



Toxicity of real-world PM_{2.5} road tunnel emissions using a mobile air-liquid interface system and submerged exposure[☆]

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ABSTRACT

Traffic-related air pollution is a major public health concern, contributing to respiratory and cardiovascular diseases worldwide. The aim of this study was to investigate the feasibility of using a mobile Air-Liquid Interface (ALI) system to assess the cytotoxicity and inflammatory potential of freshly generated PM_{2.5} (particle matter with aerodynamic diameter <2.5 μm) in a road tunnel in Stockholm. We hypothesized that cellular effects would be detectable at lower doses compared to submerged exposures. The mean particle dose in ALI was 1.4 ± 0.8 μg/cm², whereas a wide range of doses was used for submerged exposures. ALI and submerged results showed that PM_{2.5} from the road tunnel did not affect the viability of A549 cells, whereas a significant and dose-dependent decrease in viability of dTHP-1 (in submerged exposure) was observed. Furthermore, in A549 in ALI a slight increase in inflammatory response (IL-8, IL-6, and IL-1β) was observed. In submerged exposure, the inflammatory response was clearer, particularly in the dTHP-1 cells. In conclusion, this study presents the first successfully conducted *in situ* ALI exposure in a road tunnel. The results demonstrate that dTHP-1 cells exhibit clear cytotoxic and inflammatory responses, while A549 show only weak effects. These findings suggest that co-cultures of A549 and dTHP-1 may be valuable in future ALI studies.

1. Introduction

Road traffic is a major source of particulate matter (PM) in urban areas (Amato et al., 2016; Manousakas et al., 2017; Sousa Santos et al., 2021). Since the Harvard Six Cities study, researchers have demonstrated a strong relationship between elevated air pollution concentration and increased mortality, particularly in urban areas with high PM concentrations (Chen and Hoek, 2020; Dockery et al., 1993; Laden et al., 2006).

Particulate matter is a mixture of airborne particles categorized by size, where PM_{2.5} refers to the mass concentration of fine particles (aerodynamic diameter <2.5 μm). Among the primary sources of PM_{2.5} in urban environments are diesel and gasoline exhaust, both major contributors to traffic-related air pollution. In addition to exhaust emissions, traffic pollution also includes non-exhaust emissions sources,

e.g., wear of tires, clutches, brakes, and road materials. Various aspects, including vehicle maintenance, engine age and technology, and the driving style, can further influence the size, concentration, and composition of emitted particles (Alves et al., 2020; Gustafsson et al., 2019; Matejka et al., 2017; Nosko and Olofsson, 2017; Singh et al., 2020).

When inhaled, traffic-related fine particles can settle in the respiratory system, potentially causing inflammation and several diseases such as fibrosis, asthma, and COPD (European Environment Agency, 2023; Thompson, 2018). Furthermore, diesel and gasoline emissions can promote inflammation and oxidative stress in human lung cells (Crobeddu et al., 2017; Hwang et al., 2021; Wen et al., 2023) and are classified as carcinogenic and potentially carcinogenic to humans (DeMarini and Linak, 2022).

The toxicity of PM_{2.5} traffic emissions can be tested by *in vivo* and *in*

[☆] This paper has been recommended for acceptance by Admir Créso Targino.

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in vitro methods. In animal studies, PM_{2.5} from traffic and urban sources was found to increase the concentration of ROS and activate the immune system (Li et al., 2019; Xu et al., 2019). However, although animal studies provide insight into systemic effects, they are not optimal models since their physiology of the respiratory system, respiration rate, and biological mechanisms differ from those of humans (Curbani et al., 2019). Alternatively, *in vitro* models can be used, such as submerged exposure systems, where human lung cells are exposed to a suspension of filter-collected particles. A great advantage of this method is the ease with which multiple doses can be tested in a dose-response manner. However, the way particles are introduced in this model may alter their physicochemical properties compared to those of airborne particles (Yang et al., 2014). In addition, it can be hard to assess the effective dose administered to the cells in submerged models (Glaubitz et al., 2023; Zavala et al., 2020). These limitations can be addressed using an Air-Liquid Interface (ALI) system. In this system, cultured lung cells are exposed directly to airborne particles in a way that more closely resembles human inhalation. No liquid medium is present on top of the cells, allowing the particles to deposit directly onto the cells. Unlike submerged exposures, this approach preserves the properties of airborne particles, resulting in a more realistic assessment of inhalation toxic responses. It also enables comparison between the full exhaust (including both particles and gases) and the gas fraction. Furthermore, the particle dose reaching the cells can be assessed in real time by measuring particle concentrations (Juárez-Facio et al., 2024). Some submerged *in vitro* toxicity studies of traffic PM_{2.5} observed inflammatory responses, mitochondrial dysfunction, and apoptosis (Li et al., 2008; Rahmatinia et al., 2021; Xu et al., 2019; Zheng et al., 2018). Several ALI studies have been performed with single source traffic-related emissions generated in the laboratory (e.g., fuel combustion) (Hakkaraianen et al., 2023; Holder et al., 2008, 2007; Kooter et al., 2013; Lichtveld et al., 2012; Mühlhopt et al., 2016; Stoehr et al., 2015); however, only a few studies have been conducted outdoors using a mobile ALI system (Augustin et al., 2020; Bisig et al., 2018; Ding et al., 2019; Gualtieri et al., 2018; Rossner et al., 2025; Vojtisek-Lom et al., 2025) and none in a road tunnel. We recently developed a mobile ALI exposure system named ELLIE (ELectrostatic air-Liquid Interface Exposure system) (Juárez-Facio et al., 2024). The present study uses this novel ELLIE system to examine the cytotoxicity and inflammatory response (in A549) of airborne particles (PM_{2.5}) in a road tunnel in Stockholm. We hypothesized that this approach would enable the detection of cellular effects at lower exposure doses compared to conventional submerged exposures. Furthermore, we also collected particles for parallel submerged *in vitro* toxicity testing (A549, dTHP-1) for a more extensive investigation of the particles' toxic effects. Finally, a comprehensive characterization of the particle content was conducted to provide insights for future correlation studies between the chemical composition of PM_{2.5} and its cytotoxic and inflammatory responses at different sites, as particle organic (Låg et al., 2020; Takam et al., 2024) and metal content (Padgett et al., 2013) has been shown as highly relevant in particle toxicity assessment.

2. Material and methods

2.1. Sampling location and ALI exposure

Exposure experiments and sampling of road tunnel PM_{2.5} were performed in the Söderledstunnel in Stockholm (1580 m long, 4.5 m in height, speed limit 70 km/h, 60000 vehicles/week) from March to May 2022 (Kristensson et al., 2004). The asphalt mixture used in Sweden typically consists of approximately 95 % crushed stone and 5 % bitumen. Measurements were performed 500 m from the north entrance in a room next to the tunnel (Fig. S1 in supplementary material). Road tunnel emissions can differ from day to day due to, e.g., the number and type of vehicles, asphalt type, and ventilation system. Therefore, the ALI exposures and collection of PM_{2.5} from the road tunnel for submerged

experiments were conducted during the same seasonal period.

The mobile ALI system, called ELLIE, facilitated *in vitro* toxicological studies of aerosols directly sampled from the tunnel via stainless steel tubing (Juárez-Facio et al., 2024). A concentrator with a sample flow of 8.5 L/min was used prior to the ALI system (see section 2.2), passing a 1.5 L/min flow to the ALI. After the concentrator, a gas dryer (MD-110-12S-4, Perma Pure, Lakewood, USA) eliminated humidity, and a neutralizer (⁶³Ni) charged the aerosol particles. Finally, the aerosol was re-humidified (>90 %RH, MH-110-12S-4; Perma Pure, Lakewood, USA) and distributed to the seven ALI exposure chambers (37 °C). Four chambers exposed A549 cells to gases and particles, while two chambers exposed cells to only the gases (filtered aerosol). The last chamber measured particle concentrations before and after the chamber to enable estimation of the exposure dose. Critical orifices, located after the exposure chamber, controlled the flow (214 mL/min) through each chamber. An alternated electrostatic field (±1 kV) was applied to enhance particle deposition efficiency up to 79 % (Latvala et al., 2016).

2.2. Concentrator

A concentrator increased the PM_{2.5} concentration by a factor of 5 prior to the ALI system. It was composed of a growth tube (Model GTC50- Aerosol Dynamics, Inc.) (Hering and Stolzenburg, 2005) followed by a virtual impactor (cut-size of 1 μm) (Rostedt et al., 2006). The concentration factor was calibrated with monodispersed particles using two condensation particle counters (CPCs, 7–300 nm, TSI models 3752 and 3789). The procedure is described and illustrated in the supplementary material (Section S1, Fig. S2). During the toxicity testing in the road tunnel, the sample flow of the virtual impactor was 8.5 L/min, and the minor flow to the ALI was 1.5 L/min, resulting in a concentration factor of 5. The growth tube temperatures were: 6.9 °C (Conditioner), 49.1 °C (Heater), and 17.1 °C (Moderator). After the concentrator, a slightly heated stainless-steel tube (few cm, 18.4 °C) was used to evaporate excess water from the particles.

2.3. Particle size selection and dose

A PM_{2.5} cyclone (SCC 1.062 Triplex cyclone, BGI by Mesa Labs, USA, 1.5 L/min) was used to size select road tunnel particles prior to the ALI system.

The particle number concentration was measured before and after the ALI system with an Optical Particle Counter (OPC, aerosol spectrometer 1.109, Grimm Technologies, USA, aerosol flow 1.2 L/min). To maintain the total flow of 1.5 L/min through the ALI system, the flow sampled by the OPC was diluted four times (0.3 mL/min road tunnel aerosol from ALI system, 0.9 mL/min filtered air, Parker-Balston, 9933-05-AQ). Data extracted from the OPC were adjusted by the dilution factor. The difference between the mean particle number concentrations measured before and after the ALI exposure chamber was used to estimate the particle number dose deposited on the cells during the exposure (particle sizes 0.25–2.5 μm). This number was divided by the insert surface area to get the number of particles per cm².

The deposited mass dose onto the cells was calculated by multiplying the particle volume (from OPC data) and effective density of road tunnel PM_{2.5} (0.916 g/cm³, assessed by a similar procedure as Tu and Olofsson (2021)). The OPC and a Dekati PM₁₀ impactor (ISO23210:2009 standard) were measuring in the same seasonal period (8h, May 24, 2022). Pre-greased polycarbonate filters collected particles on three impactor stages (1, 2.5 and 10 μm) of the Dekati impactor (10 L/min), and a PTFE (Polytetrafluoroethylene) end filter collected smaller particles. The total air volume passing through the impactor during the test and the mass differences of the filters before and after the test was used to calculate the particle mass concentration of different particle sizes. Thereafter, the effective density of road tunnel PM_{2.5} could be assessed.

2.4. PM_{2.5} characterization

2.4.1. Organic analysis

Organic compounds in PM_{2.5} collected on a PTFE filter were analyzed according to EPA methods (US EPA, 1994; 1999, 2010); the summary of analyzed compounds together with their detailed list and instrumental methodology is available in the supplementary material (Section S2, Table S1–S8). Briefly, samples spiked with ¹³C-labelled standards (Polychlorinated Biphenyls (PCBs), and Polybrominated Diphenyl Ethers (PBDEs), Wellington Laboratories, Canada) and deuterated Polycyclic Aromatic Hydrocarbons (PAHs), and Oxygenated Polycyclic Aromatic Hydrocarbons (OPAHs) were submitted to a single Pressurized Liquid Extraction (PLE), followed by Gel Permeation Chromatography (GPC) and Solid Phase Extraction (SPE) to provide distinct fractions for analysis.

Separate runs were carried out by a Thermo Scientific Orbitrap Exploris GC 60K system equipped with a Thermo Scientific Instant Connect Split/Splitless (SSL) Injector, and a programmable temperature (PTV) injector. An automatic sample injection was performed using a Thermo Scientific TriPlus RSH autosampler and Thermo Scientific TRACE 1310 GC system, using Thermo Scientific Chromeleon software.

A capillary BPX-DXN (60 m length, 0.25 mm i.d., 0.25 film thickness) column was used for PAH, OPAH and PCB separation, while for PBDEs a Rtx-1614 capillary column (15 m length, 0.10 mm i.d., 0.10 film thickness) was used. Acquisition was performed with selected ion monitoring (SIM), and compounds were identified by retention time, comparison with mass spectra, and isotopic ratio in the case of the halogenated contaminants.

Calibration curves were made for all target compounds (concentration range 1–1000 pg/μL for PAHs and OPAHs, 0.1–200 pg/μL for PCBs, and 1–500 pg/μL for BDEs).

2.4.2. Inorganic analysis

SEM-EDS analysis.

Particles collected in the road tunnel (PM_{2.5}) were analyzed by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS) to examine particle morphology and elemental composition, respectively. Particles were removed from the collection filter (half) by sonication in 5 mL of isopropanol (35 kHz, 5 min). The filter piece was removed and the suspension precipitated overnight. The next day, 4 mL of the alcohol was removed, and the remaining volume was evaporated at mild vacuum (20 mbar). The dry powder was collected with a non-magnetic metallic spatula and put on a SEM aluminum stub covered with carbon tape. SEM-EDS analysis was carried out with a Zeiss EVO MA10 scanning electron microscope ($V = 20$ kV and $I = 300$ pA, LaB6 filament, 10 mm² active area INCA X-act silicon-drift detector, Oxford Instrument). Five areas (400 × 300 μm) were analyzed (acquisition time 500 s). In the supplementary material, the elemental composition (wt%) with corresponding standard deviation (STD) is reported (Table S9).

XRD analysis.

The crystalline fraction of collected particles was investigated by using X-Ray Diffraction (XRD, Rigaku SmartLab diffractometer, rotating anode and Copper target ($K\alpha_1Cu = 0.154$ nm) in Bragg-Brentano parafocusing geometry) directly on the collected filters. XRD pattern was acquired in the angular 2θ range of 5–90° (step size 0.02°, scan speed 0.1°/min, 15 h measurement). Diffracted beam was collected by a OD scintillator detector mounted after a dedicated monochromator. During measurement, the sample was rotated at 10 rpm to reduce any eventual preferential orientation effect. The phase identification was carried out via the PDF4+ 2023 crystallographic database (ICDD) (Gates-Rector and Blanton, 2019).

2.5. Cell culture

A549 cells were selected for ALI exposures due to their established

robustness as a respiratory epithelial model. To ensure consistency, they were also used in submerged exposures, while THP-1 cells were included for their role in inflammation (Brown et al., 2007). For ALI exposure, cell line A549 (ATCC® CCL-185™) was cultured in DMEM culture medium (GIBCO) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS GIBCO), 1 % (v/v) sodium pyruvate 100 mM (GIBCO), and 1 % (v/v) Penicillin/Streptomycin mixture (GIBCO) at standard culture conditions (37 °C, 5 % CO₂). Cells were seeded (60,000 cells/cm² in 1.5 mL) at the apical side of the cell inserts and placed in a 6-well plate with 2 mL of basal culture medium. After 24h incubation, the cells reached around 80–90 % confluency. Thereafter, the cells were put at ALI condition prior to transport and exposure, i.e., the medium from the apical side was removed, and medium in the basal chamber was changed to 2 mL DMEM enriched with 25 μM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid - GIBCO).

A549 and dTHP-1 cells were used in the submerged exposure experiments. They were seeded in 96 well plates (A549 cells: 1.0 × 10⁴ cells/well, total well volume 100 μL, 24 h incubation, THP-1: 55 × 10⁴ cells/well, volume 100 μL (with PMA 50 ng/mL)). The THP-1 cells were allowed to differentiate for 48 h (hereafter called dTHP-1). Thereafter, cells were washed gently with 100 μL PBS (GIBCO). For both cell cultures, medium was removed, and cells were exposed to 100 μL particle suspensions of different concentrations (10, 50, 100, 150, and 200 μg/mL, approximately 3.1–62.5 μg/cm² considering the suspension volume of 100 μL, a surface area of 0.32 cm² and assuming a 100 % deposition efficiency) for 24 h. After exposure, medium was collected, centrifuged at 1500 rpm/5min to remove residual particles and stored at –80 °C until use (for cytokine analysis). Further, the cells were processed for the viability assessment (Alamar Blue assay). Cobalt nanoparticles (Co NPs, 50 μg/mL) were used as the positive control for submerged exposure.

2.6. Exposure to road tunnel particles

2.6.1. Exposure at ALI

Cells were transported in inserts at ALI condition for approximately 20 min from the laboratory to the road tunnel in a thermal container. Before placing the inserts into the mobile ALI system, 3.3 mL of fresh DMEM (GIBCO) medium enriched with 25 μM HEPES (GIBCO) was added to each exposure chamber (basal side of inserts). During experiments, no medium was present on top of the cells to keep them at the air-liquid interface. Cells were exposed for 2 h to PM_{2.5} road tunnel aerosol or filtered tunnel air (internal control). Incubator controls were kept at standard conditions in the laboratory to monitor the effects of the transport and the exposure. After exposure, cell inserts were placed in a new 6-well plate with 2 mL fresh completed medium enriched with 25 μM HEPES (GIBCO) at the basal side and transported back to the laboratory, where they were incubated together with incubator controls for 24 h.

2.6.2. Filter sampling and submerged exposure

Filter sampling of PM_{2.5} (PTFE filters) for submerged toxicity testing was conducted using a Dekati PM₁₀ impactor in accordance with the ISO 23210:2009 standard (ISO 23210:2009). The 1 μm cut-off stage was removed to allow PM_{2.5} collection on the PTFE end filter (GRIMM 1.113A, pore size 1.2 μm). The upper stages were greased to prevent particle bouncing and contamination of larger particles into the PM_{2.5} sample. Particle collection was done during the same seasonal period as ALI exposures. The airflow during measurement was maintained at 10 L/min and regulated using a mass flow controller (Bronkhorst Mass-View). The PTFE filters were conditioned for 24 h at 20 °C and 50 % RH before being weighed. PM_{2.5} particle suspensions were prepared for submerged toxicity testing by adding 1.0 mL of sterile Milli-Q water on top of weighted filters (quarters) in a Schott bottle vigorously vortex (3–5 min) to release most of the particles into the water. The filters were removed, and FBS was added to the particle suspensions (final concentration 5 % FBS, GIBCO). After drying (37–40 °C, 2 h) and conditioning

(20°C, 50 %RH, 24 h) the filters were weighted again. Subsequently, the particle suspension concentration could be calculated. Suspensions of the negative control filters were prepared in the same manner as the exposed samples. After 20 min sonication of the stock suspensions, different particle concentrations (10, 50, 100 and 200 µg/mL) were immediately prepared in medium and cells were exposed.

2.7. Biological endpoints

2.7.1. Cell viability

Alamar blue assay measured cell viability after both ALI and submerged exposure experiments according to manufacturer instructions. Briefly, a solution of 10 % Alamar Blue (Alamar Blue™ HS Cell Viability Reagent - Invitrogen™) in DMEM (GIBCO) and 25 µM HEPES (GIBCO) was prepared, and 0.5 and 1 mL of the solution was administered in the basal and apical chamber, respectively, of each cell insert. Cells were incubated with the Alamar Blue 10 % solution protected from light in the incubator at standard culture conditions. After 3h incubation, 100 µL of apical solution from each insert was transferred in quadruplicates to a 96-well plate. Triton was used as positive control for the cell viability assay. For ALI exposures, before stopping the 24h incubation period of the exposed cells, Triton 0.1 % (Sigma-Aldrich) was administered to a control insert with cells and incubated at standard conditions for 30 min. Cell viability in the positive control was tested in the same manner as for exposed samples. The absorbance was read at 570 nm using a spectrophotometer (CLARIOstar®). The values for each condition were expressed as the percentage of cell viability compared to the cells kept in the incubator, which served as negative controls.

After submerged exposure, supernatant was removed and 10 % Alamar Blue prepared in fresh medium was added to dTHP-1 and A549 cells and incubated for 2h at standard conditions. Separate wells containing only Alamar blue (10 %), and only road tunnel particle suspensions, were included in the viability test to rule out particle interference in the test. Fluorescence was measured using a Tecan Infinite F200 plate reader (Tecan, Magellan 7.2 Software, Grodig, Austria) at 540/590 nm excitation and emission.

2.7.2. Cytokine release

Cytokine release from cells was tested for both ALI and submerged exposure (10 and 100 µg/mL) experiments. The medium on top of the cells was collected after the 24 h incubation with particles in 1.5 mL individual tubes, centrifuged at 1500 rpm/5min and stored at -20°C (ALI samples) or -80°C (submerged samples). The inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α were investigated with the V-PLEX Proinflammatory Panel II (human) Kit (4-Plex). Before the analysis, supernatants were thawed, centrifuged at 1500 rpm for 5min, and diluted. For A549 exposures, the samples were diluted 2 times, and for dTHP-1 exposures 20 times for IL-1β, IL-6, and TNF-α, and 500 times for IL-8 due to high background. Lipopolysaccharide (LPS 1 µg/mL, from *Escherichia coli* Type O111:B4) was used as a positive control both for ALI (LPS administered in the basal chamber of one incubator control prior to the 24h incubation) and submerged (24 h) exposure experiments.

2.8. Statistical analyses

The statistical analyses (one-way ANOVA and unpaired *t*-test) were conducted in GraphPad Prism (v. 9.0.3), and the *p*-value significance level chosen was $p \leq 0.05$. Results are presented as mean ± STD of at least 3 independent experiments.

3. Results

3.1. PM_{2.5} from road tunnel

In-depth characterization was performed to provide insight into the

chemical composition of PM_{2.5}, as understanding its constituents is important for evaluating its potential cytotoxic and inflammatory effects.

3.1.1. Organic compounds

The organic compounds of the road tunnel PM_{2.5} are summarized in [Table S1](#). Twenty-four PAHs were measured, including lower molecular weight PAHs (LMW-PAHs) containing 2- and 3-ring PAHs, middle molecular weight PAHs (MMW-PAHs) containing 4-ring PAHs, and higher molecular weight PAHs (HMW-PAHs) containing 5-, 6- and 7-ring PAHs. Based on the air sampled volume, LMW-PAHs, MMW-PAHs, HMW-PAHs, and total PAHs were detected at concentrations of 3.1, 5.0, 6.1 and 14.2 ng/m³, while mass fractions on the basis of the amount of PM_{2.5} collected were 13.3, 21.4, 26.2 and 60.9 µg/g, respectively. LMW-PAHs, MMW-PAHs, and HMW-PAHs represent 22, 35, and 43 % of the total PM_{2.5} bound PAHs, respectively. Generally, MMW-PAHs and HMW-PAHs are mainly adsorbed to the particles ([Harrad et al., 2004](#)), while LMW-PAHs are mainly distributed in the gas phase ([Ho et al., 2009](#)). High content of HMW-PAHs suggests a strong influence of gasoline vehicle emissions in PM_{2.5}, while LMW-PAH are normally linked with emissions of diesel engine vehicles ([Ho et al., 2009](#); [Keyte et al., 2016](#)). Fluoranthene and pyrene were the most abundant PAHs, accounting for 12 % each, followed by benzo[e]pyrene and benzo[ghi]perylene, which account for 10 % each of the total PAH concentration. The total concentration of the OPAHs measured in this experiment was 0.2 ng/m³ and 0.7 µg/g, with 9-fluorenone and anthraquinone as the most abundant compounds, accounting for almost 76 % of the total OPAHs.

Regarding PCBs, several congeners containing from 3 to 7 chlorine atoms, including six indicator congeners (PCB 28, 52, 101, 138, 153, 180) and eleven other toxicologically significant congeners (PCB 77, 81, 105, 114, 118, 123, 156, 157, 167, 169, 189) were analyzed. The total PCB concentration was 0.2 ng/m³ and mass fraction 0.9 µg/g. The concentration of PCBs in PM_{2.5}, expressed as the sum of six PCB indicator congeners, was 0.3 ng/m³ and mass fraction 1.1 µg/g. The highest concentrations were determined for PCB 28, followed by PCB 52. These two congeners largely contribute to the sum of the total PCB concentrations in PM_{2.5}.

Several PBDE congeners containing 2 to 10 bromine atoms were detected. They are expressed as the sum of six congeners (\sum 6PBDEs sum of BDE 28, 47, 99, 100, 154, and 153) or eleven congeners (\sum 11PBDEs sum of 6PBDEs, BDE 17, 66, 85, 99, and 183). The most abundant PBDE was BDE 209. \sum 6PBDEs, \sum 11PBDEs and \sum PBDEs concentrations expressed as concentration per amount air volume collected were 0.006, 0.007, and 0.3 ng/m³, while when expressed as mass fraction per amount particulate collected, were 0.024, 0.031, and 1.1 ng/m³, respectively.

3.1.2. Inorganic composition

Oxygen, C, and Si were the most abundant elements in the collected road tunnel PM_{2.5} particles, contributing to the total mass with 36.7, 21.9, and 12.7 wt%, respectively. The particles also contained Cl (8.45 wt%), Na (6.12 wt%), Fe (5.02 wt%), Al (3.73 wt%), K (1.76 wt%) and Ca (1.60 wt%). Finally, minor and trace amounts of Mg, S, Ti, Cu, and Mn (<1 wt%), see [Table S9](#) (Supplementary material).

The crystalline fractions of PM_{2.5} were dominated by silica (SiO₂) polymorphs and NaCl. In addition, several diffraction peaks corresponding to alumino-silicates were also observed. These observations are in good agreement with the elemental analysis.

3.1.3. Particle dose onto the cells in the ALI system

The dose of particles onto the cells was calculated using the deposited particle number (of particles smaller than 2.5 µm) during 2 h, and the effective density of 0.916 g/cm³ assessed by gravimetric measurement. The mean dose during the exposure experiments was 1.4 ± 0.8 µg/cm² (insert area with cells 4.2 cm²).

3.2. Cell viability in ALI and submerged exposures

In ALI experiments, A549 cell viability tests indicated that the experiments, including the transport and the exposure, did not affect cell viability in filtered air and exposed cells compared to incubator controls (Fig. 1A).

In submerged exposure of A549, cell viability was not affected at any of the used particle doses compared to control (Fig. 1B). In contrast, the dTHP-1 showed no change in cell viability at 10 and 50 $\mu\text{g}/\text{mL}$, whereas a significant and dose-dependent decrease in cell viability in comparison to the control was observed in the higher doses (100, 150, and 200 $\mu\text{g}/\text{mL}$), see Fig. 1C.

3.3. Cytokine release at ALI and submerged exposures

In the ALI exposure experiments, a slight increase of IL-8, IL-6, and IL-1 β cytokines was observed in A549 cells exposed to the road tunnel PM_{2.5} compared to the filtered aerosol and the incubator control (Fig. 2). However, no statistical significance was observed in any of the measured cytokines. TNF- α values are not presented since they were below the detection limit.

Under submerged exposures, PM_{2.5} from road tunnel emissions at different doses induced a more evident inflammatory response compared to ALI results. After 24h of submerged exposure, A549 cells exhibited a small but significant increase in the release of TNF- α and IL-6 cytokines at 10 and 100 $\mu\text{g}/\text{mL}$, respectively, compared to the control (Fig. 3).

In dTHP-1 cells, the increase of IL-8 and TNF- α was statistically significant compared to the control already at the lowest exposure dose (10 $\mu\text{g}/\text{mL}$). Furthermore, after exposure to 100 $\mu\text{g}/\text{mL}$ of road tunnel emissions, the treated cells induced a statistically significant increase in all investigated cytokines (IL-8, IL-6, IL-1 β , and TNF- α). For example, at the dose of 100 $\mu\text{g}/\text{mL}$, IL-8 cytokine was approximately 18 times higher compared to the control (Fig. 4).

4. Discussion

The main aim of this study was to investigate the feasibility of using a mobile ALI system to assess the cytotoxicity and inflammatory potential of freshly generated PM_{2.5} in a road tunnel in Stockholm. To date, only a limited number of studies have applied ALI systems in outdoor environments to expose cells directly to real-world particles. A study in Rome, Italy, exposed lung cells at the ALI to urban background air, observing only minor toxic effects (Gualtieri et al., 2018). Another study

in Fribourg, Switzerland, used ambient air to expose lung cells, finding that air during winter, characterized by higher pollutant concentrations than during summer, triggered oxidative stress and proinflammatory markers during long (12 h) or repeated exposures (Bisig et al., 2018). An additional outdoor ALI study examined the effects of sea breeze in an urbanized and industrialized coastal area in northern France (Augustin et al., 2020). This exposure increased oxidative stress and inflammation in BEAS-2B lung cells. Firework particles and gases during the Chinese spring festival in Beijing were also examined *in situ* using pseudo ALI, showing that emitted PM (and sometimes acidic gases) induce apoptosis and inflammation in macrophages (Ding et al., 2019). While conducting outdoor ALI experiments is labor-intensive and requires several competencies, these studies demonstrated the feasibility of advanced *in vitro* systems outdoors to test the toxicity of aerosols with the same properties as those inhaled by humans in everyday environments.

We hypothesized that cellular effects would be detectable at lower doses in ALI compared to submerged exposures (Diabaté et al., 2021; Lenz et al., 2013; Loret et al., 2016; Wilkinson et al., 2011). It is important to note that these two *in vitro* models differ in methodology and sensitivity, which should be considered when using them in parallel. For example, ALI and submerged models differ in the mode of particle deposition, i.e., via airborne particles and particles suspended in a liquid, respectively. The method to assess the dose in our ALI system was by counting particles before and after the exposure chamber. In contrast, in submerged conditions, the effective dose is calculated considering the deposition efficiency of the particle in the liquid suspension. If assuming 50 % deposition (Cappellini et al., 2020), the lowest used dose in our submerged experiments approximately equals the dose achieved in the ALI exposures. However, A549 cell viability was not impacted upon exposure to road tunnel PM_{2.5} emissions either in ALI ($1.4 \pm 0.8 \mu\text{g}/\text{cm}^2$) or in the submerged model (10–200 $\mu\text{g}/\text{mL}$). The fresh aerosol did not even show a significant effect when a concentrator was used to increase PM_{2.5} concentrations within the ALI system. This aligns with previous studies investigating PM_{2.5} toxicity from similar sources, which also reported low or no cytotoxicity in A549 using ALI or pseudo ALI models. These sources include jet fuel combustion (Kaur et al., 2022), ship diesel engine emissions (Mühlhopt et al., 2016), and PM_{2.5} in urban cities (Rossner et al., 2025; Wang et al., 2020), often with exposure concentrations higher than those used in our study. In contrast, some other studies have reported cytotoxic effects in A549 following exposure to similar sources, both in ALI and submerged exposures. To minimize stress on the cells due to prolonged periods outside the incubator, the ALI exposures conducted in the road tunnel were limited to a single 2-h exposure. This limitation restricted us to examining only a low dose. In

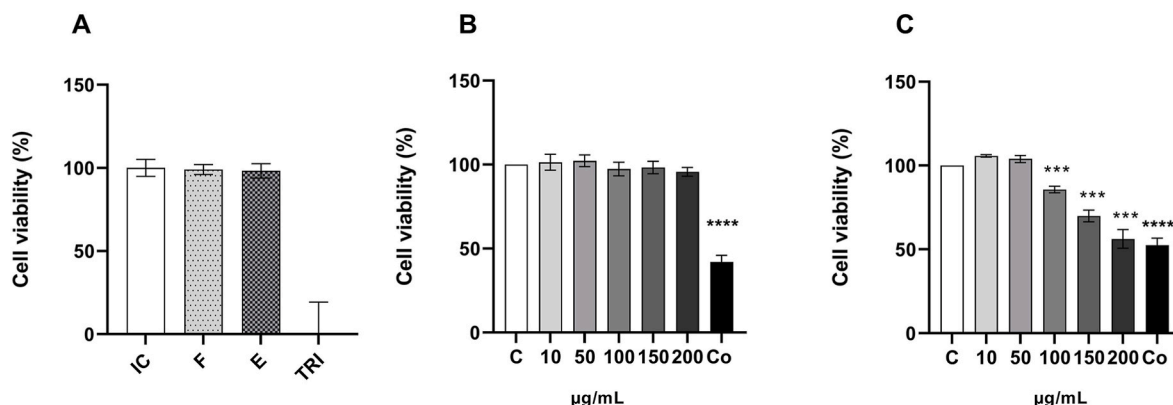


Fig. 1. Cell viability of cells exposed to PM_{2.5} from a road tunnel in central Stockholm (Söderledstunneln). A) A549 cells at ALI exposure conditions compared to control exposed for 2 h and incubated for 24 h. IC: Incubator control, F: Filtered, E: Exposed TRI: Triton 0.1 %. Estimated mean exposure dose: $1.4 \pm 0.8 \mu\text{g}/\text{cm}^2$. A549 (B) and dTHP-1 (C) in submerged exposure (24 h) to 10, 50, 100, 150, 200 $\mu\text{g}/\text{mL}$ of PM_{2.5} from filters sampled in the Söderledstunnel in Stockholm compared to control. Results are expressed as mean \pm STD of at least 3 independent experiments. Statistically significant differences were reported with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. C: Control.

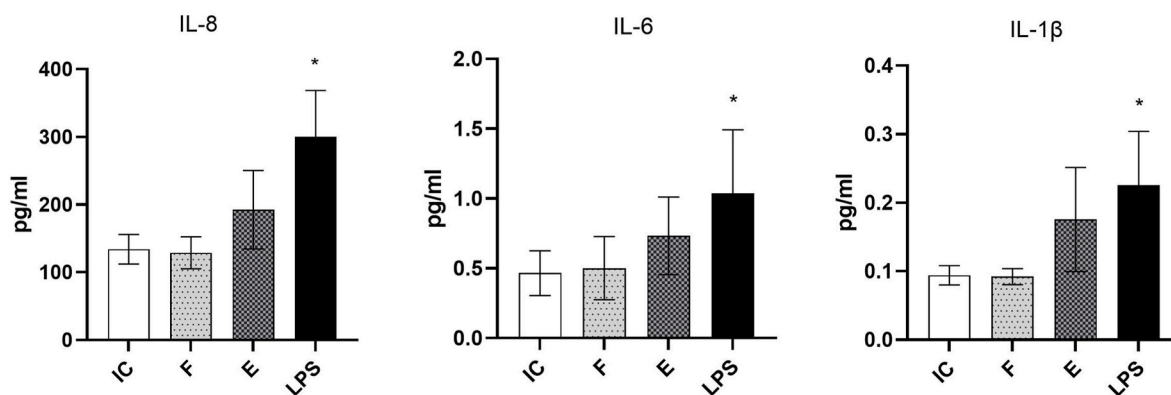


Fig. 2. Cytokine release from A549 cells exposed to PM_{2.5} road tunnel emissions (at Söderledstunneln) at ALI conditions. After 2 h exposure and 24 h incubation, the basal medium was collected to analyze the concentration of IL-8, IL-6, and IL-1β. LPS (1 μg/mL) was used as positive control. Results were expressed as mean ± STD of at least 4 independent experiments and presented as released cytokine content per treated group (pg/mL). The estimated exposure dose of road tunnel emission onto the cell cultures at ALI was $1.4 \pm 0.8 \mu\text{g}/\text{cm}^2$. Statistically significant differences were reported with $p \leq 0.05$. IC: Incubator control, F: Filtered, E: Exposed, LPS: Positive control.

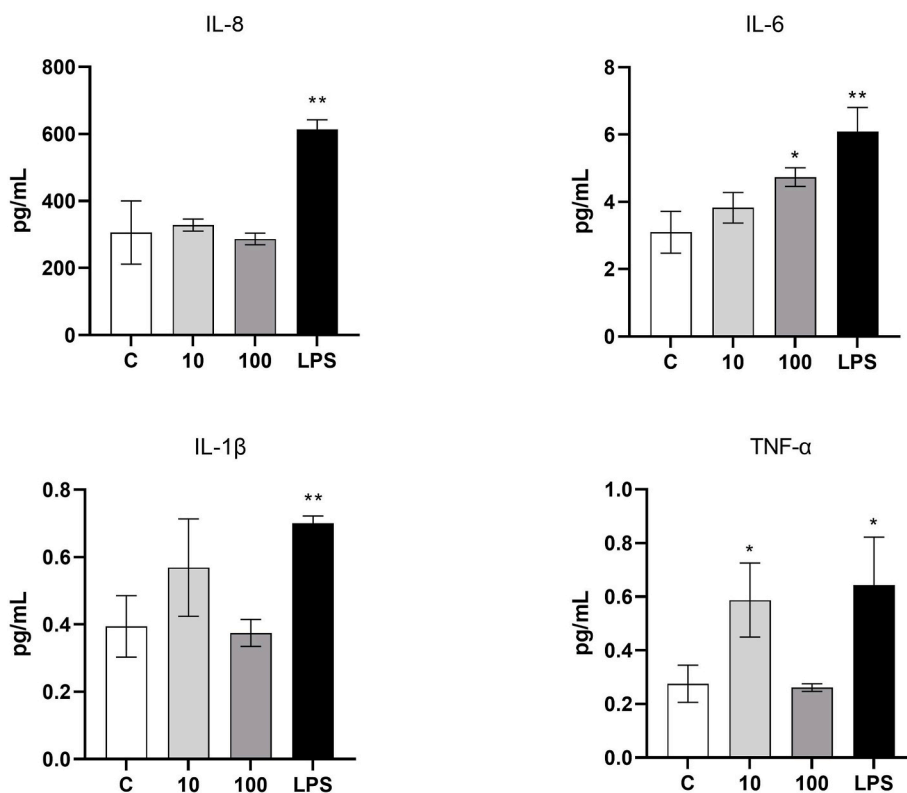


Fig. 3. Cytokine release from A549 cells exposed to PM_{2.5} collected in a road tunnel (Söderledstunneln) after submerged exposure. After 24 h exposure to 10 and 100 μg/mL to PM_{2.5}, supernatants were collected to analyze the concentrations of IL-8, IL-6, IL-1β and TNF-α. LPS (1 μg/mL) was used as positive control. Results were expressed as mean ± STD of 3 independent experiments and presented as released cytokine content per treated group (pg/mL). Statistically significant differences were reported with * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. C: Control.

contrast, the submerged model was exposed to doses up to 20 times higher than the ALI dose, yet no cell death was observed in A549 cells. Consequently, our experiments could not determine the relative sensitivity of the ALI and submerged models. Similar submerged studies have instead found a dose-dependent cytotoxicity in A549 after exposure to ambient PM_{2.5} collected from Tehran and Seoul (MohseniBandpi et al., 2017; Shim et al., 2021). A slight decrease in cell viability was also observed in ALI exposure to a low dose ($0.005 \mu\text{g}/\text{cm}^2$) of heavy truck exhaust particles (Kooter et al., 2013), and Yan et al. (2023) found a dose-dependent reduction in cell viability in ALI exposure to PM_{2.5} from

traffic in China (25–100 μg). These results suggest that urban particles from different locations can lead to varying toxic effects, probably due to differences in particle composition, size, and morphology.

Model sensitivity is also dependent on the cell type used, influencing the outcome of the particle toxicity testing. Although a recent study by Friesen et al. (2022) reported greater sensitivity in the submerged model compared to the ALI, several other studies have demonstrated that alveolar epithelial cells, such as A549, exhibit a stronger response to aerosolized particles in ALI experiments when compared to submerged culture systems (Diabaté et al., 2021; Lenz et al., 2013; Lore et al., 2016;

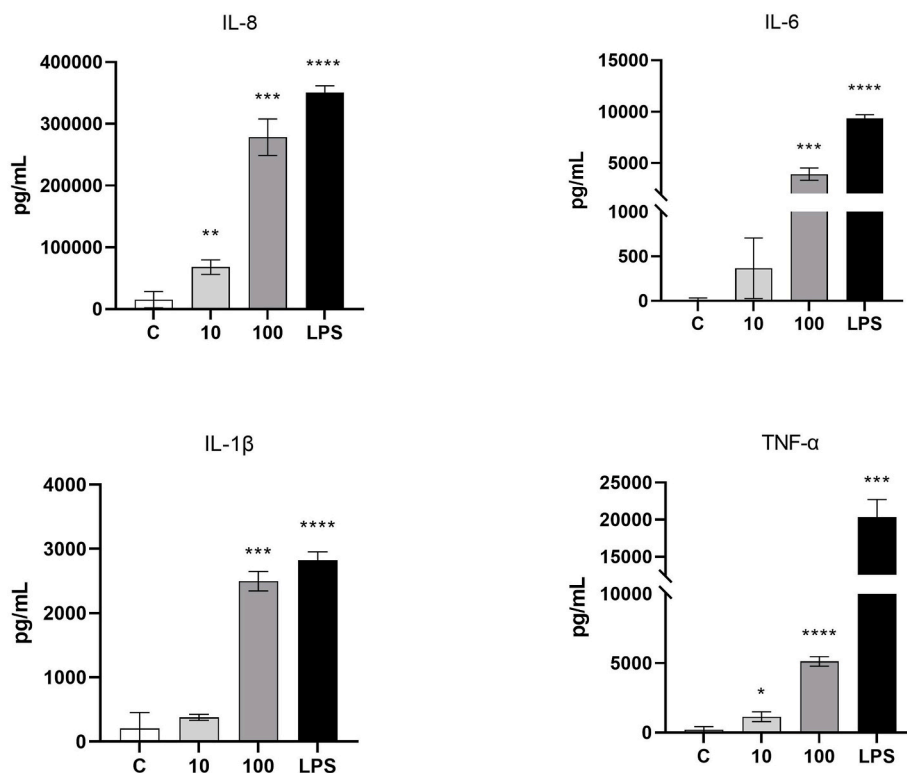


Fig. 4. Cytokine release from dTHP-1 cells exposed to PM_{2.5} collected in a road tunnel (Söderledstunneln) after submerged exposure. After 24 h exposure to 10 and 100 µg/mL to PM_{2.5}, supernatants were collected to analyze the concentrations of IL-8, IL-6, IL-1β and TNF-α. LPS (1 µg/mL) was used as positive control. Results were expressed as mean ± STD of 3 independent experiments and presented as released cytokine content per treated group (pg/mL). Statistically significant differences were reported with * p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001, ****p ≤ 0.0001. C: Controls.

Wilkinson et al., 2011). This increased sensitivity under ALI conditions is believed to arise from two main factors. First, the presence of airflow in ALI systems induces a baseline oxidative stress, thereby enhancing the cellular responsiveness to particulate exposure. Second, due to the lower viscosity of air compared to liquid media, particle deposition occurs more rapidly, resulting in prolonged and more effective particle–cell interactions over an equivalent exposure period (Upadhyay and Palmberg, 2018). However, despite the hypothesized increased sensitivity of the ALI model, the exposure was not sufficiently potent to discern effects at the tested dose.

In our submerged exposures, in addition to A549, we also used dTHP-1 (immune cells). In contrast to the result using A549, a dose-dependent cytotoxicity was observed in dTHP-1. At the highest concentration (200 µg/mL), the viability in dTHP-1 cells was only 50 %. This increased sensitivity of dTHP-1 cells compared to A549 has been noted in other studies as well. For instance, a study examining the toxicity of ambient PM_{2.5} collected in Shanghai, China, also found a significant dose-dependent reduction in cell viability for dTHP-1, but not in A549 (60–500 µg/mL) (Wang et al., 2019).

The higher sensitivity of dTHP-1 to road tunnel emissions in our study was also seen in the analysis of inflammatory markers (IL-6, IL-8, IL-1β, TNF-α). In both ALI and submerged experiments, A549 expressed low cytokine concentrations. In contrast, dTHP-1 in submerged exposure secreted significantly higher concentrations of these markers, showing a clear dose-dependent response. This suggests that dTHP-1 is useful for assessing the inflammatory response to air pollutants. While dTHP-1 cells have been utilized in pseudo-ALI conditions (Ding et al., 2019), they cannot be used alone in ALI experiments because they don't homogeneously adhere to the semipermeable membrane of the ALI cell inserts (Kletting et al., 2018). Overall, the combination of low exposure concentrations in ALI and the reduced sensitivity of A549 compared to immune cells suggests that alternative cell models may be advantageous

in ALI studies. One option would be to use co-cultures in ALI experiments, such as A549 in combination with dTHP-1. This approach is supported by previous ALI studies of urban PM_{2.5}. No inflammatory response was seen in A549, but when co-cultured with THP-1, there was a notable increase in IL-6, TNF-α, and IL-8 (60 and 180 µg/mL) in pseudo-ALI (Wang et al., 2020, 2019). However, other studies exposing A549 to similar sources but at higher doses have seen increased cytokine concentrations, for example, in ALI exposures to traffic-related particles (Lichtveld et al., 2012), submerged exposures to PM_{2.5} from road tunnels and PM_{2.5} in urban air (Cheng et al., 2021; Niu et al., 2024). These findings emphasize that air pollutant concentration is also crucial for the usability of A549. Nevertheless, the results from the present study and others suggest that a co-culture of A549 and dTHP-1 is likely a more sensitive model, and more suitable to detect effects and lower more realistic concentrations (Loret et al., 2016).

Although the concentration of secreted cytokines was low, our ALI study did show a slight increase in IL-6, IL-8, and IL-1β in exposed groups compared to control and filtered groups. While this increase was not statistically significant, two factors are important to consider: 1) PM_{2.5} particle concentration in the road tunnel was relatively low compared to concentrations typically used in the laboratory, and 2) there was a considerable day-to-day fluctuation in PM_{2.5} concentration in the road tunnel. The variable concentration was caused by parameters such as different vehicle types, transit frequency, number of cars, and weather conditions, which limited the replicability of the particle dose during the different ALI experiments. The variable concentration is a limitation in outdoor experiments, where direct sampling does not allow control of source emissions. In our study, the mean particle concentration was used to calculate the exposure dose for the ALI experiments, preventing any direct correlation between daily doses and specific toxicity outcomes. Such challenges have also been highlighted previously; for example, the fact that pooling cell samples from different days makes it impossible to

distinguish the toxicity of different particle compositions obtained during different days (Gualtieri et al., 2018). A dose assessment specific to each ALI experiment would improve studies where variable concentrations occur. A more straightforward approach is feasible when higher and more stable particle concentrations are present in the air, such as those produced in controlled laboratory environments. (Müller et al., 2012; Fromell et al., 2023).

Several studies suggest that the toxicity of PM_{2.5} can be a consequence of the combined effects of PAHs, metals, and the particles themselves (Deng et al., 2013; Garçon et al., 2006; Gualtieri et al., 2011). We detected PAHs (mainly fluoranthene, pyrene, benzo[e]pyrene, and benzo[ghi]perylene), OPAHs (mainly 9-fluorenone and Antraquinone), PCBs (mainly PCB 28 and 52), and PBDEs (mainly BDE 209) in our PM_{2.5} samples. At the cellular level, PAHs activate the aryl hydrocarbon receptor (AhR) and are metabolized mainly by cytochrome P450 enzymes, producing reactive metabolites that generate ROS. This leads to oxidative stress, cellular damage, and the activation of inflammatory pathways, including the NF-κB pathway, which regulates proinflammatory cytokines such as IL-6 and TNF-α (Holme et al., 2023). Thus, PAHs (mainly phenanthrene, fluoranthene, pyrene, chrysene, benzo[b]fluoranthene, and benzo[ghi]perylene) in PM_{2.5} from a road tunnel can be linked to an increased IL-6 and TNF-α concentrations in A549 (Chen et al., 2019). In contrast, Gualtieri et al. (2010) found no association between PAHs in PM_{2.5} and cytokine release in submerged exposure of A549 cultures (1, 10 and 25 μg/cm²). However, it was suggested that the lack of inflammatory markers could be an artifact due to particles absorbing the cytokines. Additionally, PAHs in traffic PM_{2.5} activate mutagenic and genotoxic pathways (Castorena-Torres et al., 2008; Gualtieri et al., 2012, 2010; Mahadevan et al., 2004). However, these studies used PAHs doses at least ten times higher than those found in our Stockholm road tunnel PM_{2.5} exposure experiments. The total PAH mass fraction of our PM_{2.5} sample was also lower compared to road tunnels in Portugal (Oliveira et al., 2011), France (Keyte et al., 2016), and China (Zhao et al., 2020).

The concentration of OPAHs in the road tunnel in Stockholm was lower than those detected in traffic-related PM_{2.5} samples collected from a tunnel (Zhao et al., 2020), and urban and rural areas in China (W. Li et al., 2015a), and several cities in Southern Europe (Alves et al., 2017). However, concentrations of PCBs in Stockholm were higher than those recently detected in several other countries (Barhoumi et al., 2018; Degrendele et al., 2014; Kim et al., 2011; Kim and Masunaga, 2005; Kuzu, 2016; Trinh et al., 2018). ΣPBDEs in PM_{2.5} were more than ten times higher than the mean concentration detected at a traffic site in Thessaloniki (Besis et al., 2015) and a rural area in China (Y. Li et al., 2015b), while Σ₆PBDEs and Σ₁₁PBDEs in PM_{2.5} were comparable to those measured in urban and industrialized areas of the United Kingdom (Harrad et al., 2004), Japan (Hayakawa et al., 2004), Canada (Shoeib et al., 2004) and Turkey (Cetin and Odabasi, 2008), but lower than concentrations measured in USA (Strandberg et al., 2001) and China (Chen et al., 2006).

Metals were also present in the road tunnel PM_{2.5} samples, mainly Fe (5.0 wt%) and Al (3.8 wt%). The main sources of metals in traffic PM_{2.5} are the wear of brake pads, brake discs, and street materials. Brake pads are primarily made of Fe, Cu, Zn, and Sn and may also contain trace amounts of Co, Cr, Pb, Ba, Al, Si, Mn, and Cd. (Dante, 2015). Brake discs, on the other hand, are typically composed of cast iron (Hulskotte et al., 2014). Several of these metals, particularly Fe, Mn, and Zn, are known to induce inflammation by increasing intracellular ROS through the Fenton reaction (Bonetta et al., 2009; Gandhi et al., 2022; Paunovic et al., 2020). The elevated ROS levels contribute to the secretion of proinflammatory cytokines, including TNF-α, IL-6, and IL-1β, particularly in immune cells (Padgett et al., 2013; Simeonova et al., 1997). Compared to other environments (e.g., subway and railway), the road tunnel and street particulates have less metal content (Kam et al., 2013); nevertheless, studies have measured higher oxidative stress due to street particles compared to subway particles, suggesting that organic

compounds are also involved in these mechanisms (Seaton et al., 2005; Spagnolo et al., 2015). Niu et al. (2024) linked Fe (4 wt%) in road tunnel PM_{2.5} in China to increased ROS production in A549 cells (100 μg/mL). Several other studies have found increased oxidative stress and reduced cell viability following exposure to PM_{2.5} with Fe and Al as the predominant metals at similar exposure doses to ours, though with significantly higher metal concentrations (Deng et al., 2013; Dergham et al., 2015; Dieme et al., 2012; Goudarzi et al., 2019; Michael et al., 2013; Rahmatinia et al., 2021; Song et al., 2019). Furthermore, chemical analyses of the road tunnel particles revealed the presence of NaCl (from road salt used for de-icing roads during winter and springtime in Sweden) and crystalline silica and aluminosilicates from sand, soil, and road wear. Carbon accounted for 22 wt% of the sample, but the absence of crystalline carbon suggests that it is amorphous and/or present in nanometer-sized particles. Therefore, carbon most likely originates from elemental black and organic brown carbon produced by fuel combustion, as well as from other organic compounds. These findings underscore that different compositions of traffic aerosols may lead to differences in toxic effects (MohseniBandpi et al., 2017; Shim et al., 2021; Wang et al., 2019).

In this study, we mainly observed effects in cells cultured in submerged exposures. This is likely mainly explained by the notably higher doses in submerged exposures, as well as the use of dTHP-1 cells. Furthermore, in ALI experiments, cells are directly exposed to airborne particles and gases, while in submerged exposures, suspended particles require time to settle on the cell layer. This time lag indicates that incubation periods should be adjusted not only to the tested endpoint but also to account for the particle sedimentation time. Specifically, we noted that shorter incubation times sometimes are suitable to see effects in ALI experiments, while a minimum 24-h incubation period is preferred for assessing cytotoxicity and cytokine release in submerged exposures (Wang et al., 2020). Although direct ALI exposure in the road tunnel aims to preserve the particles' properties, the humidification and drying processes in the mobile ALI system could potentially represent a limitation, altering the original morphology and the semi-volatile partitioning of the aerosol. Furthermore, outdoor experiments limited the ability to maintain a constant and sufficient dose to observe clear effects. Nevertheless, A549 cells also showed a weak effect in submerged exposures. Finally, due to the use of a single filter for both submerged exposures and chemical analyses, correlation studies between toxicity endpoints and PM_{2.5} composition were not possible. Future studies should analyze particle characteristics for each exposure to be able to correlate individual components of PM_{2.5} and toxicological outcomes.

5. Conclusions

This study presents the first successfully conducted *in situ* ALI exposure in a road tunnel. We hypothesized that cellular effects would be detectable at lower doses compared to submerged exposures. However, the comparison was difficult since no or only weak effects were observed for A549 in ALI and submerged. Furthermore, accurate comparison was also hindered by dose differences despite using a concentrator that increased cellular doses fivefold. An additional limitation was represented by the impossibility of assessing the effects of tunnel microclimate and traffic variability on the PM_{2.5} particle composition. The limitation related to traffic variability could be overcome in the future by estimating the dose of each individual exposure. Clear effects were observed for dTHP-1 in submerged exposures, suggesting that co-cultures of A549 and dTHP-1 may be useful in future ALI studies. Despite the challenges with ALI, it is worth noting that, in contrast to submerged exposures, it enables assessing both particulate and gaseous phases of the aerosol. This is clearly a benefit that encourages the use of ALI to assess real-world emissions.

CRedit authorship contribution statement

Micol Introna: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Teresa Juárez-Facio:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Naga Veera Srikanth Vallabani:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ming Hui Tu:** Writing – review & editing, Investigation. **Paavo Heikkilä:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Andrea Colombo:** Writing – review & editing, Methodology, Formal analysis. **Valentina Liboni:** Methodology, Formal analysis. **Bozhena Tsyupa:** Investigation. **Alessandro Mancini:** Writing – review & editing, Investigation. **Jorma Keskinen:** Writing – review & editing, Supervision, Resources, Methodology. **Ulf Olofsson:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Sarah Sulamith Steimer:** Writing – review & editing, Supervision, Resources, Conceptualization. **Hanna Lovisa Karlsson:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Karine Elihn:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Funding

This research was supported by the European Commission's Horizon 2020 research and innovation programme: nPETS (grant agreement No 954377, <https://www.npets-project.eu/>) aimed at studying the sub 100 nm particles emitted from transport.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2025.126486>.

Data availability

Data will be made available on request.

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