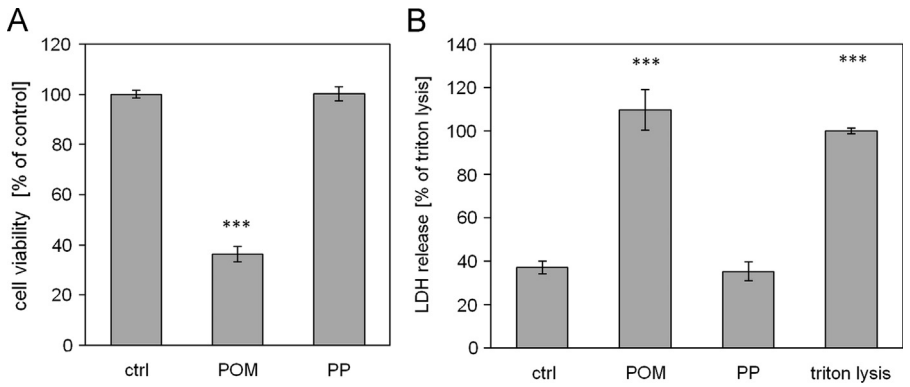

Fig. 5. Isolator cups made of polyoxymethylene (POM), but not polypropylene (PP) release toxic components into the cell culture medium after excessive contact periods. RPMI cell culture medium was put into isolator cups made of POM and PP or standard cell culture-compliant conical tubes made of PP (ctrl) for 24 h. Isolator cups were completely assembled, including electrodes and sealings in the way they are used for cell exposure experiments. After treatment of A549 cells with the conditioned media for 24 h, effects on cell vitality were measured by (A) AlamarBlue® reduction and (B) LDH release. LDH released by non-treated cells lysed with 0.1% Triton-X 100 for 30 min was used as a reference for 100% LDH release. Data are reported as the mean ± StdDev of 6 samples from two independent experiments (***$p < 0.001$ compared to control).

However, we observed clear biological responses when the tested aerosols were analysed in our system. Therefore, it was not necessary to further increase the particle dose and we decided against additional particle charging. Installing one big, or 18 individual radioactive sources to achieve diffusional charging is not applicable. Using a corona charger, inducing a plasma in the gas phase, leads to byproducts, like ozone, which are not compatible with cell exposure.

3.2. Biological validation of the exposure system

3.2.1. Material compatibility with cell culture

For exposure in the ALI exposure system, the Transwell inserts with cells have to be placed into isolator cups that contain cell culture medium, which supplies the cells with nutrients during the exposure period. For construction of the isolator cup, a durable plastic with good machinability properties was favoured. Due to its suitability for food contact, polyoxymethylene (POM) was selected as the primary material. However, cell culture medium, after 24 h contact with isolator cups made of POM, was clearly toxic to A549 lung epithelial cells, as was shown by a decrease of cell viability (Fig. 5A) and increase of LDH release (Fig. 5 B) compared to control cells.

When isolator cups were instead manufactured from polypropylene (PP), a material routinely used in cell culture applications, but with poor machinability, no toxicity could be observed. We conclude that POM cups probably released toxic components which induced adverse effects and decided to use PP cups instead. Even though the properties of POM are desirable for engineering purposes, this material appears to be not suitable for use in cell culture applications. Indeed,

compromised biocompatibility of POM was reported before, presumably due to leaching of formaldehyde by degradation (Kusy & Whitley, 2005; LaIuppa, McAdams, Papoutsakis & Miller, 1997). Apart from polymers, other materials also have the potential to leach toxic components, e.g. metals (Rachet al., 2013). The tested isolator cups were all assembled in the same manner as they are used for cell experiments. Therefore, toxicity from other materials that get into contact with the cell culture medium can be excluded.

3.2.2. *Effects of two separated humidification systems*

Cells kept under ALI conditions, i.e. without a liquid layer on the apical side, are extremely susceptible to dehydration. Even a short period of dry air blowing onto the cells when inserting them into the ALI system caused heavy damage, which could be avoided by protecting the cells from the airstream (Fig. S1). The ALI system described here uses two different approaches to provide humidification for either aerosol or clean air. We wanted to be sure that both humidification systems provide sufficient humidity, thus not impairing cell vitality during exposure experiments. We exposed A549 cells to HEPA-filtered room air directed through the conditioning reactor normally used for the aerosol experiments as well as through the second independent humidification system used for clean air controls. All other procedures and settings were kept the same as for normal aerosol exposures. Both exposures to clean air resulted in no loss of cell viability (Fig. 6A) and no elevated release of LDH (Fig. 6B) compared to “lab control” cells. However, A549 cells were damaged when exposed to dry air (artificial toxicity, Fig. 6A). The possibility to expose cells to clean air and an aerosol in parallel is a major advancement of the

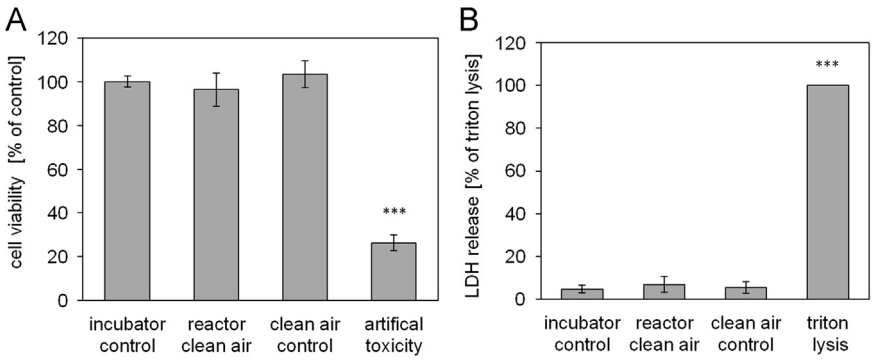

Fig. 6. A549 cells were exposed to HEPA-filtered ambient air for 4 h, humidified by passing either through the conditioning reactor (reactor clean air) or through the second humidification system for control positions (clean air control). After exposure, cell viability was measured by reduction of AlamarBlue® (A) and cell membrane integrity by LDH release (B). Cells kept in a standard cell culture incubator without CO_2 supplementation (incubator control) served as negative control. The positive controls for the AlamarBlue assay were cells that were intentionally challenged by exposure to non-humidified air (artificial toxicity). Cells that were lysed with 1% Triton-X 100 were used as a reference for maximum LDH release (100%). Reported are the means ± StdDev of 9 samples from 3 independent experiments (A) or 3 samples from one representative experiment (B) (***$p < 0.001$ compared to control).

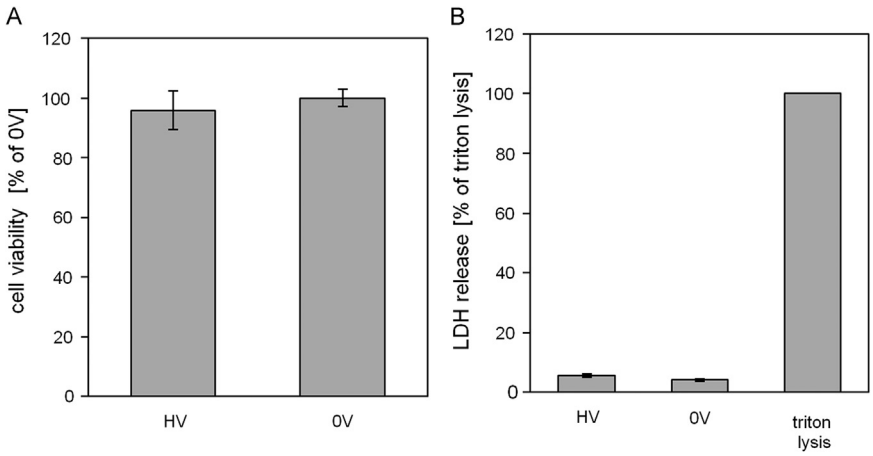

Fig. 7. Application of an electrostatic field has no influence on cell viability and cell membrane integrity. A549 cells were exposed to humidified HEPA-filtered ambient air for 4 h, with either a high voltage (HV, −1000 V) or no voltage (0 V) applied to the electrodes. After exposure, cell viability was measured by reduction of AlamarBlue® (A) and cell membrane integrity by LDH release (B). AlamarBlue® reduction was normalised to the sample without electrostatic field (100%) and LDH release to the sample lysed with Triton X-100 (100%). Reported are the means ± StdDev of 6 samples from two independent experiments.

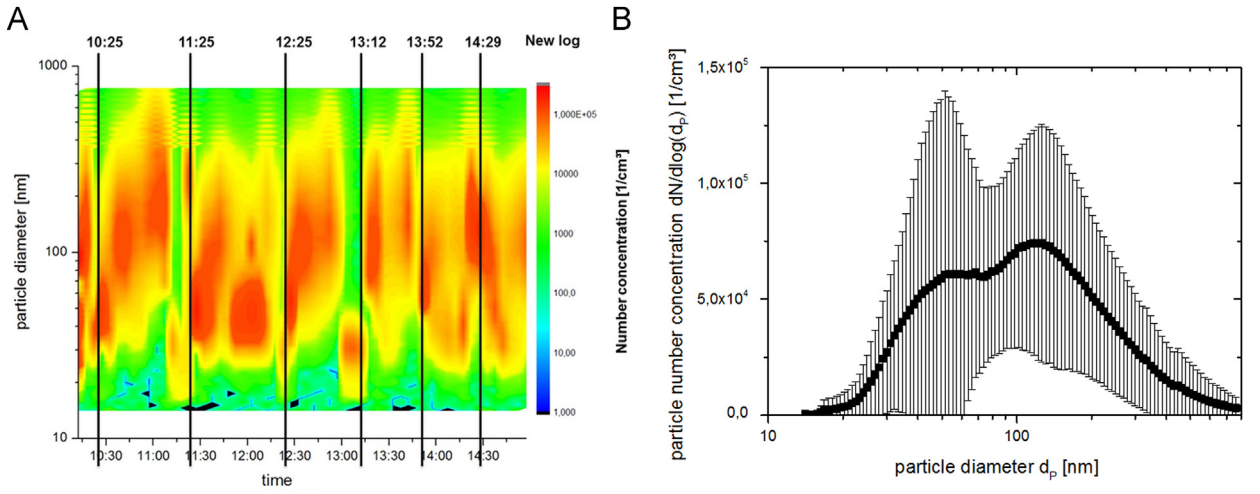

Fig. 8. Particle number size distribution dN/dlog(d_P) of wood combustion aerosol in the reactor of the exposure system determined by SMPS. A: each measurement of 5 min. duration plotted versus exposure time. Black markers indicate the input of a new wood log in the stove. B: mean of 53 measurements with standard deviation in each channel.

described system. Only with a dedicated humidification system for exposure to clean air, it is possible to properly investigate aerosols with potentially toxic gas phase constituents.

3.2.3. *Influences of an electrostatic field on cell vitality*

The effect of the electrostatic field which can be applied to enhance particle deposition efficiency (see Fig. 4) has been tested with A549 cells by exposure to clean air with and without high voltage. No influence on cell viability (Fig. 7A) or LDH release (Fig. 7 B) was observed after 4 h at the positions where the electrical potential was set to −1000 V when compared to positions without an electrostatic field. Other groups working with ALI exposure systems that use electrostatic deposition performed similar experiments. In agreement with our results, they consistently report no effects on cell viability or other toxicological endpoints (Hawley, McKenna, Marchese & Volckens, 2014; Zavala et al., 2014). The effect of the electrical field was only tested with regard to cell viability. Recent studies (Panas et al., 2014) did not indicate an influence of the electrical field on selected signal transduction pathways or the expression of proteins. Yet, more subtle sub-lethal effects cannot be ruled out at this time but will be further studied in the future by applying omics approaches as described in Fig. 11. The same holds true for the two different humidification approaches which might also trigger different biological responses.

3.3. *Application examples: exposures of cells to wood stove exhaust and ship diesel aerosol*

3.3.1. *Aerosol properties of wood stove exhaust*

The described ALI system was used to expose cultured cells to log wood stove exhaust. During the exposure period, the particle size distribution in the diluted exhaust was determined by SMPS. The number size distribution of one representative experiment over the exposure period of 4 h is shown in Fig. 8. The particle size distribution as well as the geometric particle diameter and the number concentration were dependent on the burning phase. Time points when a new wood log was added to the fireplace are indicated in the figure. The total number concentration varies between 1.0E+04 1/cm³ and 1.2E+05 1/cm³, the geometric mean of the particle diameter d_P varies between 30 and 120 nm.

Evaluating TEM images (Fig. 9), a dose on the Transwells® of 0.34 ± 0.12 μg/cm² without HV, and 1.33 ± 0.29 μg/cm² with HV, was determined for a 4 h exposure. The increase factor of the deposition efficiency f is determined from the TEM analysis to 3.9 for flame-ionised wood combustion particles, which is lower than factor 9 determined for fluorescein-sodium particles (see Fig. 4) by fluorescence spectroscopy. It is assumed, that there is a difference in the particle charge probability which leads to these different increase factors. As pointed out in 3.1 it was decided not to influence the particle charge so the deposition efficiency by electrostatic forces depends on the particle charging by the formation process. The fluorescein sodium particles are formed by drying a sprayed solution whereas the wood stove aerosol is formed in a flame process and diluted by a factor of 10 which is expected to lead to other charging conditions.

The airborne particle mass c_m is calculated according Eq. 1 and used to calculate the surface dose $RID_{SMPS,diff}$ by Eq. 3. The deposition efficiency of fluorescein-sodium is applicable as the size distributions are similar and the diffusional deposition mainly depends on the size.

$$RID_{SMPS,diff} = c_m * t_{exposure} * V_{exposure} * f/A \quad (3)$$

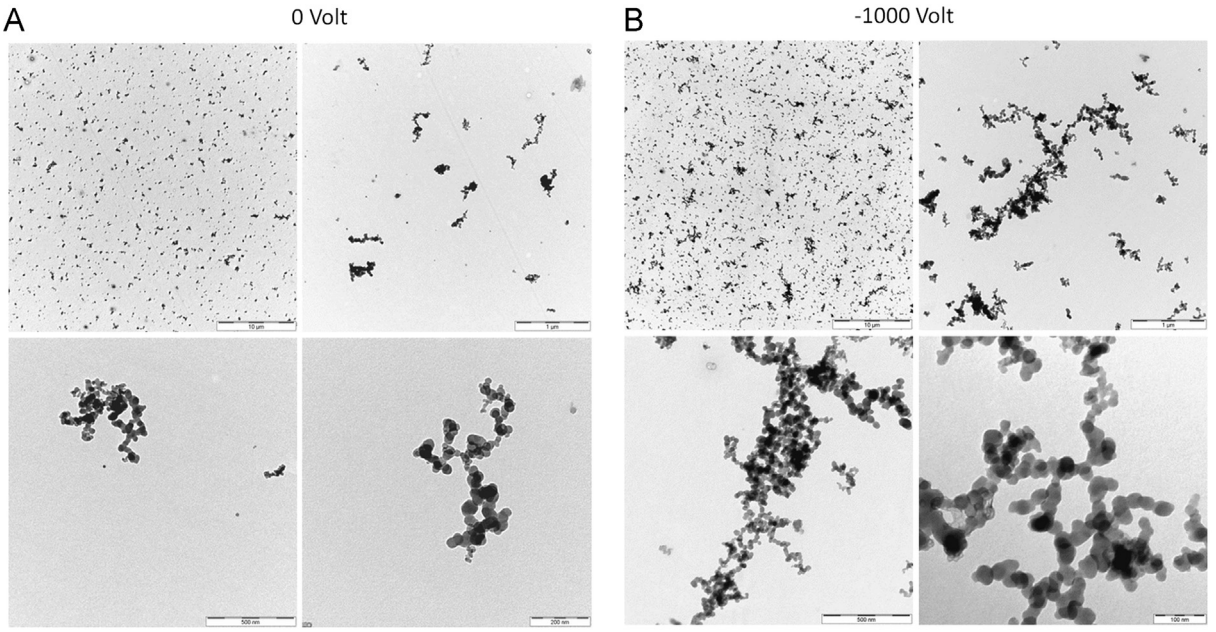

Fig. 9. Enhanced deposition of wood smoke particles in the presence of an electrostatic field. Copper grids of 200 mesh coated with Formvar film are exposed to complete 1:10 diluted wood smoke aerosol (B) with (aerosol HV) or (A) without an electrostatic field (aerosol 0 V) for 4 h. Images taken by TEM at 7000 × magnification are evaluated by ImageJ.

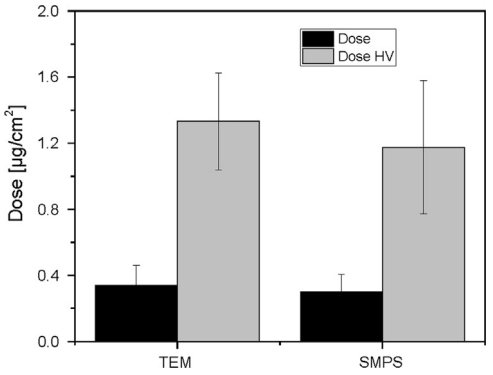

Fig. 10. Comparison of doses calculated from SMPS and TEM data for exposures with (HV) and without electrostatic deposition. Shown is the deposited particle dose on Transwell® inserts during a 4 h exposure experiment with 1:10 diluted wood smoke, determined by evaluating TEM and SMPS data.

$$RID_{SMPS,HV} = RID_{TEM,HV}/RID_{TEM,diff} * RID_{SMPS,diff} \quad (4)$$

A dose of $RID_{SMPS,diff} = 0.30 \pm 0.10\ \mu g/cm^2$ is observed without high voltage. Using the increase factor from TEM evaluation for the exposure with high voltage a dose of $RID_{SMPS,HV} = 1.18 \pm 0.40\ \mu g/cm^2$ is calculated. Dose determination by TEM and SMPS show a good agreement within the diffusional deposition. The electrostatic deposited dose calculated from the SMPS data is dependent on the TEM data (Fig. 10). Elihn et al. also observed a good agreement when comparing the spectroscopic determination of deposited Cu particles with TEM analysis (Elihn et al., 2013). As the particle deposition behaviour only depends on the particle size and on the gas phase conditions, there is no difference in deposition expected between the wet cell surface and the dry polyester membrane of the Transwell membrane insert. Once a particle in the nanometre range adheres to a surface, van der Waals and capillary forces are big enough to prevent shear forces of an aerosol flow from removing those particles. It has to be noted, however, that both our methods derive mass from measured particle numbers using a particle density which, in a complex and unsteady process as the log wood combustion, is also varying with particle composition and size. In consequence, mass calculations from number concentrations from SMPS or TEM measurements can only be regarded as approximations. The integrated QCM dose monitoring is a direct mass measurement but was not used for the wood combustion aerosol as the doses were below the detection limit.

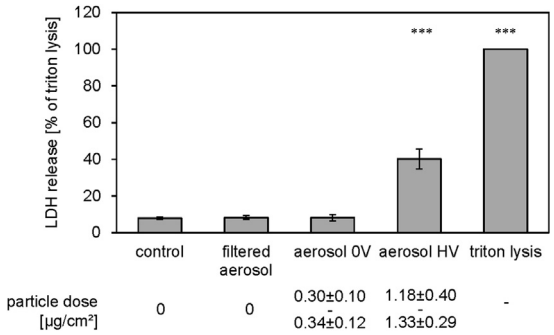

Fig. 11. Enhanced deposition of wood smoke particles by an electrostatic field leads to cell death after short exposure periods. A549 cells were exposed to 1:10 diluted wood smoke aerosol with (aerosol HV) or without (aerosol 0 V) an electrostatic field as well as to filtered aerosol for 4 h. Cells exposed to clean air served as negative control and as positive control after lysis with 1% Triton-X 100. The data were normalised to the positive control with maximum LDH release (100%). Reported are the means ± StdDev of 3 samples of one representative experiment ($^{***}p < 0.001$ compared to control). The indicated particle dose is the range from the two estimation methods using TEM and SMPS data.

3.3.2. *Cytotoxic effects of wood smoke aerosol are dependent on dose*

A549 cells were exposed to 1:10 diluted beech combustion aerosol. Medium from beneath the membrane was sampled directly after the end of the 4 h exposure period and analysed for released LDH. Exposure to the diluted wood smoke did not induce detectable cytotoxicity when the whole aerosol or filtered aerosol was tested without application of the electrostatic field. However, enhanced particle deposition by using an electrostatic field generated by a potential of −1000 V led to a significant increase of released LDH (Fig. 11). This indicates a particle-mediated toxicity of wood smoke aerosol, which is dependent on the dose. Two studies report data on wood smoke toxicity using ALI. Künzi et al. could not observe any effects, including acute toxicity, when comparing wood smoke-exposed cells to control cells. However, the authors estimated the particle dose on cells to be much lower than in the present study (Künzi et al., 2013). Interestingly, Hawley and Volckens also did not observe any alterations in acute toxicity following exposure to wood burning emissions from cook stoves, although particle doses were comparable to those in the current study due to corona charging and electrostatic deposition (Hawley & Volckens, 2013).

More data are available on the acute toxicity of wood stove emissions using submerged exposure. While the majority of researchers report several biological effects, but no acute toxicity of collected wood smoke particles (Kocbach Bølling et al., 2009), some found toxic effects on murine macrophages after high particle doses, particularly from efficient combustions (Jalava et al., 2012; Uski et al., 2014). The doses which induced toxicity under submerged conditions assuming that all particles in the suspension deposit on the cells still are a factor of 2–10 higher than particle doses achieved with our ALI experimental set-up. Therefore, it can be concluded that for wood stove emissions, the ALI exposure technique is more sensitive to acute toxicity than submerged exposure.

3.3.3. *Aerosol properties of ship diesel emissions*

The application of the ALI system for studying biological effects of the exhaust from a ship diesel engine (gas phase and complete aerosol) is given as a second example. The design and the results of the biological experiments are described in Oeder et al. (Oeder et al., 2015). The ship diesel aerosol was characterised regarding size and mass distributions as well as the chemical composition by Mueller et al. (2015). Depending on the load of the engine, the modal value of the Diesel fuel (DF) exhaust particles varies in the range of 200–600 nm, determined by SMPS. The cells were exposed for 4 h to DF which was 1:10 diluted with clean air and the estimated particle dose was 28 ± 1.5 ng/cm² (Oeder et al., 2015). The calculation of the deposited mass is based on a gravimetric filter analysis of the diluted aerosol and assuming a deposition probability of 1.5% (Comouth et al., 2013).

3.3.4. *Principle component analysis of whole-genome expression induced by ship diesel emissions*

At the chosen dilution ratio, the particle fraction of DF did not show signs of acute cytotoxicity after 4 h exposure (Oeder et al., 2015). Additionally to the results presented in Oeder et al., the variance of gene expression on the whole-genome level was analysed by principle component analysis (PCA) after the 4 h exposure to filtered or unfiltered combustion aerosol of diesel fuel and clean air, which served as the control condition. Exposure triplicates of treatment conditions clustered together. A clustering of samples within one group indicates that there was only little variance due to the exposure system. Instead, variance was caused clearly by the exposure aerosol. Component 1 of the PCA separated samples from complete aerosol, filtered aerosol and clean air controls from each other, while component 2 mainly separated samples from filtered aerosol from clean air and complete aerosol (Fig. 12). This indicates that the treatment with the complete aerosol led to greater differences in gene regulation compared to control than the filtered aerosol. Additionally, there was no overlap between samples from differently treated groups, leading to the conclusion that for every condition, most of the variance was caused by aerosol treatment rather than by the exposure system itself. This makes the ALI system applicable for reproducible measurements of distinct exposure conditions.

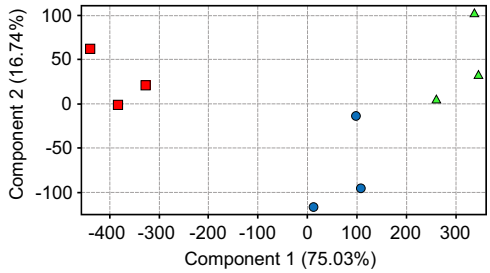

Fig. 12. Principle component analysis (PCA) of whole-genome expression data after 4 h exposure to combustion aerosols from a ship diesel engine using diesel as a fuel. PCA contains > 3-fold regulated genes. Squares = aerosol, circles = filtered aerosol, triangles = clean air. Component 1 on *x*-axis explains 75.03%, component 2 on *y*-axis explains 16.74% of variance.

4. Conclusions

This report describes a modified automated exposure station that has several advantages over its predecessor model:

1. Optional electrostatic field to achieve a higher particle deposition.
2. Internal control exposure to humidified clean air.
3. Software-controlled leakage test.

To our knowledge, this is the first mobile ALI exposure device that provides these features as an integrated system. The possibility to expose control cell cultures to clean air is a particularly important progress, as only the comparison to clean air allows for a proper investigation of potentially bioactive volatile compounds within a single system. We also showed that the new implemented features are compatible with cell viability. The system has been successfully applied in studies addressing wood burning and ship diesel engine emissions. The results indicate a high sensitivity and applicability of the system to real-world exhaust.

Nevertheless, the system also has its limitations, e.g. that only one aerosol together with its gaseous fraction can be tested at the same time. On the other hand, with 18 positions for cell cultures, also complex biological studies with simultaneous determination of different endpoints are possible.

Improvements towards a more realistic cell system are possible by using more complex co- or triple-cell cultures, which include other cell types occurring in the lung such as endothelial cells and macrophages or by using primary human lung cells. Considering the effect of the lung lining fluid is beneficial for the relevance of in vitro studies. This can be achieved in ALI exposure studies by either addition of surfactant on top of the cells or by using cells which produce surfactant.

In conclusion, the further development of the ALI system contributes to the establishment of a standardized test method which would simulate the *in vivo* situation during inhalation much better than submerged *in vitro* tests using collected particles. The method significantly increases the validity of *in vitro* tests and may contribute to further reduce the number of animal testing.

Supplementary material: Fig Bio2C_suppl: an example of detection of artefacts

An active ventilation system was installed as part of the air conditioning within the housing of the exposure system. When inserting new Transwell inserts with cells, the fan was on and unintentionally blew dry air over the cells, which resulted in cell death (fan, no cover). When the cells were protected by a cover (fan, covered) or cells were unprotected, but far away from the fan (no fan, no cover), no cytotoxicity was detected. After insertion into the exposure unit, all samples were exposed to humidified, HEPA-filtered ambient air for 4 h and subsequently analysed for LDH release. The control cells remained in a laboratory incubator (control). Cells, which were lysed with 0.2% Triton-X 100, were used as a reference for maximal LDH release. Results are from one experiment.

Acknowledgements

We thank Marco Mackert, Sonja Schaaf, and Silvia Andraschko for their excellent technical support, including the operation of the ALI exposure system, aerosol characterisation by SMPS, and the dose determination by fluorescence and TEM analyses.

The financial support by the Helmholtz Association via the Virtual Institute HICE (grant number VH-VI-418) and by the KIT Innovation Fund is gratefully appreciated.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jaerosci.2016.02.005.

References

Abe, S., Takizawa, H., Sugawara, I., & Kudoh, S. (2000). Diesel exhaust (DE)-induced cytokine expression in human bronchial epithelial cells: A study with a new cell exposure system to freshly generated DE in vitro. *American Journal of Respiratory Cell and Molecular Biology*, *22*, 296–303, http://dx.doi.org/10.1165/ajrcmb.22.3.3711.

Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics international*, *11*(7), 36–43.

Anderson, J. O., Thundiyil, J. G., & Stolbach, A. (2012). Clearing the air: a review of the effects of particulate matter air pollution on human health. *Journal of medical toxicology: official journal of the American College of Medical Toxicology*, *8*, 166–175, http://dx.doi.org/10.1007/s13181-011-0203-1.

AshaRani, P. V., Low Kah Mun, G., Hande, M. P., & Valiyaveettil, S. (2009). Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS nano*, *3*, 279–290, http://dx.doi.org/10.1021/nn800596w.

Aufderheide, M. (2005). Direct exposure methods for testing native atmospheres. *Experimental and Toxicologic Pathology*, *57*, 213–226, http://dx.doi.org/10.1016/j.etp.2005.05.019.

Aufderheide, M., Halter, B., Möhle, N., & Hochrainer, D. (2013). The CULTEX RFS: a comprehensive technical approach for the in vitro exposure of airway epithelial cells to the particulate matter at the air–liquid interface. *BioMed Research International*, *2013*, 1–15, http://dx.doi.org/10.1155/2013/734137.

Aufderheide, M., Ritter, D., Knebel, J. W., & Scherer, G. (2001). A method for in vitro analysis of the biological activity of complex mixtures such as sidestream cigarette smoke. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft für Toxikologische Pathologie*, *53*, 141–152.

Aufderheide, M., Scheffler, S., Möhle, N., Halter, B., & Hochrainer, D. (2011). Analytical in vitro approach for studying cyto- and genotoxic effects of particulate airborne material. *Analytical and Bioanalytical Chemistry*, *401*(10), 3213–3220, http://dx.doi.org/10.1007/s00216-011-5163-4.

Bakand, S., & Hayes, A. (2010). Troubleshooting methods for toxicity testing of airborne chemicals in vitro. *Journal of Pharmacological and Toxicological Methods*, *61*(2), 76–85, http://dx.doi.org/10.1016/j.vascn.2010.01.010.

Bitterle, E., Karg, E., Schroeppel, A., Kreyling, W. G., Tippe, A., Ferron, G. A., Schmid, O., Heyder, J., Maier, K. L., & Hofer, T. (2006). Dose-controlled exposure of A549 epithelial cells at the air–liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere*, *65*(10), 1784–1790, http://dx.doi.org/10.1016/j.chemosphere.2006.04.035.

Blank, F., Rothen-Rutishauser, B. M., Schurch, S., & Gehr, P. (2006). An optimized in vitro model of the respiratory tract wall to study particle cell interactions. *Journal of Aerosol Medicine*, *19*(3), 392–405, http://dx.doi.org/10.1089/jam.2006.19.392.

Broßell, D., Tröller, S., Dziurowitz, N., Plitzko, S., Linsel, G., Asbach, C., Azong-Wara, N., Fissan, H., & Schmidt-Ott, A. (2013). A thermal precipitator for the deposition of airborne nanoparticles onto living cells—Rationale and development. *Journal of Aerosol Science*, *63*, 75–86, http://dx.doi.org/10.1016/j.jaerosci.2013.04.012.

Cohen, J., Teeguarden, J., & Demokritou, P. (2014). An integrated approach for the in vitro dosimetry of engineered nanomaterials. *Particle and Fibre Toxicology*, *11*(1), 20.

Comouth, A., Saathoff, H., Naumann, K.-H., Mülhopt, S., Paur, H.-R., & Leisner, T. (2013). Modelling and measurement of particle deposition for cell exposure at the air liquid interface. *Journal of Aerosol Science*, *63*, 103–114, http://dx.doi.org/10.1016/j.jaerosci.2013.04.009.

Cooney, D. J., & Hickey, A. J. (2011). Cellular response to the deposition of diesel exhaust particle aerosols onto human lung cells grown at the air-liquid interface by inertial impaction. *Toxicology in Vitro: An International Journal Published in Association With BIBRA*, *25*, 1953–1965, http://dx.doi.org/10.1016/j.tiv.2011.06.019.

Corbett, J. J. (2003). Updated emissions from ocean shipping. *Journal of Geophysical Research*, *108*, 4650, http://dx.doi.org/10.1029/2003JD003751.

de Bruijne, K., Ebersviller, S., Sexton, K. G., Lake, S., Jetters, J., Walters, G. W., Doyle-Eisele, M., Woodside, R., Jeffries, H. E., & Jaspers, I. (2009). Design and testing of electrostatic aerosol in vitro exposure system (EAVES): an alternative exposure system for particles. *Inhalation Toxicology*, *21*, 91–101, http://dx.doi.org/10.1080/08958370802166035.

Dockery, D. W. (2009). Health effects of particulate air pollution. *Annals of Epidemiology*, *19*, 257–263, http://dx.doi.org/10.1016/j.annepidem.2009.01.018.

Dockery, D. W., & Pope, C. A. (1994). Acute respiratory effects of particulate air pollution. *Annual Review of Public Health*, *15*, 107–132, http://dx.doi.org/10.1146/annurev.pu.15.050194.000543.

Elihn, K., Cronholm, P., Karlsson, H. L., Midander, K., Wallinder, I. O., & Möller, L. (2013). Cellular dose of partly soluble Cu particle aerosols at the air–liquid interface using an in vitro lung cell exposure system. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, *26*(2), 84–93, http://dx.doi.org/10.1089/jamp.2012.0972.

Fukano, Y., Ogura, M., Eguchi, K., Shibagaki, M., & Suzuki, M. (2004). Modified procedure of a direct in vitro exposure system for mammalian cells to whole cigarette smoke. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft für Toxikologische Pathologie*, *55*, 317–323, http://dx.doi.org/10.1078/0940-2993-00341.

Fukano, Y., Yoshimura, H., & Yoshida, T. (2006). Heme oxygenase-1 gene expression in human alveolar epithelial cells (A549) following exposure to whole cigarette smoke on a direct in vitro exposure system. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft für Toxikologische Pathologie*, *57*, 411–418, http://dx.doi.org/10.1016/j.etp.2005.12.001.

Hawley, B., McKenna, D., Marchese, A., & Volckens, J. (2014). Time course of bronchial cell inflammation following exposure to diesel particulate matter using a modified EAVES. *Toxicology in Vitro*, *28*(5), 829–837, http://dx.doi.org/10.1016/j.tiv.2014.03.001.

Hawley, B., & Volckens, J. (2013). Proinflammatory effects of cookstove emissions on human bronchial epithelial cells. *Indoor Air*, *23*, 4–13, http://dx.doi.org/10.1111/j.1600-0668.2012.00790.x.

Herzog, F., Clift, M., Picapietra, F., Behra, R., Schmid, O., Petri-Fink, A., & Rothen – Rutishauser, B. (2013). Exposure of silver-nanoparticles and silver-ions to lung cells in vitro at the air–liquid interface. *Particle and Fibre Toxicology*, 1–14.

Holder, A. L., Lucas, D., Goth-Goldstein, R., & Koshland, C. P. (2008). Cellular response to diesel exhaust particles strongly depends on the exposure method. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, *103*, 108–115, http://dx.doi.org/10.1093/toxsci/kfn014.

Holder, A. L., & Marr, L. C. (2013). Toxicity of silver nanoparticles at the air–liquid interface. *BioMed Research International*, *2013*, 1–11, http://dx.doi.org/10.1155/2013/328934.

Jalava, P. I., Happo, M. S., Kelz, J., Brunner, T., Hakulinen, P., Mäki-Paakkanen, J., Hukkanen, A., Jokiniemi, J., Obernberger, I., & Hirvonen, M.-R. (2012). In vitro toxicological characterization of particulate emissions from residential biomass heating systems based on old and new technologies. *Atmospheric Environment*, *50*, 24–35, http://dx.doi.org/10.1016/j.atmosenv.2012.01.009.

Joeng, L., Hayes, A., & Bakand, S. (2013). Validation of the dynamic direct exposure method for toxicity testing of diesel exhaust in vitro. *ISRN Toxicology*, *2013*, 139512, http://dx.doi.org/10.1155/2013/139512.

Kelly, F. J., & Fussell, J. C. (2012). Size, source and chemical composition as determinants of toxicity attributable to ambient particulate matter. *Atmospheric Environment*, *60*, 504–526, http://dx.doi.org/10.1016/j.atmosenv.2012.06.039.

Kim, J. S., Peters, T. M., O'Shaughnessy, P. T., Adamcakova-Dodd, A., & Thorne, P. S. (2013). Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials. *Toxicology in Vitro*, *27*(1), 164–173, http://dx.doi.org/10.1016/j.tiv.2012.08.030.

Knebel, J. W., Ritter, D., & Aufderheide, M. (2002). Exposure of human lung cells to native diesel motor exhaust–development of an optimized in vitro test strategy. *Toxicology In Vitro: An International Journal Published in Association With BIBRA*, *16*, 185–192.

Kocbach Bølling, A., Pagels, J., Yttri, K. E., Barregard, L., Sallsten, G., Schwarze, P. E., & Boman, C. (2009). Health effects of residential wood smoke particles: The importance of combustion conditions and physicochemical particle properties. *Particle and Fibre Toxicology*, *6*, 29, http://dx.doi.org/10.1186/1743-8977-6-29.

Kooter, I. M., Alblas, M., Jedynska, A. D., Steenhof, M., Houtzager, M. M. G., & Ras, M. v (2013). Alveolar epithelial cells (A549) exposed at the air–liquid interface to diesel exhaust: First study in TNO's powertrain test center. *Toxicology in Vitro*. http://dx.doi.org/10.1016/j.tiv.2013.10.007.

Künzi, L., Mertes, P., Schneider, S., Jeannet, N., Menzi, C., Dommen, J., Baltensperger, U., Prévôt, A. S. H., Salathe, M., Kalberer, M., & Geiser, M. (2013). Responses of lung cells to realistic exposure of primary and aged carbonaceous aerosols. *Atmospheric Environment*, *68*, 143–150, http://dx.doi.org/10.1016/j.atmosenv.2012.11.055.

Kusy, R. P., & Whitley, J. Q. (2005). Degradation of plastic polyoxymethylene brackets and the subsequent release of toxic formaldehyde. *American Journal of Orthodontics And Dentofacial Orthopedics: Official Publication of the American Association of Orthodontists, Its Constituent Societies, and The American Board of Orthodontics*, *127*, 420–427, http://dx.doi.org/10.1016/j.ajodo.2004.01.023.

Laluppa, J. a, McAdams, T. a, Papoutsakis, E. T., & Miller, W. M. (1997). Culture materials affect ex vivo expansion of hematopoietic progenitor cells. *Journal of Biomedical Materials Research*, *36*, 347–359.

Lanzerstorfer, C. (2015). Chemical composition and physical properties of filter fly ashes from eight grate-fired biomass combustion plants. *Journal of Environmental Sciences (China)*, *30*, 191–197, http://dx.doi.org/10.1016/j.jes.2014.08.021.

Lenz, A.-G., Karg, E., Brendel, E., Hinze-Heyn, H., Maier, K. L., Eickelberg, O., Stoeger, T., & Schmid, O. (2013). Inflammatory and oxidative stress responses of an alveolar epithelial cell line to airborne zinc oxide nanoparticles at the air-liquid interface: A comparison with conventional, submerged cell-culture conditions. *BioMed Research International*, *2013*, 1–12, http://dx.doi.org/10.1155/2013/652632.

Lenz, A.-G., Stoeger, T., Cei, D., Schmidmeir, M., Pfister, N., Burgstaller, G., Lentner, B., Eickelberg, O., Meiners, S., & Schmid, O. (2014). Efficient bioactive delivery of aerosolized drugs to human pulmonary epithelial cells cultured at air–liquid interface conditions. *American Journal of Respiratory Cell and Molecular Biology*, *51*(4), 526–535, http://dx.doi.org/10.1165/rcmb.2013-0479OC.

Li, X., Nie, C., Shang, P., Xie, F., Liu, H., & Xie, J. (2013). Evaluation method for the cytotoxicity of cigarette smoke by in vitro whole smoke exposure. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft fur Toxikologische Pathologie*, *66*, 27–33, http://dx.doi.org/10.1016/j.etp.2013.07.004.

Lichtveld, K., Ebersviller, S. M., Sexton, K. G., Vizuete, W., Jaspers, I., & Jeffries, H. (2012). In vitro exposures in diesel exhaust atmospheres: Resuspension of PM from filters verses direct deposition of PM from air. *Environmental Science Technology*, *46*, 9062–9070, http://dx.doi.org/10.1021/es301431s.

Linsel, G., Bauer, M., Berger-Preiß, E., Gräbsch, C., Kock, H., Liebsch, M., Pirow, R., Ritter, D., Smirnova, L., & Knebel, J. (2011). Prävalidierungsstudie zur Prüfung der toxischen Wirkung von inhalativ wirksamen Stoffen (Gase) (pp. 1–43).

Maier, K. L., Alessandrini, F., Beck-Speier, I., Josef Hofer, T. P., Diabaté, S., Bitterle, E., Stöger, T., Jakob, T., Behrendt, H., Horsch, M., Beckers, J., Ziesenis, A., Hültner, L., Frankenberger, M., Krauss-Etschmann, S., & Schulz, H. (2008). Health effects of ambient particulate matter—Biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhalation Toxicology*, *20*(3), 319–337, http://dx.doi.org/10.1080/08958370701866313.

Mathis, C., Poussin, C., Weisensee, D., Gebel, S., Hengstermann, A., Sewer, A., Belcastro, V., Xiang, Y., Ansari, S., Wagner, S., Hoeng, J., & Peitsch, M. C. (2013). *Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air–liquid interface resemble bronchial epithelium from human smokers* (Vol. 304).

Monopoli, M. P., Wan, S. H. A., Bombelli, F. B., Mahon, E., & Dawson, K. A. (2013). Comparisons of nanoparticle protein corona complexes isolated with different methods. *Nano LIFE*, *3*(4), 1–9, http://dx.doi.org/10.1142/s1793984413430046.

Mueller, L., Jakobi, G., Czech, H., Stengel, B., Orasche, J., Arteaga-Salas, J. M., Karg, E., Elsasser, M., Sippula, O., Streibel, T., Slowik, J. G., Prevot, A. S. H., Jokiniemi, J., Rabe, R., Harndorf, H., Michalke, B., Schnelle-Kreis, J., & Zimmermann, R. (2015). Characteristics and temporal evolution of particulate emissions from a ship diesel engine. *Applied Energy*, *155*, 204–217, http://dx.doi.org/10.1016/j.apenergy.2015.05.115.

Mülhopt, S., Diabaté, S., Krebs, T., Weiss, C., & Paur, H. R. (2009). Lung toxicity determination by in vitro exposure at the air liquid interface with an integrated online dose measurement. Journal of Physics: Conference Series, 170, S.012008/012001-012004. http://dx.doi.org/10.1088/1742-6596/170/1/012008.

Mülhopt, S., Paur, H. R., Diabaté, S., & Krug, H. F. (2008). In vitro testing of inhalable fly ash at the air liquid interface. In Y. J. Kim, & U. Platt (Eds.), *Advanced Environmental Monitoring* (pp. 402–414). Dordrecht: Springer Netherlands.

Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A. C. R., Gehr, P., Burtscher, H., Morin, J.-P., Konstandopoulos, A., & Rothen-Rutishauser, B. (2010). New exposure system to evaluate the toxicity of (scooter) exhaust emissions in lung cells in vitro. *Environmental Science Technology*, *44*, 2632–2638, http://dx.doi.org/10.1021/es903146g.

Naeher, L. P., Brauer, M., Lipsett, M., Zelikoff, J. T., Simpson, C. D., Koenig, J. Q., & Smith, K. R. (2007). Woodsmoke health effects: a review. *Inhalation Toxicology*, *19*, 67–106, http://dx.doi.org/10.1080/08958370600985875.

Nara, H., Fukano, Y., Nishino, T., & Aufderheide, M. (2013). Detection of the cytotoxicity of water-insoluble fraction of cigarette smoke by direct exposure to cultured cells at an air-liquid interface. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft für Toxikologische Pathologie*, *65*, 683–688, http://dx.doi.org/10.1016/j.etp.2012.08.004.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z., & Zhang, H. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. *Accounts of Chemical Research*, *46*(3), 607–621.

Nemmar, A., Holme, J. a, Rosas, I., Schwarze, P. E., & Alfaro-Moreno, E. (2013). Recent advances in particulate matter and nanoparticle toxicology: a review of the in vivo and in vitro studies. *BioMed Research International*, *2013*, 1–22, http://dx.doi.org/10.1155/2013/279371.

Oeder, S., Kanashova, T., Sippula, O., Sapcariu, S. C., Streibel, T., Arteaga-Salas, J. M., Passig, J., Dilger, M., Paur, H.-R., Schlager, C., Mülhopt, S., Diabaté, S., Weiss, C., Stengel, B., Rabe, R., Harndorf, H., Torvela, T., Jokiniemi, J. K., Hirvonen, M.-R., Schmidt-Weber, C., Traidl-Hoffmann, C., BéruBé, K. A., Wlodarczyk, A. J., Prytherch, Z., Michalke, B., Krebs, T., Prévôt, A. S. H., Kelbg, M., Tiggesbäumker, J., Karg, E., Jakobi, G., Scholtes, S., Schnelle-Kreis, J., Lintelmann, J., Matuschek, G., Sklorz, M., Klingbeil, S., Orasche, J., Richthammer, P., Müller, L., Elsasser, M., Reda, A., Gröger, T., Weggler, B., Schwemer, T., Czech, H., Rüger, C. P., Abbaszade, G., Radischat, C., Hiller, K., Buters, J. T. M., Dittmar, G., & Zimmermann, R. (2015). Particulate matter from both heavy fuel oil and diesel fuel shipping emissions show strong biological effects on human lung cells at realistic and comparable in vitro exposure conditions. *PLoS One*, *10*(6), e0126536, http://dx.doi.org/10.1371/journal.pone.0126536.

Okuwa, K., Tanaka, M., Fukano, Y., Nara, H., Nishijima, Y., & Nishino, T. (2010). In vitro micronucleus assay for cigarette smoke using a whole smoke exposure system: a comparison of smoking regimens. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft für Toxikologische Pathologie*, *62*(4), 433–440, http://dx.doi.org/10.1016/j.etp.2009.06.002.

Panas, A., Comouth, A., Saathoff, H., Leisner, T., Al-Rawi, M., Simon, M., Seemann, G., Dössel, O., Mülhopt, S., Paur, H.-R., Fritsch-Decker, S., Weiss, C., & Diabaté, S. (2014). Silica nanoparticles are less toxic to human lung cells when deposited at the air–liquid interface compared to conventional submerged exposure. *Beilstein Journal of Nanotechnology*, *5*, 1590–1602, http://dx.doi.org/10.3762/bjnano.5.171.

Panas, A., Marquardt, C., Nalcaci, O., Bockhorn, H., Baumann, W., Paur, H.-R., Mülhopt, S., Diabaté, S., & Weiss, C. (2013). Screening of different metal oxide nanoparticles reveals selective toxicity and inflammatory potential of silica nanoparticles in lung epithelial cells and macrophages. *Nanotoxicology*, *7*(3), 259–273, http://dx.doi.org/10.3109/17435390.2011.652206.

Paur, H.-R., Cassee, F. R., Teeguarden, J., Fissan, H., Diabate, S., Aufderheide, M., Kreyling, W. G., Hänninen, O., Kasper, G., Riediker, M., Rothen-Rutishauser, B., & Schmid, O. (2011). In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung-A dialog between aerosol science and biology. *Journal of Aerosol Science*, *42*, 668–692, http://dx.doi.org/10.1016/j.jaerosci.2011.06.005.

Paur, H.-R., Mülhopt, S., Weiss, C., & Diabaté, S. (2008). In vitro exposure systems and bioassays for the assessment of toxicity of nanoparticles to the human lung. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, *3*, 319–329, http://dx.doi.org/10.1007/s00003-008-0356-2.

Raemy, D. O., Grass, R. N., Stark, W. J., Schumacher, C. M., Clift, M. J. D., Gehr, P., Rothen, P., 8211, & Rutishauser, B. (2012). Effects of flame made zinc oxide particles in human lung cells—A comparison of aerosol and suspension exposures. *Particle and Fibre Toxicology*, *9*, 33, http://dx.doi.org/10.1186/1743-8977-9-33.

Reda, A. A., Schnelle-Kreis, J., Orasche, J., Abbaszade, G., Lintelmann, J., Arteaga-Salas, J. M., Stengel, B., Rabe, R., Harndorf, H., Sippula, O., Streibel, T., & Zimmermann, R. (2014). Gas phase carbonyl compounds in ship emissions: Differences between diesel fuel and heavy fuel oil operation. *Atmospheric Environment*, *94*(0), 467–478, http://dx.doi.org/10.1016/j.atmosenv.2014.05.053.

Savi, M., Kalberer, M., Lang, D., Ryser, M., Fierz, M., Gaschen, A., Rička, J., & Geiser, M. (2008). A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environmental Science and Technology*, *42*(15), 5667–5674.

Sayes, C. M., Reed, K. L., & Warheit, D. B. (2007). Assessing toxicity of fine and nanoparticles: Comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicological Sciences*, *97*(1), 163–180, http://dx.doi.org/10.1093/toxsci/kfm018.

Schwarze, P., Øvrevik, J., Låg, M., Refsnes, M., Nafstad, P., Hetland, R., & Dybing, E. (2006). Particulate matter properties and health effects: consistency of epidemiological and toxicological studies. *Human Experimental Toxicology*, *25*(10), 559–579.

Seagrave, J., Dunaway, S., McDonald, J. D., Mauderly, J. L., Hayden, P., & Stidley, C. (2007). Responses of differentiated primary human lung epithelial cells to exposure to diesel exhaust at an air–liquid interface. *Experimental Lung Research*, *33*, 27–51, http://dx.doi.org/10.1080/01902140601113088.

Sippula, O., Stengel, B., Sklorz, M., Streibel, T., Rabe, R., Orasche, J., Lintelmann, J., Michalke, B., Abbaszade, G., Radischat, C., Gröger, T., Schnelle-Kreis, J., Harndorf, H., & Zimmermann, R. (2014). Particle emissions from a marine engine: Chemical composition and aromatic emission profiles under various operating conditions. *Environmental Science Technology, A-I.* http://dx.doi.org/10.1021/es502484z.

Soderholm, S. C. (1979). Analysis of Diffusion Battery Data. *Journal of Aerosol Science*, *10*, 163–175.

Steiner, S., Czerwinski, J., Comte, P., Müller, L. L., Heeb, N. V., Mayer, A., Petri-Fink, A., & Rothen-Rutishauser, B. (2013). Reduction in (pro-)inflammatory responses of lung cells exposed in vitro to diesel exhaust treated with a non-catalyzed diesel particle filter. *Atmospheric Environment*, *81*, 117–124, http://dx.doi.org/10.1016/j.atmosenv.2013.08.029.

Stevens, J. P., Zahardis, J., MacPherson, M., Mossman, B. T., & Petrucci, G. A. (2008). A new method for quantifiable and controlled dosage of particulate matter for in vitro studies: The electrostatic particulate dosage and exposure system (EPDExS). *Toxicology in Vitro*, *22*(7), 1768–1774, http://dx.doi.org/10.1016/j.tiv.2008.05.013.

Subramanian, R., Khlystov, A. Y., Cabada, J. C., & Robinson, A. L. (2004). Positive and negative artifacts in particulate organic carbon measurements with denuded and undenuded sampler configurations. Special issue of aerosol science and technology on findings from the fine particulate matter supersites program. *Aerosol Science and Technology*, *38*(Suppl. 1), S27–S48, http://dx.doi.org/10.1080/02786820390229354.

Teeguarden, J., Hinderliter, P. M., Orr, G., Thrall, B. D., & Pounds, J. G. (2007). Particokinetics in vitro: Dosimetry considerations for in vitro nanoparticle toxicity assessments. *Toxicological Sciences*, *95*(2), 300–312, http://dx.doi.org/10.1093/toxsci/kfl165.

Tippe, A., Heinzmann, U., & Roth, C. (2002). Deposition of fine and ultrafine aerosol particles during exposure at the air/cell interface. *Journal of Aerosol Science*, *33*(2), 207–218, http://dx.doi.org/10.1016/S0021-8502(01)00158-6.

Tsukue, N., Okumura, H., Ito, T., Sugiyama, G., & Nakajima, T. (2010). Toxicological evaluation of diesel emissions on A549 cells. *Toxicology In Vitro: An International Journal Published in Association With BIBRA*, *24*, 363–369, http://dx.doi.org/10.1016/j.tiv.2009.11.004.

Uski, O., Jalava, P. I., Happo, M. S., Leskinen, J., Sippula, O., Tissari, J., Mäki-Paakkanen, J., Jokiniemi, J., & Hirvonen, M.-R. (2014). Different toxic mechanisms are activated by emission PM depending on combustion efficiency. *Atmospheric Environment*, *89*, 623–632, http://dx.doi.org/10.1016/j.atmosenv.2014.02.036.

Weber, S., Hebestreit, M., Conroy, L. L., & Rodrigo, G. (2013). Comet assay and air–liquid interface exposure system: A new combination to evaluate genotoxic effects of cigarette whole smoke in human lung cell lines. *Toxicology in Vitro: An International Journal Published in Association With BIBRA*, *27*, 1987–1991, http://dx.doi.org/10.1016/j.tiv.2013.06.016.

Zavala, J., Lichtveld, K., Ebersviller, S., Carson, J. L., Walters, G. W., Jaspers, I., Jeffries, H. E., Sexton, K. G., & Vizuete, W. (2014). The Gillings Sampler – An electrostatic air sampler as an alternative method for aerosol in vitro exposure studies. *Chemico-Biological Interactions*, *220*(0), 158–168, http://dx.doi.org/10.1016/j.cbi.2014.06.026.