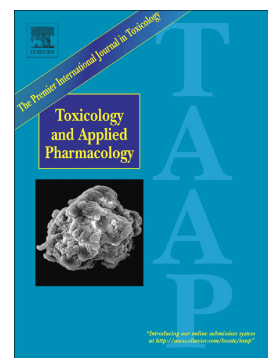


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Oxidative potential and cellular toxicity of carbonaceous aerosols undergoing aging in an atmospheric simulation chamber

Virginia Vernocchi^{a,*}, Marco Brunoldi^{a,e}, Silvia Canepari^{b,c}, Emanuela Corsini^d, Tommaso Isolabella^{a,e}, Lorenzo Massimi^{b,c}, Federico Mazzei^{a,e}, Gloria Melzi^d, Marco Paglione^f, Sofia Pantaleoni^d, Paolo Prati^{a,e}, Marco Rapuano^f, Matteo Rinaldi^f, Caterina Tiraboschi^{b,g}, Dario Massabò^{a,e*}

^a Istituto Nazionale di Fisica Nucleare, Sezione di Genova, via Dodecaneso 33, 16146 Genoa (Italy)

^b Department of Environmental Biology, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

^c National Research Council of Italy - Institute of Atmospheric Pollution (CNR-IIA), Via Salaria km 29.300, 00015 Monterotondo (Roma), Italy

^d Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan (Italy)

^e Dipartimento di Fisica, Università degli Studi di Genova, Via Dodecaneso 33, 16146 Genoa (Italy)

^f National Research Council of Italy - Institute of Atmospheric Sciences and Climate (CNR-ISAC), Bologna, 40129 Italy

^g Department of Public Health and Infectious Diseases (DSPMI), Sapienza University of Rome, P.le Aldo Moro, 5, Rome 00185, Italy

**Corresponding author: Virginia Vernocchi (vvernocchi@ge.infn.it), Dario Massabò (dario.massabo@ge.infn.it)*

Email address of each author: MB (Marco.Brunoldi@unige.it), SC (silvia.canepari@uniroma1.it), EC (emanuela.corsini@unimi.it), TI (tommaso.isolabella@ge.infn.it), LM (l.massimi@uniroma1.it), FM (federico.mazzei@ge.infn.it), GM (gloria.melzi@unimi.it), MP (m.paglione@isac.cnr.it), SP (sofia.pantaleoni@unimi.it), PP (Paolo.Prati@ge.infn.it), MRa (m.rapuano@isac.cnr.it), MRi (m.rinaldi@isac.cnr.it), CT (caterina.tiraboschi@uniroma1.it)

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Abstract:

This study investigates how chemical composition, atmospheric aging, and environmental conditions affect the oxidative potential (OP) and cellular toxicity of soot particles using an atmospheric simulation chamber (ASC). In the ASC ChAMBRé were simulated real-world summer and winter scenarios, exposing pure soot particles (generated by using the mini-inverted soot generator) and various secondary aerosol precursors (i.e., toluene, 2,5-dimethylfuran and α -pinene) alternatively to light or dark conditions and different oxidants. OP was assessed using multiple assays (namely, 2',7'-dichlorofluorescein – DCFH, Dithiothreitol - DTT and Ascorbic Acid – AA), revealing that soot particles exposed to light, especially in presence of toluene, exhibited higher OP. The presence of toluene also increased cellular reactive oxygen species (ROS) production, leading to elevated cytotoxicity, DNA damage, and release of the proinflammatory cytokine interleukin-8 (IL-8) in BEAS-2B cells. Ammonium sulfate addition reduced OP and the associated do not enhance toxicological responses, suggesting that non-toxic components in aged particulate matter (PM) may mitigate some harmful effects. Toxicological assessment showed increased cytotoxicity, genotoxicity, oxidative stress, and inflammatory responses in soot generated under high irradiance conditions typical of summer and traffic environments, compared to low irradiance winter scenarios. Strong correlations were observed between OP and toxicological endpoints, such as ROS formation, LDH release, micronuclei formation, and

IL-8 secretion underscoring the role of chemical composition and environmental aging in determining PM toxicity. The study highlights OP assays as a reliable tool for predicting PM-induced oxidative stress and potential health effects, emphasizing the importance of considering soot chemical composition and aging processes in urban air pollution assessments.

1. Introduction

Although it has been widely recognized that atmospheric aerosols cause adverse health effects (Lelieveld et al., 2015) there is still a gap of knowledge on how different aerosol physical-chemical properties (concerning both primary and secondary particulate matter, PM) can act and interact to influence PM toxicity in real-world conditions (Rohr and Wyzga, 2012). Ambient air pollution was ranked the seventh highest risk factor for human health (WHO, 2024). Among the most relevant air pollutants, PM represents a major challenge due to the vast number of possible both anthropogenic and natural sources involved. Identifying and quantifying the influences of specific components on assessing health-related impacts, therefore, represents one of the most challenging areas of environmental health research (Kelly and Fussell, 2012). The direct emission of particles in the atmosphere (so-called “primary” PM) accounts for only a fraction of ambient PM in outdoor environments, being the remaining fraction (“secondary” PM) formed in the atmosphere through complex chemical and physical reactions involving gas-to-particle conversion processes (e.g., photochemical reactions). The discovery of secondary organic aerosols (SOA) exhibiting a toxicological potential and causing oxidative stress in lung cells (Künzi et al., 2015) demonstrated that ambient aerosols responsible for potential impacts on human health are not only emitted from pollution hotspots and subsequently dispersed to background areas, but are also formed at background sites as a function of environmental conditions, such as temperature, solar irradiance (Jimenez et al., 2009), and availability of liquid water in form of fog/cloud droplets or deliquesced aerosols (Ervens et al., 2011).

Soot particles are included in the definition of Black Carbon (BC), the carbonaceous aerosol generated by the incomplete combustion of fossil fuel and biomass burning, is recognized to have an important role in climate change (Bond et al., 2013; Ramanathan and Carmichael, 2008) and health effects (Cassee et al., 2013; Olstrup et al., 2016). During the atmospheric stay, BC interacts with the other atmospheric constituents: aging processes, including coagulation, wetting, photo-ageing, and inorganic and organic coating formation, can affect soot properties (Colbeck et al., 1990; Hallett et al., 1989; Liu et al., 2023; Wang et al., 2017). During transport and aging, fresh soot particles mix with organic and inorganic aerosols, which changes their morphology and compactness and further leads to variations in their optical properties and radiative forcing (Wang et al., 2017). So, understanding the ageing processes of BC in the atmosphere is important for assessing its environmental and climatic impacts.

Several studies have reported that the primary mechanism by which PM can cause adverse health effects is by triggering oxidative stress phenomena (Albano et al., 2022; Quezada-Maldonado et al., 2021). Consequently, in recent years, cellular and acellular assays have been widely employed to assess PM-induced oxidative stress (Barzgar et al., 2023; Bates et al., 2019; Botero et al., 2020; Khoshnamvand et al., 2022; Molina et al., 2020). Cellular assays measure the alteration of various biological responses (e.g., DNA damage, antioxidant enzyme activity, non-enzymatic antioxidant metabolites) resulting from the generation of oxidative stress in cell lines exposed to PM. Conversely, acellular oxidative potential (OP) assays, such as the ascorbic acid (AA) and the dithiothreitol (DTT) OP assays (OP^{AA} and OP^{DTT} , respectively), estimate the ability of PM to generate reactive oxygen species (ROS) by measuring the depletion of chemical proxies of cellular antioxidants (i.e., AA and DTT), or directly estimate particle-bound reactive oxygen species content, as in the case of the dichlorofluorescein (DCFH) OP assay (OP^{DCFH}), which measures the fluorescence signal intensity of DCF over time due to enzyme-catalyzed oxidation of non-fluorescent DCFH by ROS.

It is important to note that both cellular and acellular assays are typically applied to soluble extracts of PM sampled on filters, thereby excluding insoluble particles from the assessment of PM-induced oxidative stress (Frezzini et al., 2022b, 2022a; Vaccarella et al., 2025). The differing solubility properties of PM chemical components and the potential instability of redox-active compounds under these conditions may limit the representativeness of the results (Frezzini et al., 2022b, 2022a; Massimi et al., 2022). Furthermore, a significant knowledge gap persists regarding the varying sensitivity of the different cellular and acellular assays to PM components, hindering a clear understanding of the relationships between PM physicochemical composition and its ability to generate oxidative stress (Daellenbach et al., 2020; Grange et al., 2022; Massimi et al., 2020). In recent years, several studies have attempted to link different OP values and/or cellular responses to specific PM components and sources (Dominutti et al., 2023; Massimi et al., 2024; Melzi et al., 2024), however, the results have often been inconsistent across studies, likely due to the potential synergistic or competitive interactions between the diverse redox-active species present in PM (Ngoc Thuy et al., 2024; Pietrogrande et al., 2022; Vaccarella et al., 2025). Consequently, the specific PM components and the chemical-physical reactions that induce the formation of particles most responsible for generating oxidative stress are still unclear. In a recent review paper, (Liu and Chan, 2022) highlighted that the OP of elemental carbon-containing particles (ECCAPs) is significantly altered by environmental conditions, with atmospheric aging (e.g., ozone exposure) generally increasing OP and the formation of secondary particles or coatings (e.g., quinones, sulfates) having diverse effects on their capacity to induce oxidative stress. Although a significant role of SOAs in inducing oxidative stress has been recognized (Künzi et al., 2015), the mechanisms that drive ROS formation in secondary aerosol at background sites as a function of environmental conditions and their ability to generate oxidative stress remain poorly understood.

The present study was carried out in the context of the RHAPS (Redox-activity and Health-effects of Atmospheric Primary and Secondary aerosol) project, which was launched in 2019 (Costabile

et al., 2022). The major objective of RHAPS was to identify the specific properties (and combinations of them) of PM₁ (particulate matter with an aerodynamic diameter less than 1 μm) that are responsible for toxicological effects and can be used as new metrics for health-related outdoor pollution studies. Previous findings of RHAPS suggest that urban nanoparticles may have high pro-inflammatory and oxidative responses at low PM₁ levels, probably associated with the enrichment in traffic-related reactive compounds otherwise scavenged by larger particles (which are more abundant at higher PM₁ concentrations)(Costabile et al., 2023; Gualtieri et al., 2024; Melzi et al., 2024).

The specific aim of this work was to investigate how the properties of soot particles (an important component of traffic-related and combustion aerosol) are modified depending on different atmospheric conditions and on the interaction with specific atmospheric constituents. For this reason, the use of an atmospheric simulation chamber (ASC) was the best experimental solution. Conducting experiments in an ASC allowed us to generate atmospheric conditions that can be controlled and monitored, and to mimic the desired scenario while knowing all the components involved. Compounds specifically selected because representative of anthropogenic and biogenic emissions were introduced and aged under different controlled conditions, and the resulting PM loadings were collected on filters and off-line analyzed for the determination of their OP and toxicological properties.

2. Materials and methods

2.1 ChAMBRé

Experiments were conducted inside the confined volume of the ASC ChAMBRé (Chamber for Aerosol Modelling and Bio-aerosol Research, (Massabò et al., 2018)). Several online instruments were connected to the chamber; the most relevant for the project were:

- Scanning Mobility Particle Sizer (SMPS; TSI Inc.; model 3938) composed of a differential mobility analyzer (DMA; TSI Inc.; model 3081A) and a water condensation particle

counter (w-CPC; TSI Inc.; model 3789), filled with technical demineralized water (conductivity at 20 °C: max 1.5 $\mu\text{S cm}^{-1}$; Avantor). The SMPS measured particle size distribution in the range 15 - 900 nm.

- Optical particle size (OPS; TSI Inc.; model 3330), measuring particle size distribution in the range 0.3–10 μm .
- Two photoacoustic extinctionmeters (PAXs; Droplet Measurement Technologies, Boulder, CO, USA), measuring aerosol optical properties (i.e., absorption, extinction and scattering coefficients, single scattering albedo - SSA) at $\lambda = 870$ and 405 nm. PAXs provided the BC concentration inside ChAMBRé too. Details in (Vernocchi et al., 2022).
- Gas monitors for NO/NO₂, O₃ and CO/CO₂ (AC32e, O342e and CO12e respectively, manufactured by ENVEA®). Details in (Vernocchi et al., 2023).

2.2 Aging procedures

Different BC aging processes were investigated, starting from the simplest situation of pure soot up to BC exposed to both inorganic and organic compounds and/or simulated solar radiation and combinations of these. Each component required a specific procedure to be generated.

Soot particles (BC) were generated by using the mini-inverted soot generator (MISG; Argonaut Scientific Corp., model MISG-2), fed by 80 mLpm of propane and 7 Lpm of air. The injection lasted 1 minute and was performed according to what was described in (Vernocchi et al., 2022). Since the focus of the RHAPS project is on the fine fraction of PM, a PM1 cyclone (M1 Sharp Cut Cyclone – SCC 2.229; MesaLabs) was inserted between MISG and ChAMBRé to avoid the possible injection of super-aggregates.

Solar radiation is simulated with a solar simulator (SoSi) (Sciencetech Inc. TM), mounting the AM1.5G filter to reproduce the optical absorption of the atmosphere. Details on the SoSi can be found in (Vernocchi et al., 2023).

Salt seeds of ammonium sulfate or ammonium nitrate were generated starting from a liquid solution, prepared by dissolving 0.2 g of salt in 50 mL of MilliQ water. The solution was nebulized

with the Sparging Liquid Aerosol Generator (SLAG, by CH Technologies, Inc.) nebulizer, working with 3.5 lpm of air, provided by a Mass Flow Controller (MFC, by Bronkhorst), for 600 sec. SLAG is fed with salt solution using an automatic syringe pump working at 0.4 mlpm.

NO₂ was provided by a specific bottle, and the concentration (i.e., 500 – 600 ppb) inside the chamber was kept constant thanks to the feedback control system described in (Vernocchi et al., 2023). Ozone was generated via photoreaction by a UV lamp ($\lambda < 240$ nm) inserted through an ISO-K25 flange in the lateral volume (i.e., no UV radiation illuminates particles in the main volume) of the chamber. In this way, about 500 ppb of ozone was reached into the chamber volume. NO₂ and Ozone were injected as precursors of the NO₃ radical in order to simulate oxidation in dark conditions (Kodros et al., 2020).

Nitrous acid (HONO) was produced by the reaction between H₂SO₄ and Na₂NO₂ and injected in order to feed the system of OH radicals under light conditions; HONO photolysis as a source of hydroxyl radical has been widely used in many atmospheric simulation chambers (Aktypis et al., 2024), and its advantages and disadvantages have been well documented previously (Bell et al., 2023). Reagents were injected consecutively by using a glass flask and a MFC-controlled air flow: first 4 mL of H₂SO₄, then 2 mL of Na₂NO₂ flowed with an air flow of 1 lpm.

Organic compounds were injected with the glass jar and the 1 lpm air flow. The amount of each compound was: Toluene (ThermoFischer Scientific) 2 μ L, 2,5-Dimethylfuran (2,5-DMF; ThermoFischer Scientific) 3 μ L and (+)- α -pinene (ThermoFischer Scientific) 4 μ L.

The injection order of each component is summarized in Table 1. For all the experiments (except BC dark), 2 hours of BC exposure were considered.

Table 1: Injection order of each component: most reactive ones were introduced last, to activate the reaction when all components were in the chamber.

Component	Injection order
------------------	------------------------

<i>Ammonium Nitrate</i>	<i>Simultaneous with BC injection</i>
<i>NO₂</i>	<i>Simultaneous with BC injection</i>
<i>O₃</i>	<i>Introduced 3 minutes after the injection of the last compound</i>
<i>2,5-dimethylfuran</i>	<i>Injected at the end of BC (or salt) injection</i> <i>i.e.: BC (+ Amm.Nitr. + NO₂) → 2,5-dimethylfuran → O₃</i>
<i>Ammonium Sulfate</i>	<i>Simultaneous with BC injection</i>
<i>HONO</i>	<i>3 minutes after the injection of the last compound</i> <i>i.e.: BC (+ Amm.Sulf.) → HONO → Light ON</i>
<i>Toluene</i>	<i>3 minutes after the BC (or salt) injection</i> <i>i.e.: BC (+ Amm.Sulf.) → Toluene → HONO → Light ON</i>
<i>Light</i>	<i>ON at the end of the last compound injection</i>
<i>α - pinene</i>	<i>3 minutes after the BC injection</i> <i>i.e.: BC → α - pinene → O₃</i>

To evaluate the possible effect of different factors, experiments were conducted, increasing the complexity step by step. In the following Table (2), all the investigated conditions are summarized.

Table 2: Reagents involved in each specific experiment, grey cells indicate dark experiments while yellow cells indicate light experiments (i.e., SoSi switched on). Experiments 2) to 5) simulate a simplified winter situation with biomass burning while experiments 7) to 10) simulated a summer situation dominated by traffic emissions with the presence of ammonium sulfate.

			WINTERTIME CONDITIONS			SUMMERTIME CONDITIONS			BIOGENIC SPECIES	
			DARK			LIGHT - SoSi ON			LIGHT - SoSi ON	
EXP ID		BC	NO ₂ + O ₃	AMM. NITR.	2,5- DMF	HONO	AMM. SULF.	TOLUENE	O3	α-pinene
1 - REF	BC DARK	✓								
2	BC NO2 O3 DARK	✓	✓							
3	BC AMM.NITR. NO2 O3 DARK	✓	✓	✓						
4	BC NO2 2,5-DMF O3 DARK	✓	✓		✓					
5	BC NO2 AMM.NITR. 2,5- DMF O3 DARK	✓	✓	✓	✓					
6 - REF	BC LIGHT	✓								
7	BC HONO LIGHT	✓				✓				
8	BC AMM.SULF. HONO LIGHT	✓				✓	✓			
9	BC TOLUENE HONO LIGHT	✓				✓		✓		
10	BC AMM.SULF. TOLUENE HONO LIGHT	✓				✓	✓	✓		
11	BC O3 α-PINENE LIGHT	✓							✓	✓

The purpose of the approach was to mimic different scenarios, representative of ambient conditions similar to what was observed during the RHAPS field campaigns carried out in Po

Valley in different seasons and with different dominant PM sources (Costabile et al., 2022). 1) to 6) were considered as reference experiments, simulating the presence of pure generated soot particles (BC) not exposed to any additional oxidants and just subjected to two different irradiation conditions, one more representative of wintertime (dark conditions) and the other of summertime (light conditions). Experiments 2) to 5) aimed at simulating a simplified winter situation with biomass burning (BB) emissions as the main source of carbonaceous aerosol (BC + OA) and ammonium nitrate as the most abundant inorganic species (typical of Po Valley wintertime, (Crova et al., 2024; Paglione et al., 2020)). Dark oxidation was considered the most important wintertime ageing mechanism and simulated by the injection and reaction of NO_2 and O_3 to form NO_3 radical (Kodros et al., 2020). 2,5-dimethylfuran was selected as a representative compound of BB-OA emissions reacting with NO_3 in dark conditions (Newland et al., 2022). Experiments 7) to 10) simulated a summer situation dominated by traffic emissions and ammonium sulfate (as the most abundant summertime inorganic species in Po Valley), subjected to OH radical oxidation (provided by injection of HONO) under light. In contrast with the previous experiments based on anthropogenic emissions, the last experiment (11) was conducted by injecting together with BC a biogenic species, namely α -pinene, producing SOA via O_3 oxidation under light conditions. Average ambient parameters for each experiment were reported in the Supplementary Material (Table S1).

The experimental sequence aimed to:

- study if different anthropogenic emission sources of carbonaceous aerosol (specifically biomass burning and traffic) and different oxidation/ageing pathways lead to different levels of oxidative potential and toxicity of the PM burden;
- investigate if ageing of carbonaceous aerosol contributes to the transfer of oxidative/toxic species in the water-soluble extracts (thanks to water-soluble oxidative potential assays - WSOP), as well as in the total extracts (total oxidative potential - TOP), and looking at the WSOP/TOP ratios of the different experiments;

- confirm that concentrations of non-toxic components such as ammonium-sulfate and ammonium-nitrate lead to a reduction of the oxidative potential and toxicity of the PM collected on the filters (a “dilution” of the toxicological effects).

2.3 Filters and offline compositional analyses

Particles from the chamber were collected on quartz fiber filters (47 mm Pallflex Tissuquartz 2500 QAO-UP) to undergo several offline analyses. A stainless-steel filter holder connected to a sequential sampler (Gemini - DadoLab Srl) was used to simultaneously collect filters at 20 lpm for 12 min, resulting in an air sampled volume of 0.24 m³. Sampling started 2 hours after the last reagent injection or light turning on, to allow reactions to evolve inside ChAMBRé. Since no aging processes occur, the sampling in the experiment with only dark soot started after 3 minutes of mixing time.

For each sample, optical analysis was performed to quantify the aerosol absorption coefficient by the Multi-Wavelength Absorbance Analyzer (MWAA, (Massabò et al., 2015, 2013)), a laboratory instrument performing analysis at five wavelengths (850, 635, 532, 405, and 375 nm). Carbonaceous composition (Elemental Carbon – EC, Organic Carbon – OC, Total Carbon TC) was determined for each sample by Sunset Laboratory Inc. Sunset EC/OC analyzer performing thermal–optical transmittance analysis (TOT) with the NIOSH 5040 protocol (National Institute for Occupational Safety and Health: NIOSH, 1999) corrected for temperature offsets.

2.4 Assays for oxidative potential assessment

Polytetrafluoroethylene (PTFE) membrane filters (Pall R2PJ047, Pall Life Sciences, Ann Arbor, MI, USA) were sampled at 20 lpm for 12 min, resulting in an air sampled volume of 0.24 m³, to perform OP assays. PM collected on PTFE membrane filters was subjected to toothbrush detachment and suspension in water by applying a previously optimized method (Frezzini et al., 2022b; Massimi et al., 2022). Each PTFE filter was put in a Petri dish of 50 mm diameter and overlaid with 10 mL of deionized water. Subsequently, the filter was brushed for 2 min using an electric toothbrush with a sensitive brush head (Braun, Germany, Oral-B Vitality Sensitive) as

extensively described in (Massimi et al., 2022). Then, the water suspension was split into two equal 5 mL aliquots, each diluted 1:4 with deionized water and rotationally agitated for 30 min. One of the two resulting 20 mL samples was filtered through a 0.45 μm pore nitrocellulose filter to separate the insoluble fraction of PM from the water-soluble one, which was then analyzed in two replicate samples (10 mL each) for water-soluble OP (WSOP) by applying the OP^{DCFH} , OP^{DTT} and OP^{AA} methods reported in (Massimi et al., 2020) and (Frezzini et al., 2022a) to measure $\text{WSOP}^{\text{DCFH}}$, WSOP^{DTT} and WSOP^{AA} . The other 20 mL sample (both soluble and insoluble fraction of PM) was instead analyzed in two replicates (10 mL each) for total OP (TOP) applying the same methods on the water-soluble and insoluble particles together (TOP^{DCFH} , TOP^{DTT} , and TOP^{AA}) as detailed in (Frezzini et al., 2022b).

OP^{DCFH} was measured by adding 125 μL of DCFH (5 μM) and 5 mL of HRP (0.5 units mL^{-1}) to 1.5 mL of the sample in a water bath at 37 $^{\circ}\text{C}$ for 5 minutes. DCF fluorescence (excitation at 427 nm, emission at 530 nm) was measured using fluorescent spectroscopy. An external standard calibration curve using H_2O_2 solutions was used to convert fluorescence intensity to H_2O_2 equivalents, and OP^{DCFH} was expressed as nmol H_2O_2 per sampled PM volume ($\text{nmol H}_2\text{O}_2 \text{ m}^{-3}$) and per PM mass concentration ($\text{nmol H}_2\text{O}_2 \mu\text{g}^{-1}$). OP^{DTT} was measured using three 0.7 mL aliquots of each sample incubated at 37 $^{\circ}\text{C}$ with 0.1 mL of DTT (1 mM) and 0.2 mL of potassium phosphate buffer (1 M). The DTT reaction was stopped at 0, 10, and 20 minutes by adding 1 mL of 10% TCA. An aliquot of the reaction mixture was mixed with tris-buffer, EDTA, and DTNB to form TNB, and DTT absorbance was measured at 412 nm by UV-Vis spectrophotometry. OP^{DTT} was expressed as the DTT consumption rate per sampled PM volume ($\text{nmol DTT min}^{-1} \text{ m}^{-3}$) and per PM mass concentration ($\text{nmol DTT min}^{-1} \mu\text{g}^{-1}$). OP^{AA} was determined by adding 300 μL of phosphate buffer (0.5 mM) and 100 μL of AA (2 mM) to 2.5 mL of the sample. AA absorbance was measured at 265 nm using a UV-Vis spectrophotometer at 0, 10, and 20 minutes, and OP^{AA} was expressed as the AA consumption rate per sampled PM volume ($\text{nmol AA min}^{-1} \text{ m}^{-3}$) and per PM mass concentration ($\text{nmol AA min}^{-1} \mu\text{g}^{-1}$).

2.5 Study design for toxicological assessment

PTFE membrane filters (Pall R2PJ047, Pall Life Sciences, Ann Arbor, MI, USA) were sampled at 50 lpm for 30 min, resulting in a volume of 1.5 m³, for toxicological investigation. The toxicological evaluation was performed using the BEAS-2B cell line (Sigma Aldrich, Darmstadt, Germany, cod. 95102433), treated at a non-cytotoxic concentration of PM. The cells were treated by diluting 1:10 the PM extracted in water in complete medium (LHC-9, Gibco, Life Technologies, Monza, Italy). The level of cytotoxicity was determined by analysing the lactate-dehydrogenase (LDH) activity with the MAK066 kit (Sigma-Aldrich, Darmstadt, Germany). Once determined that no cytotoxic concentrations are used, the toxicological assessment is done by analysing eventual DNA damage induced by the treatment (i.e., tail moment of alkaline comet assay, immunofluorescence for γ -H2AX, and micronucleus) and the modulation of main genes involved in DNA repair and oxidative stress pathways. Oxidative stress was also evaluated with the analysis of reactive oxygen species (ROS) formation. Lastly, inflammatory status is analysed by studying the secretion of the interleukin (IL-) 8. Detailed methods can be found in the supplementary materials.

Each experiment (*n*) was repeated at least three times. Data are reported normalizing the mean for the mass of the filter (mg filter), and as mean $\pm$ standard deviation (SD). Statistical analysis was performed using the software GraphPad Prism 10.4.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using a one-way ANOVA test followed by Dunnett's Multiple Comparison post hoc test. Results were considered significant at $p < 0.05$.

3. Results and Discussion

3.1 Aerosol characterization

Particle number size distribution (PNSD) was measured during all the experiments by a SMPS (see Par. 2.1). During all the experiments, the size distribution was almost constant with an average mode diameter of 168 ± 9 nm. All the uncertainties reported in this paragraph refer to the

standard deviation calculated on the variability of the average values of each experiment. This allows us to affirm that, in all the experiments we are comparing, OP and toxicological impacts depend on the different chemical composition (since the distribution is quite constant); that means, the responsible of the properties observed on filter analysis is the bulk mass of the particulate matter. In the supplementary materials (Fig. S1 and S2), we reported the mean size distribution as representative for each experiment.

Concentration of typical atmospheric gases was monitored in real time during all the experiments (see Par. 2.1), and specific reactions for each experiment can be observed. During the BC dark reference experiment, a constant time trend is observed: typical gas species produced by combustion and specific to MISG (i.e., CO_2 , NO , and NO_2) increased as a consequence of the injection, and then they remained constant (no chemical reactions happening). After BC injection, the CO_2 level increased by about 100 ppm with respect to the ambient concentration. NO and NO_2 emissions were about 74 ± 2 and 11 ± 6 ppb, respectively. On the other hand, in the BC light reference experiment, the photoreaction of NO - NO_2 could be observed (see Fig. S3 in Supplementary material) suggesting photo-oxidation was active in the chamber. Another interesting example is the reaction between O_3 and NO_2 observed in the experiment ($\text{BC} + \text{NO}_2 + \text{O}_3$, dark condition), when a sharp NO_2 concentration decrease occurred after O_3 introduction into the chamber volume (see Fig. S4 in Supplementary material) suggesting NO_3 radical formation. This is useful to confirm the correct occurrence of the expected reactions during the experiment.

Optical properties were monitored in real time during all the experiments by two PAX units (see Par. 2.1), here we summarize them in terms of single scattering albedo (SSA) variation. During both the reference experiments (i.e., BC dark and light), SSA was constant, and no differences were observed due to the light exposure. The IR-PAX average SSA values were 0.19 ± 0.01 and 0.20 ± 0.01 , in agreement with (Moallemi et al., 2019; Vernocchi et al., 2022), while the BLUE-PAX were 0.24 ± 0.01 and 0.24 ± 0.02 , for dark and light experiments, respectively. Instead, in the other

experiments the behavior of SSA was specific to the components involved; anyway, once all the injections had been completed, the value of SSA then remained constant. The [BC + O₃ + α -pinene, light] resulted to be the only experiment showing a significant increase in SSA values. The IR-SSA increased from 0.21 ± 0.01 to 0.26 ± 0.01 , while the BLUE-SSA increased from 0.24 ± 0.01 to 0.31 ± 0.01 .

Samples collected on quartz fiber filters underwent the following offline analysis (see Par. 2.3). The carbonaceous composition was determined by Sunset analysis. No significant differences in the EC:TC ratio as the black carbon aging conditions varied could be observed. Average concentration values over the whole sample set were: 274 ± 84 (EC), 166 ± 29 (OC), and 440 ± 106 (TC) in $\mu\text{g m}^{-3}$ while the average EC:TC was 0.62 ± 0.04 .

An offline characterization of optical properties was also performed by the MWAA, determining the aerosols' absorption coefficient (b_{abs}) at five different wavelengths (see Par. 2.3) and consequently the spectral dependence of b_{abs} . The dependence can be expressed in terms of Ångström absorption exponent (AAE; (Moosmüller et al., 2011)), according to the power law: $b_{\text{abs}}(\lambda) \approx \lambda^{-\text{AAE}}$. Average AAEs resulted equal to 0.92 ± 0.04 for the dark experiments series and to 0.85 ± 0.05 for the light experiments series. Values agree with values reported in (Vernocchi et al., 2022). Absorption spectra were reported in the Supplementary material (Fig. S5).

Coupling information by MWAA and Sunset, MACs (Mass Absorption Coefficients) were determined by the relation $b_{\text{abs}}(\lambda) = \text{MAC} \times \text{EC}$, with b_{abs} (Mm^{-1}), MAC ($\text{m}^2 \text{g}^{-1}$), and EC ($\mu\text{g m}^{-3}$). MAC values are reported in Table 3. These values agree with what was obtained in (Vernocchi et al., 2022) for the same pure soot.

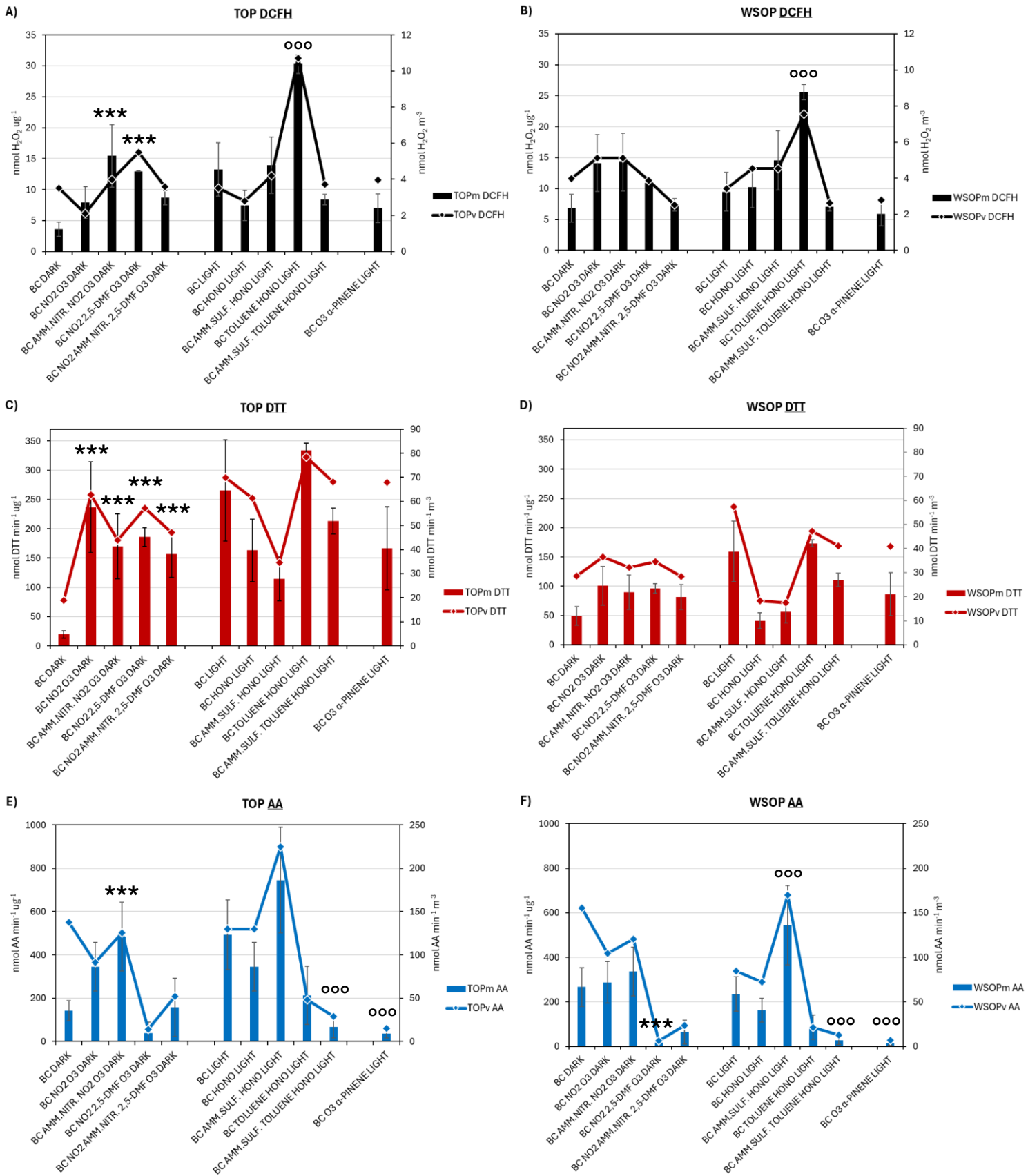
Table 3: Summary of the retrieved MAC values ($\text{m}^2 \text{g}^{-1}$).

PAX	
870 nm	405 nm
6.3 ± 0.3	14.7 ± 0.5
MWAA	

850 nm	635 nm	532 nm	405 nm	375 nm
5.57 ± 0.06	7.4 ± 0.1	8.9 ± 0.1	11.0 ± 0.1	11.0 ± 1

3.2 Oxidative potential assessment

Figure 1 shows that OP^{DCFH} , OP^{DTT} , and OP^{AA} exhibited different responses to the PM samples generated under different chamber conditions; the OP refers to oxidative potential per mass of PM (OPm), representing the PM's intrinsic reactivity. Notably, all OP values were much higher than those typically observed on field PM samples (Bates et al., 2019; Daellenbach et al., 2020; Grange et al., 2022; Molina et al., 2020). TOP and WSOP showed comparable variability across chamber-generated PM samples, with TOP generally displaying higher values than WSOP, particularly for OP^{DTT} and OP^{AA} , in agreement with (Frezzini et al., 2022b; Verma et al., 2012). Less pronounced differences were observed between TOP^{DCFH} and $WSOP^{DCFH}$, likely because particle-bound ROS are predominantly water-soluble.



*Figure 1: OP of the chamber-generated PM samples expressed per sampled PM volume and PM mass concentration: TOP^{DCFH} (panel A), $WSOP^{DCFH}$ (panel B), TOP^{DTT} (panel C), $WSOP^{DTT}$ (panel D), and TOP^{AA} (panel E), $WSOP^{AA}$ (panel F). Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; *** $p < 0.001$ vs. BC DARK and °°° $p < 0.001$ vs. BC LIGHT.*

Figure 1 shows that BC exposed to typical summer season irradiance conditions, characterized by high solar radiation (sample BC LIGHT), exhibited considerably higher WSOP and TOP values for all OP assays, especially for OP^{DTT} , compared to BC samples generated under typical winter season conditions, i.e., low irradiance (sample BC DARK). This result aligns with recent literature (Liu and Chan, 2022) demonstrating that ozone aging significantly increases the OP of various soot types (e.g., lab soot, diesel particles), an effect further amplified by combined exposure to visible light due to an increase in active oxygen-containing functional groups. Consequently, the higher irradiation typical of the warmer season and the subsequent photo-transformation of soot particles are confirmed to play a crucial role in the generation of reactive species.

For chamber-generated PM samples simulating a summer scenario dominated by traffic emissions, subjected to $OH\cdot$ radical oxidation (via HONO injection) under light conditions, the toluene-added sample (BC + HONO + toluene) exhibited significantly higher values for TOP^{DCFH} (panel A), TOP^{DTT} (panel C), $WSOP^{DCFH}$ (panel B), and $WSOP^{DTT}$ (panel D) compared to the other samples. Conversely, this sample (BC + HONO + toluene) did not show increased values for either TOP^{AA} (panel E) or $WSOP^{AA}$ (panel F), whereas the ammonium sulfate-added sample (BC + HONO + ammonium sulfate) resulted in significantly higher TOP^{AA} and $WSOP^{AA}$. This observation likely reflects the lower sensitivity of OP^{AA} to organic components from exhaust traffic compared to its higher sensitivity to inorganic components, as reported in previous studies (Massimi et al., 2024, 2020; Molina et al., 2020; Vaccarella et al., 2025). However, the significantly higher OP^{DCFH} and OP^{DTT} (highly sensitive to exhaust organic components) for the toluene-added sample (BC + HONO + toluene) highlight the role of toluene from exhaust traffic in generating ROS under $OH\cdot$

radical-induced oxidative conditions, particularly in the absence of ammonium sulfate. Indeed, the ammonium sulfate-added sample (BC + HONO + toluene + ammonium sulfate) led to a reduction in all OP values, particularly for TOP^{DCFH} and $WSOP^{DCFH}$. This result is in agreement with recent literature (Liu and Chan, 2022), which demonstrated that while organic coatings like quinones can increase the OP of soot particles, sulfates and nitrates significantly reduce OP by forming passivating layers. Overall, this finding confirms that the presence of non-toxic components characteristic of aged PM, such as ammonium sulfate, may reduce the OP of PM from exhaust traffic.

3.3 Toxicological assessment

The toxicological assessment was performed by analysing cell viability through the evaluation of LDH release. Results are shown in Figure 2. High levels of cytotoxicity were generally detected for the samples obtained in the presence of light, with statistically significant increase of cell death following treatment with the experiments performed in presence of light, and, in a statistically significant manner, after treatment with the sample BC with toluene and HONO in light condition and BC with ammonium nitrate, NO_2 , and O_3 in dark condition. However, this increase in cell cytotoxicity was observed only after normalization to the mass of PM on filters; for this reason, the tested concentrations were also used to analyze the oxidative stress, genotoxicity and inflammatory effects of the samples. Interestingly, the samples characterized by high level of cytotoxicity are the one representative of the summer conditions and traffic component, and this is in accordance with previous works that have demonstrated the increase of cytotoxicity following exposure with traffic-derived PM (Landkocz et al., 2017; MohseniBandpi et al., 2017; Niu et al., 2024).

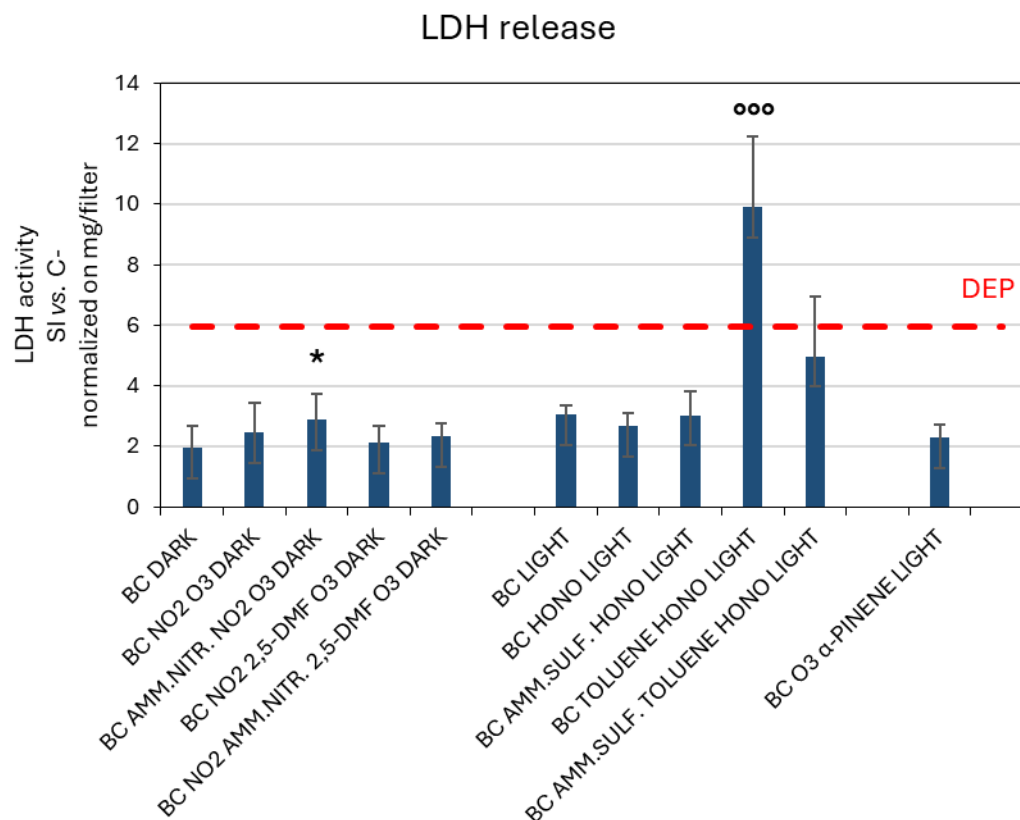


Figure 2: Cytotoxicity assessed through the evaluation of LDH release in BEAS-2B after 24 hours of treatment with PM (1:10 in medium). Data are reported as LDH activity in mU/mL normalized on the control sample and the mass of PM of the filter in graph B. Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; * $p < 0.05$ vs. BC DARK; *** $p < 0.001$ vs. BC LIGHT. Red dashed line: positive control (Diesel exhaust particles; DEP).

Due to the kinetics of oxidative stress generation, reactive oxygen species (ROS) production was investigated after 30 minutes and 1 hour. The results of the test are shown in Figure 3 (A for 30 minutes treatment, and B for 1 hour treatment). A statistically significant increase in ROS generation was detected, following the same trend at the two time points, after treatment with BC generated in presence of light with toluene and HONO. These samples were generated to mimic the traffic component of urban PM. Previous studies, also in vivo and in humans, have shown that oxidative stress acts as a biological intermediary of traffic-related alterations (Sanchez-Rodriguez et al., 2023; Zhang et al., 2022). Statistically significant increase was also

detected following the exposure with the samples of BC with ammonium nitrate, NO₂, and O₃, and NO₂, 2,5-DMF and O₃, generated in dark conditions, which is then not detected following 1 hour of treatment.

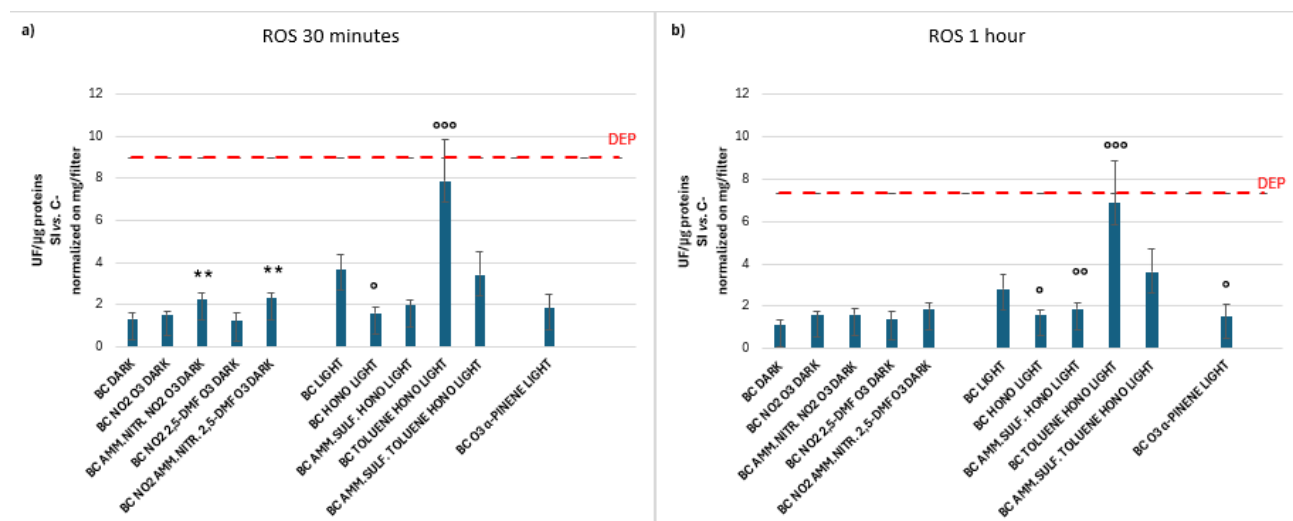


Figure 3: ROS formation in BEAS-2B detected following 30 minutes A) and 1 hour B) of treatment with PM (1:10 in medium). Data are reported as % of units of fluorescence and normalized on the μg of protein; normalization was also performed on the mg of the filter. Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; ** $p < 0.01$ vs. BC DARK, and $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ vs. BC DARK LIGHT. Red dashed line: positive control (Diesel exhaust particles; DEP).

DNA damage was evaluated using different assays to investigate the mode of action of the particles (Figure 4). High level of damage was generally detected in samples obtained under light conditions across all assays. The comet assay (Figure 4A), which highlights the presence of DNA breaks and adducts revealed increase of DNA damage following exposure to BC, and BC with ammonium sulfate and HONO in light condition. The presence of HONO, a known precursor of hydroxyl radicals, may enhance the oxidative potential of the mixture, contributing to the overall oxidative stress observed; while it seems that the other conditions tested are able to mitigate the effect of the BC generated in light condition. This is also highlighted by the presence of damage

to the double-stranded helix indicated by the presence of phosphorylated H2AX (Figure 4B), in particular the samples generated in light condition. The increased damage is detected but it is not statistically significant compared to control. Interestingly, chromosomal damage was detected in BC with toluene and HONO in light condition following micronucleus assay (Figure 4C), whereas the other samples showed no damage, and their results were comparable to the control sample. The presence of toluene, associated with the traffic component of PM, appears to contribute to the generation of micronuclei. Toluene and its oxidation products have been previously implicated in genotoxic effects, including DNA strand breaks and chromosomal aberrations, likely due to their capacity to induce cellular stress and interfere with mitotic processes (Borgie et al., 2014; Cheng et al., 2023). This is also in agreement with previous *in silico* toxicological predictions (e.g., Ames test positivity, (Decesari et al., 2018)) which already underlined that SOA compounds derived from the oxidation of substituted benzene (such as toluene) have the highest mutagenic properties among all tested species (both anthropogenic and biogenic). These findings further support the hypothesis that traffic-derived PM contains specific chemical constituents capable of inducing severe genetic damage (Borgie et al., 2014), reinforcing the need for targeted assessments of urban air pollution risks. Moreover, these results highlight the complexity of PM-induced DNA damage, where different chemical compositions and environmental conditions determine the mode and severity of genotoxic effects. The observed differences emphasize the importance of investigating not only total PM exposure but also its chemical composition, as we previously concluded (Melzi et al., 2024).

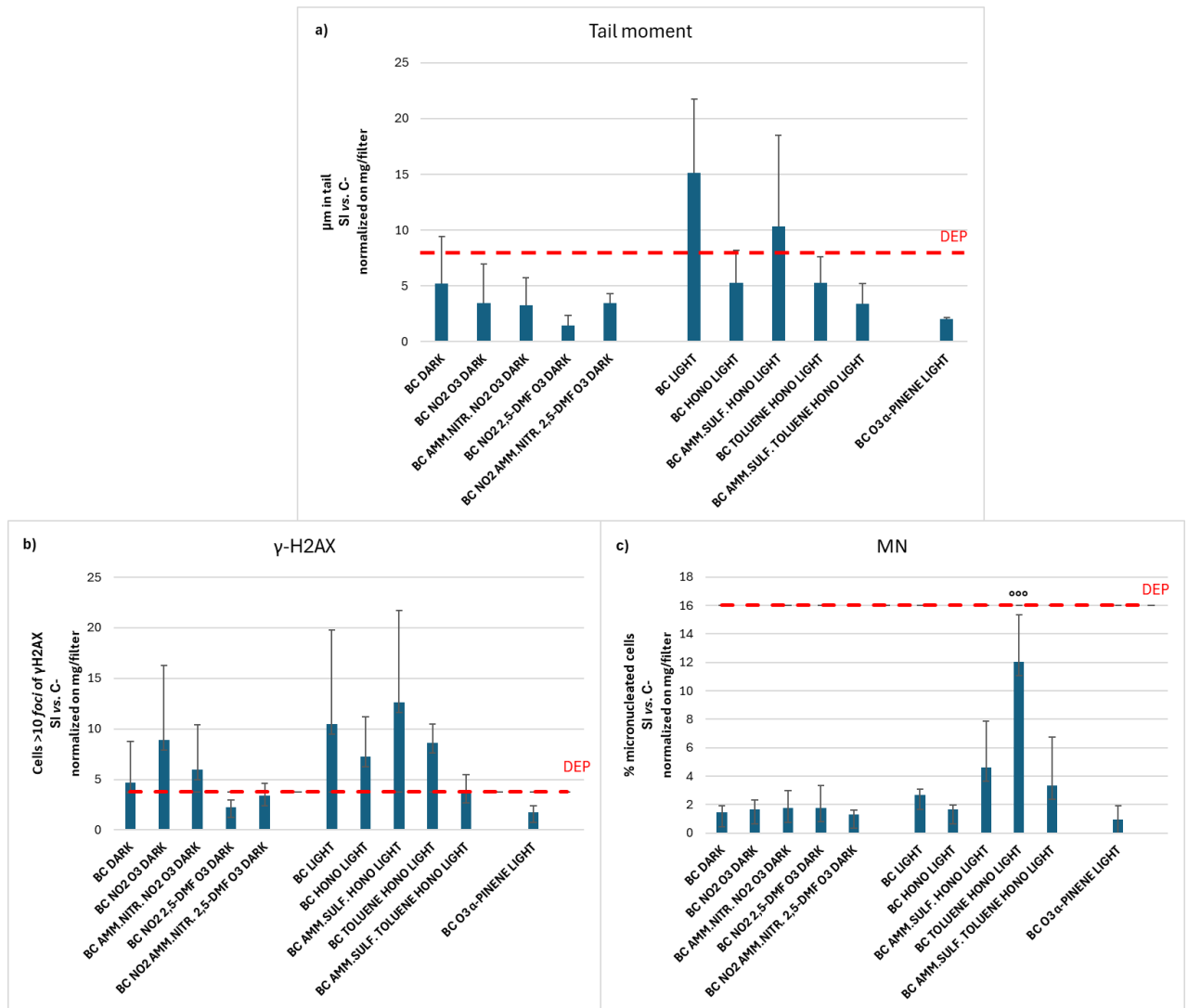


Figure 4: Genotoxicity evaluated through the comet assay, immunofluorescence for phosphorylated H2AX, and micronucleus assay in BEAS-2B after 24 hours of treatment with PM (1:10 in medium). Comet assay results are reported as the mean of the tail moment values normalized on the control and the mass on filters in Figure A). Statistical analysis: One Way ANOVA. Figure B) shows the results of the immunofluorescence for H2AX, the graph is referred to the percentage of cells with high damage (>10 foci). Statistical analysis: One Way ANOVA. The micronucleus data in Figure C) are reported as % of micronuclei (MN) normalized on the control sample and the mass of the filter in graphs. Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; *** $p < 0.001$ vs. BC LIGHT. Red dashed line: positive control (Diesel exhaust particles; DEP).

Gene expression was evaluated after 24 hours treatment to assess modulation in pathways involved in DNA repair and oxidative stress: the results are shown in Figure 5. ATM (Fig. 5A) expression showed no differences from the BC generated in light and dark condition, even though it is possible to observe a trend of increase. ATM activation primarily occurs through post-translational modifications (e.g., phosphorylation) rather than transcriptional regulation, especially in the early or acute response phases. Indeed, ATM kinase activity is well-known to be rapidly activated in response to DNA damage without requiring changes in mRNA abundance. Therefore, the lack of ATM mRNA upregulation does not contradict the presence of DNA damage but rather highlights the complexity of the cellular response, where ATM activation and signalling may be regulated at multiple levels beyond transcription. GADD45 α (Fig. 5B) expression increased after treatment with BC generated in dark conditions, although a moderate activation was observed for other samples as well, similar to the positive control activation. This gene plays a well-established role in the DNA damage response, particularly in promoting DNA repair and cell cycle arrest (Chandramouly, 2022). Therefore, its upregulation likely reflects activation of DNA repair mechanisms rather than a general stress response, supporting the interpretation of the data in the context of genotoxic effects induced by the treatments. In the context of PM exposure, both HMOX1 and NQO1 are typically upregulated as part of the Nrf2-mediated antioxidant response, reflecting the activation of protective pathways against oxidative stress triggered by PM. Evaluation of expression of HMOX1 (Fig. 5C) and NQO1 (Fig. 5D) genes was also conducted, although no statistically significant changes were observed, with the only exception of NQO1 with BC with toluene, HONO in light condition.

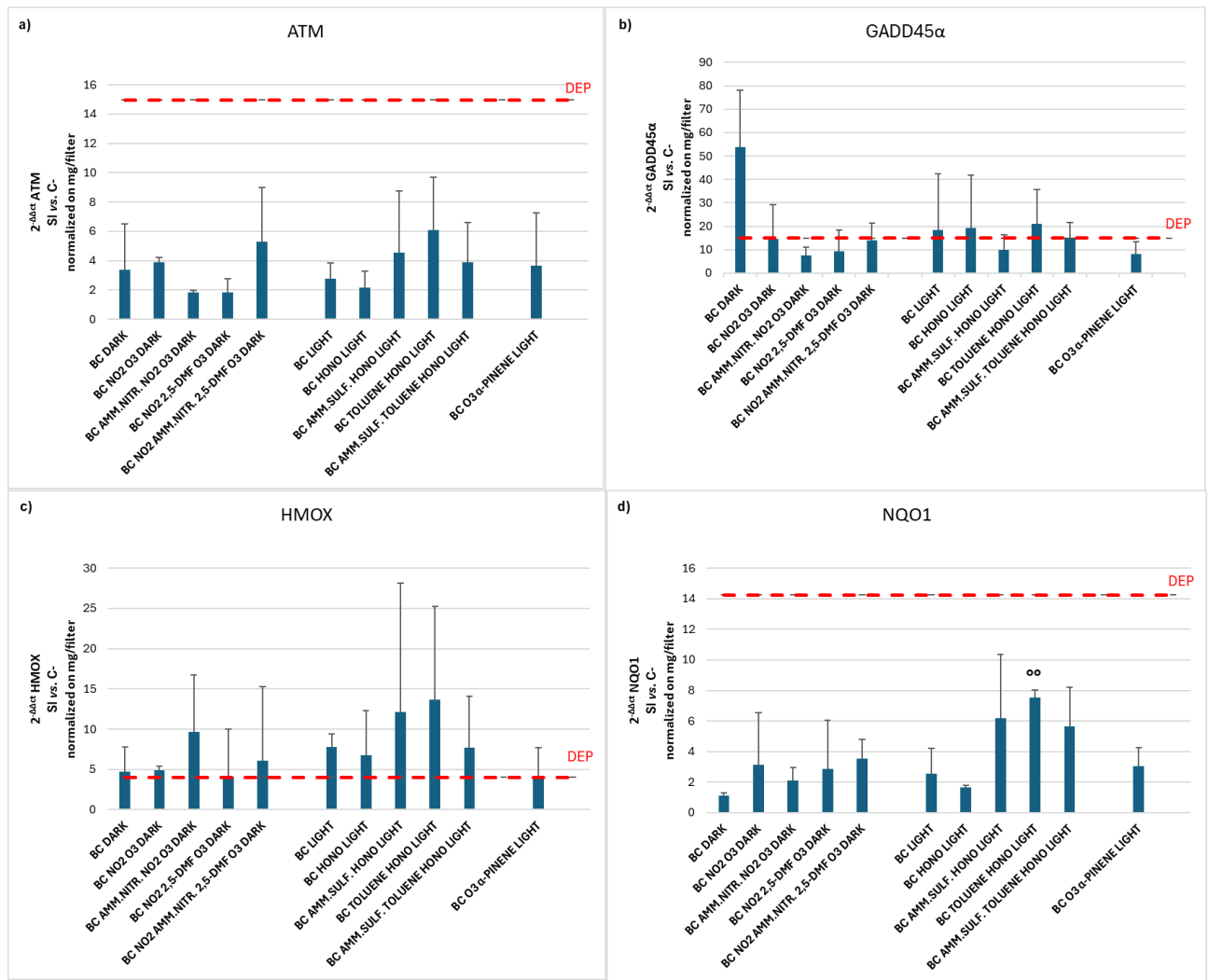


Figure 5: Modulation of the expression of genes involved in DNA repair pathways after 24 hours of treatment with PM (1:10 in medium) in BEAS-2B: A) ATM and B) GADD45α, and oxidative stress related: C) HMOX and D) NQO1. GAPDH was used as a housekeeping gene. Data are reported as $2^{-\Delta\Delta C_t}$ normalized on the mg of the filter B). Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; °°p<0.01 vs. BC LIGHT. Black dashed line: basal control (C-) and red dashed line: positive control (Diesel exhaust particles; DEP).

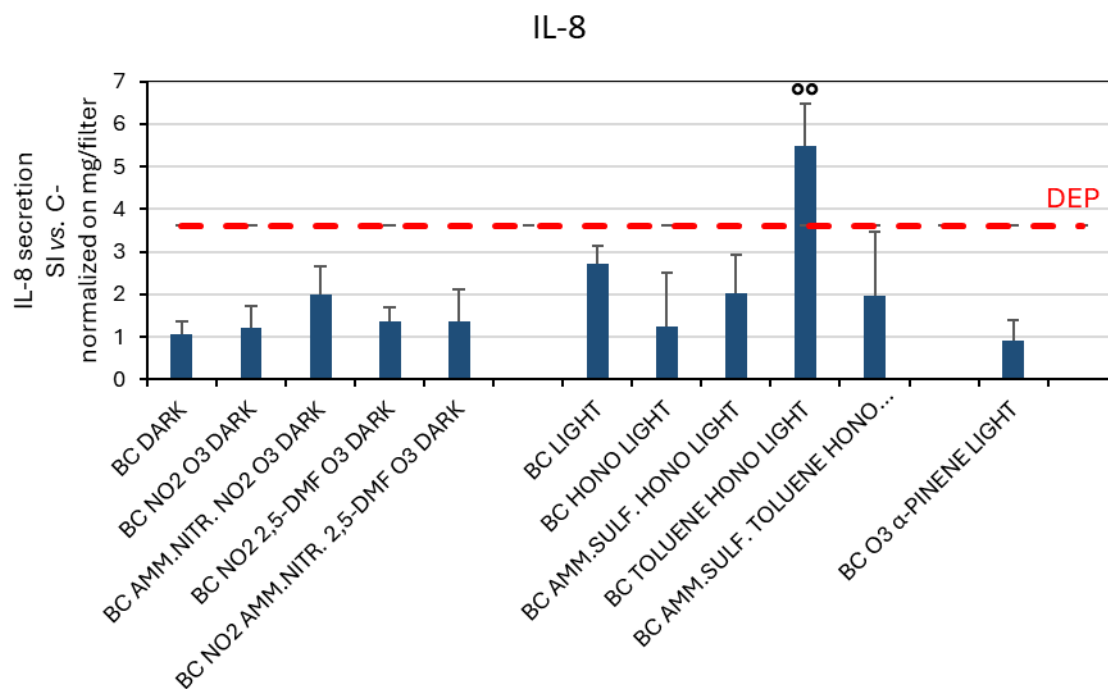


Figure 6: Interleukin-8 secretion after 24 hours of exposure of cells to 1:10 PM in medium, evaluated as proxy of inflammation. Data are reported normalized on the control sample and the mass of the filter. Statistical analysis: One Way ANOVA, followed by Dunnett's Multiple Comparison test; ^{oo} $p < 0.01$ vs. BC LIGHT. Red dashed line: positive control (Diesel exhaust particles; DEP).

Interleukin-8 release was evaluated after 24 hours treatment to assess the inflammatory potential of the samples. A statistically significant increase in interleukin-8 release was observed only for sample BC with toluene and HONO in light condition (Figure 6).

The toxicological data generally showed that our system effectively responds to stimuli associated with the traffic component. Interestingly, the response to the biomass burning component, represented by the addition of 2,5-DMF, was of minor intensity in the system. In this specific case, the biomass burning-like PM appears less toxic than traffic-derived-like PM. This is also possibly due to the fact that 2,5-dimethylfuran does not fully capture the complexity of real-world biomass burning emissions, which can be more harmful under different conditions. Moreover, the observed cytotoxicity, genotoxicity, oxidative stress, and inflammatory responses

3.4 Comparison OP-TOX

Table 4: Correlation between toxicological assessment and OP analysis, green scale indicates the correlation (R^2 of linear regression) level.

R ²	LDH	> 10 foci	MN	ROS	ROS	IL8	ATM	HMO	NQO	GADD45	Tail	TOP	TOP
				30'	1h			X	1	a	momen t	m DCF H	TOP m AA DTT
LDH		0.08	0.89	0.92		0.88	0.42						
	2	9	6	0.956	2	0	0.580	0.644	0.006	0.009			
> 10 foci			0.19	0.10		0.20	0.07						
		4	0	0.077	8	5	0.429	0.133	0.003	0.471			
MN				0.83		0.89	0.37						
			3	0.847	5	2	0.644	0.649	0.007	0.067			
ROS 30'						0.93	0.39						
				0.965	6	6	0.545	0.533	0.005	0.226			
ROS 1h						0.88	0.38						
					1	7	0.503	0.606	0.001	0.011			

IL8 ATM HMOX NQO1 GADD45 a Tail moment							0.32							
							0	0.677	0.527	0.004	0.099			
								0.324	0.608	0.044	0.014			
									0.588	0.001	0.193			
										0.026	0.063			
												0.025		
TOPm		0.15	0.74	0.72		0.84	0.22							
DCFH	0.712	5	8	4	0.687	5	7	0.655	0.486	0.030	0.0806		0.073	0.539
	0.000	0.77	0.02	0.00	0.000	0.04	0.00							
TOPm AA	2	6	4	2	2	5	6	0.359	0.032	0.015	0.3901			0.010
TOPm		0.09	0.36	0.56		0.53	0.21							
DTT	0.517	0	0	3	0.560	2	1	0.260	0.352	0.068	0.0283			

In Table 4 are reported the correlations (R^2 of linear regression) between the analysis of the oxidative potential and the toxicological assessment, here we considered only TOP (not WSOP) to be consistent with the toxicological assessment performed on the total sample. The analysis showed strong correlation between OP and toxicological parameters. In particular, correlation analysis demonstrated a relationship between OP^{DCFH} and OP^{DTT} assays results and various biological responses, including cytotoxicity, oxidative stress, inflammation, DNA damage, HMOX and NQO1 expression in BEAS-2B cells. These biomarkers are widely recognized indicators of cellular responses to redox imbalance and xenobiotic stress, suggesting that these OP metrics effectively capture biologically relevant oxidative reactivity of particulate matter. Moreover, OP^{AA} showed correlation with key toxicological endpoints, particularly those related to DNA damage and the modulation of oxidative stress pathways, evaluated to assess. Our previous work

provided early evidence of a consistent relationship between oxidative potential and adverse biological outcomes, and the present findings build upon that foundation, offering a more nuanced view by comparing multiple OP measurement methods and their predictive capacity across cellular endpoints (Melzi et al., 2024). Moreover, these experimental insights align with a growing body of epidemiological evidence demonstrating associations between oxidative potential—particularly when assessed through AA and DTT assays—and respiratory health effects. For instance, recent studies have reported that higher OP levels correlate with impaired lung function, increased frequency of emergency department visits for respiratory symptoms, and heightened risks of cardiovascular and pulmonary morbidity (Fang et al., 2016; Janssen et al., 2015). Together, these findings support the integration of OP metrics as complementary tools to traditional mass-based air pollution measures, offering enhanced resolution for health risk assessment and air quality management.

The observed correlation between OP and DNA damage endpoints underscores a critical toxicological pathway linking PM exposure to genotoxic effects. Among the OP assays evaluated, both OP^{DTT} and OP^{AA} demonstrated significant associations with markers of DNA strand breaks and oxidative DNA lesions in BEAS-2B cells, suggesting that the redox-active fraction of PM plays a central role in driving genotoxic stress (Vilas-Boas et al., 2024). These findings support the hypothesis that particle-induced oxidative stress is a key upstream event leading to DNA damage, potentially through the generation of reactive oxygen species (ROS) and subsequent interference with DNA repair mechanisms. Importantly, DNA damage is not only a biomarker of acute cellular stress but also a precursor event in mutagenesis and carcinogenesis. Therefore, the strong association between OP and genotoxicity provides a mechanistic bridge between short-term cellular toxicity and potential long-term health outcomes, such as respiratory cancer risk. This highlights the added value of incorporating OP measurements into PM risk assessments and supports their use as early-warning indicators of hazardous particle exposure (Diao et al., 2019).

4. Conclusions

In this study, we explored the influence of atmospheric conditions and ageing on the properties of soot particles, a key component of traffic-related and combustion aerosols, and their subsequent effects on oxidative stress and toxicity. Our findings underline the complexity of particulate matter composition and its ability to induce oxidative stress, which is linked to various health impacts. By utilizing an atmospheric simulation chamber, we were able to create controlled conditions that mimic real-world scenarios, allowing for a better understanding of PM-induced oxidative stress and cellular toxicity.

This study demonstrates that different PM components and photochemical aging pathways play a key role in the OP and cellular toxicity of carbonaceous aerosols. The traffic component, particularly under light conditions and in the presence of HONO and toluene, exhibited high OP and induced significant biological responses. In particular, toluene substantially increased the OP^{DTT} and OP^{DCFH} , playing also a critical role in generating ROS cytotoxicity, genotoxicity, and the release of the proinflammatory cytokine IL-8. The presence of ammonium sulfate appeared to modulate these effects, sometimes reducing the OP and most of the assessed toxicological parameters.

Although OP^{AA} exhibited a positive correlation only with the >10 foci, HMOX and tail moment toxicological endpoints, good correlations were found for OP^{DCFH} and OP^{DTT} with the majority of the assessed biological parameters, including cytotoxicity, oxidative stress, IL-8, and DNA damage. In particular, strong correlations were found between OP^{DCFH} and LDH release, % of micronuclei, IL-8 secretion, HMOX expression and ROS formation in BEAS-2B cells. This result highlights a direct link between particle-bound ROS, oxidative stress generation in cell lines and toxicological effects, confirming the reliability of OP^{DCFH} for estimating PM-induced oxidative stress and health effects, in agreement with previous findings (Costabile et al., 2023, 2019; Melzi et al., 2024).

These findings emphasize the importance of considering the chemical composition and aging processes of PM in assessing its overall toxicity and potential health risks, particularly in urban environments dominated by traffic emissions.

Author contributions

Conceptualization: MP, PP, DM; Data Curation: VV, TI, LM, GM, SP, MRi, DM; Formal analysis: VV, TI, LM, GM, SP, MRa, DM; Funding acquisition: EC, LM, PP, DM; Investigation: VV, GM, MP, SP, MRa, CT, DM; Methodology: VV, DM; Resources: PP, DM; Software: MB; Supervision: SC, EC, LM, MP, PP, DM; Writing – original draft: VV, LM, GM; MP; Writing – review and editing: VV, MB, SC, EC, LM, FM, GM, MP, PP, MRa, MRi, DM.

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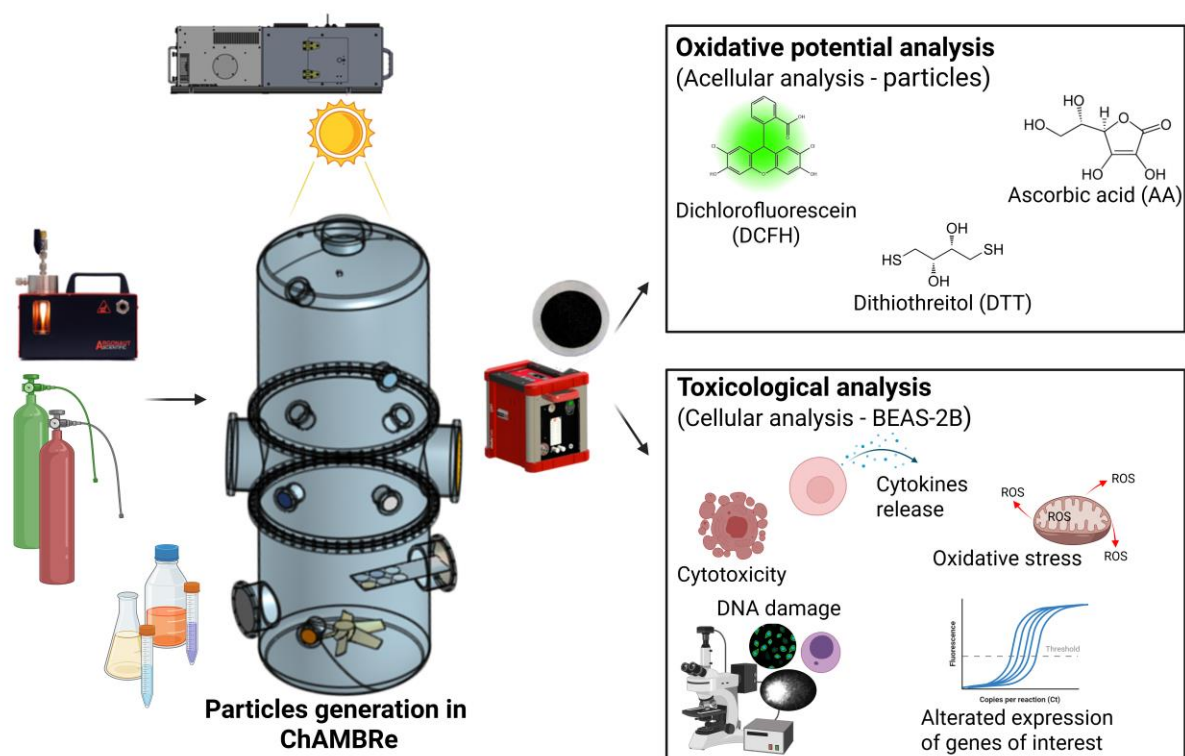
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Declaration of interests

☐ 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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Virginia Vernocchi, Marco Brunoldi, Silvia Canepari, Emanuela Corsini, Tommaso Isolabella, Lorenzo Massimi, Federico Mazzei, Gloria Melzi, Marco Paglione, Sofia Pantaleoni, Paolo Prati, Marco Rapuano, Matteo Rinaldi, Caterina Tiraboschi, Dario Massabo' reports financial support was provided by Government of Italy. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



- Carbonaceous aerosol from fossil fuel emitted by combustion of fossil fuel injected in an atmospheric simulation chamber
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