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D4.2 Toxicity results for exhaust and secondary aerosols using basal and easily measured of toxicity markers and advanced microscopic methods

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PAREMPI
Particle emission prevention and impact: from real-world emissions of traffic to secondary PM of urban air

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PAREMPI Particle emission prevention and impact: from real-world emissions of traffic to secondary PM of urban air

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Table of Contents

List of abbreviations 4
1. Executive Summary 5
2. Introduction 6
3. Methods 8
3.1 Measurement systems for PM_{fresh} and PM_{aged} 8
3.2 Description of PAREMPI measurement campaigns 9
3.3 ALI Exposure System 11
3.4 Air–Liquid Interface (ALI) Models 12
3.5 Toxicity Endpoints and Assays 14
3.5.1 Tissue barrier 15
3.5.2 Cytotoxicity Markers 15
3.5.3 Oxidative Stress Markers 15
3.5.4 Genotoxicity Markers 16
3.5.5 Toxicogenomics 16
3.5.6 Microscopy 16
4. Toxicological Findings Summary 18
4.1 Light-duty campaign 18
4.2 Heavy-duty campaign 19
4.3 Aviation and Marine campaign 20
4.3.1 Aviation campaign 21
4.3.2 Marine campaign 22
5. Robustness and Limitations 25
5.1 Methodological Robustness 25
5.2 Influence of Environmental and Operational Factors 25
6. Conclusions 27
6.1 Recommendations for Future Research 27
7. References 28

List of abbreviations

ALI	Air-Liquid Interface
AK	Adenylate Kinase
BC	Black Carbon
BEAS-2B	Human Bronchial Epithelial Cell Line
C	Cytotoxicity (in toxicity rating)
CAST	Combustion Aerosol Standard Generator
D	Different / Other Endpoints (in toxicity rating)
DEG	Differentially Expressed Gene
DMB	Diesel Marine Blend
DPF	Diesel Particulate Filter
EF	Emission factor
FAME	Fatty acid methyl ester
FID	Flame ionization detector
FTIR	Fourier transform infrared spectroscopy
G	Genotoxicity (in toxicity rating)
H_2AX	Phosphorylated Histone H2AX (marker of DNA damage)
HD	Heavy duty
HIA	Health impact assessment
HVO	Hydrotreated Vegetable Oil
LD	Light duty
NH_3	Ammonia
Nitro-PAH, NPAH	Nitrated PAHs
NO_x	Nitrogen oxides
nv	Non-volatile
OC	Organic Carbon
OF	Oxidation Flow
OFR	Oxidation flow reactor
OH	Hydroxide
Org	Sum of organic compounds (mass)
O	Oxidative Damage (in toxicity rating)
Oxy-PAHs, OPAHs	Oxygenated PAHs
PAH	Polycyclic aromatic hydrocarbons
PAM	Potential aerosol mass chamber
PEMS	Portable Emissions Measurement System
PM	Particulate matter (mass)
PM_{aged}	Particulate matter (mass), aged particles including SecA
PM_{fresh}	Particulate matter (mass), fresh particles before ageing
PN	Particle Number
PTR-MS	Proton Transfer Reaction Mass Spectrometer
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
RDE	Real Driving Emissions
SecA	Secondary aerosol, includes SOA+SIA
SOA	Secondary organic aerosol
SoC	State of Charge of the battery
T	Timepoint (e.g. T0, T24, T48)
TEER	Transepithelial Electrical Resistance
VOC	Volatile Organic Compounds

1. Executive Summary

This deliverable summarises the toxicological findings within Work Package 4 (WP4) of the PAREMPI project, which investigated the biological effects of fresh exhaust particles (PM_{fresh}) and photochemically aged engine exhaust (PM_{aged}) from transport sectors. The aim was to evaluate whether atmospheric ageing processes modify the toxicological profile of emissions from different transport sectors and fuels. Toxicological experiments were integrated directly into emission measurement campaigns covering light-duty vehicles, heavy-duty trucks, marine fuels and aviation fuels **(D2.2)**.

Exposures were performed using a portable air–liquid interface (ALI) system enabling direct exposure of human respiratory cell models to diluted exhaust or aged aerosol under controlled physiological conditions **(D2.3)**. A differentiated three-dimensional airway epithelium (MucilAir™) served as the primary model, complemented in selected campaigns by the BEAS-2B epithelial cell line to increase sensitivity under conditions of low particle mass. Biological responses were assessed using a panel of endpoints including epithelial barrier integrity, cytotoxicity, oxidative stress, genotoxicity, transcriptomic profiling and fluorescence microscopy.

Across the measurement campaigns, acute cytotoxic responses were generally low, indicating that short-term exposures at environmentally relevant dilution levels did not cause substantial cell damage. However, transcriptomic analyses revealed measurable biological responses, demonstrating that absence of cytotoxicity does not necessarily imply absence of biological activity. Environmental factors such as temperature, driving conditions and fuel composition influenced emission profiles and, in some cases, affected cellular responses.

The results also highlight the importance of considering secondary aerosol formation when evaluating the health relevance of transport emissions. Photochemical ageing alters the chemical composition of emissions and may modify their biological effects, emphasizing the need to assess both fresh and aged particles in toxicological studies.

Overall, the PAREMPI toxicological framework demonstrates that integrating air–liquid interface exposure systems into emission measurement campaigns is technically feasible and provides biologically relevant information to complement physicochemical emission characterization. The findings contribute to improved understanding of the health implications of transport emissions and support future research and regulatory assessments addressing both primary emissions and secondary aerosol formation.

2. Introduction

Air pollution remains one of the leading environmental risk factors in Europe, contributing to morbidity, premature mortality, and considerable societal costs. Despite significant progress in reducing regulated tailpipe emissions, transport sources continue to influence urban air quality. In addition to directly emitted particles, exhaust gases contain various volatile and semi-volatile compounds that can undergo atmospheric transformation, forming secondary aerosol (SecA) and contributing to PM_{aged}. These transformation processes may substantially increase the overall particulate burden associated with transport emissions, yet they are not fully reflected in current regulatory metrics and research focused primarily on primary exhaust components.

Understanding the health relevance of both fresh and aged emissions is therefore essential for a comprehensive assessment of transport-related air pollution. Toxicological evaluation provides a biological perspective that with emission characterization and modelling activities helps to interpret the physicochemical changes in terms of potential cellular responses. The **D4.2** presents a overview of toxicity results obtained within the PAREMPI measurement campaigns, focusing on general response patterns observed for fresh and aged aerosol. Detailed quantitative data are reserved for peer-reviewed publications, while the present report provides a structured overview of methodological approaches, key observations and broader relevance for health impact assessment and policy development.

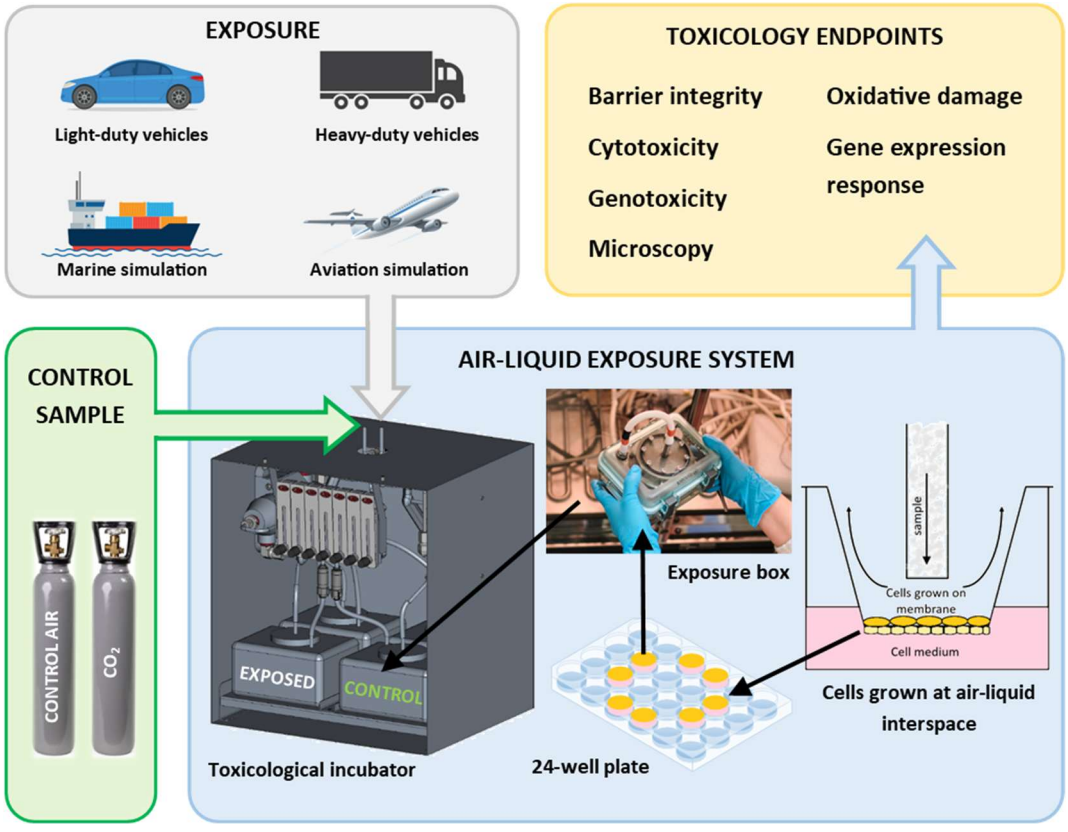

***Figure 1**. Schematic overview of toxicological assessment in WP4. Cells grown at air-liquid interspace were exposed to emissions in different campaigns from light-duty vehicles, heavy-duty vehicles, marine fuel, and aviation fuels. Samples were toxicologically evaluated using battery of simple and more in-depth assays to assess the effect of each type of exposure.*

Work Package 4 (WP4) represents the toxicological part of the PAREMPI project and provides the biological assessment needed to evaluate emission measurements and secondary aerosol formation investigated in WP2 (***Figure 1***). The objective is to evaluate if and how photochemical ageing modifies the toxicological response of transport-derived emissions. By combining exposures to PM_{fresh} and PM_{aged}, WP4 links physicochemical characterisation with biological responses in advanced *in vitro* respiratory models.

WP4 is closely integrated with the robust measurement framework developed within PAREMPI. Sampling, dilution and oxidation flow reactor (OFR) ageing procedures were aligned with the air–liquid interface (ALI) exposure system to ensure realistic exposure conditions. A key methodological advancement of the project was the adaptation of ALI exposure approaches for both laboratory and mobile measurement conditions, enabling toxicity assessment during various measurement campaigns. The results presented in this deliverable therefore summarise biological response patterns observed across transport sectors and ageing conditions, while detailed quantitative datasets will be reported in peer-reviewed publications and database.

Acknowledgements:
The authors would like to acknowledge all partners involved in the emission measurement campaigns and methodological development within the PAREMPI project. In particular, we thank the contributors to Deliverables D2.2, D2.3 and D5.3 for their essential work on emission characterisation, secondary aerosol formation and measurement methodology, which provided the basis for the toxicological assessment presented in this deliverable.

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3. Methods

3.1 Measurement systems for PM_{fresh} and PM_{aged}

The measurements were performed by international collaboration involving VTT Technical Research Center of Finland, Tampere University, Finnish Meteorological Institute, BOSMAL, Institute of Experimental Medicine CAS, ONERA and Lund University. Detailed emission characterizations were conducted during the measurement campaigns of the PAREMPI project. In all four exhaust measurement campaigns versatile research-grade instrumentation covering both gaseous and particulate pollutants as well as OFRs for PM_{aged} formation potential simulation were used (***Figure 2***). Toxicity experiments were linked with emission characterizations, described in deliverable report **D2.3** of the project.

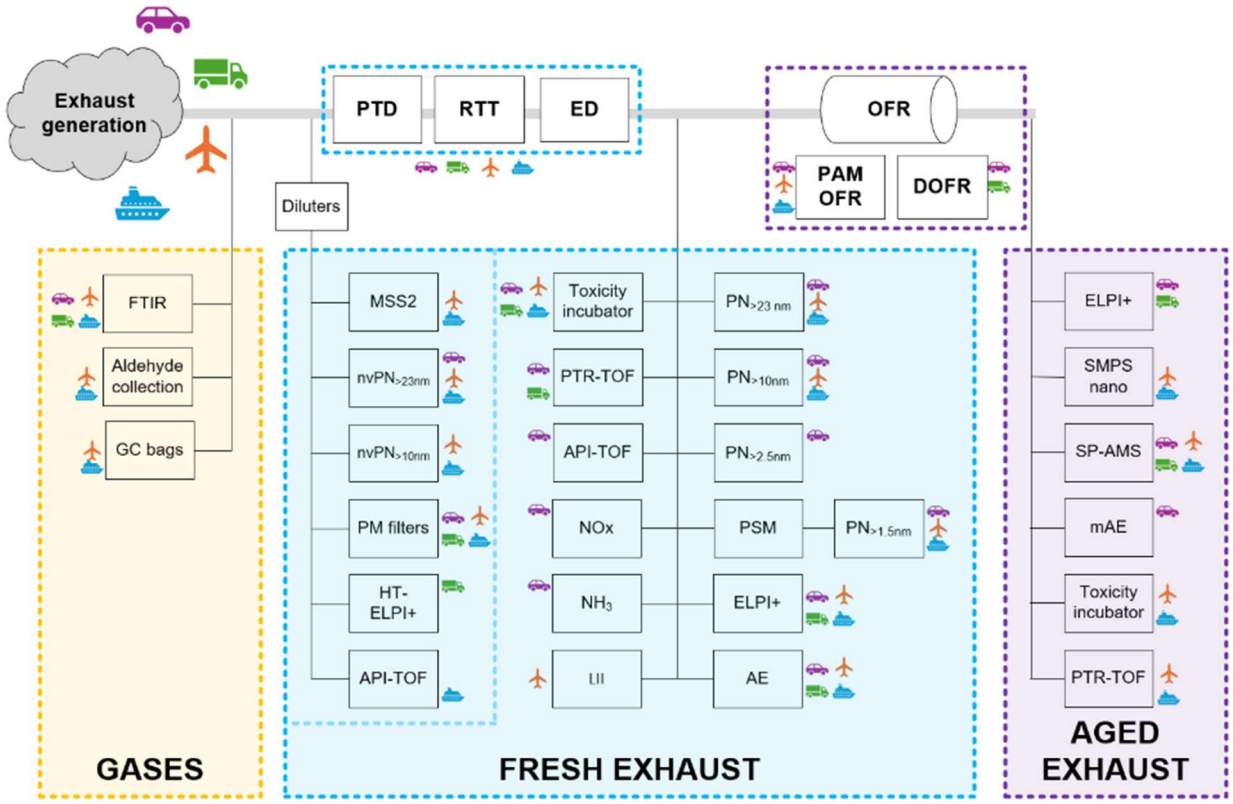

Figure 2. *Generalized schematic of PAREMPI set-ups. With each instrument/metric/part of the schematic, symbols (car, truck, airplane, ship) indicate the experiment where it was used. Abbreviations: AE = Aethalometer, API-TOF = Atmospheric Pressure Interface Time of Flight, DOFR = Dekati Oxidation Flow Reactor, ED = Ejector Diluter, ELPI = Electrical Low Pressure Impactor, FTIR = Fourier Transfer Infrared, GC = Gas Chromatograph, HT = High Temperature, LII = Laser Induced Incandescence, mAE = MicroAethalometer, MSS = Micro Soot Sensor, NH3 = Ammonia, NOx = Nitrogen Oxides, nvPN = Non-Volatile Particle Number, OFR = Oxidation Flow Reactor, PAM = Potential Aerosol Mass, PM = Particle Mass, PN = Particle Number, PSM = Particle Size Magnifier, PTD = Porous Tube Diluter, PTR-TOF = Proton Transfer Reaction Time of Flight, RTT = Residence Time Tube, SMPS = Scanning Mobility Particle Sizer, SP-AMS = Soot Particle Aerosol Mass Spectrometer.*

One of the main research topics of the experiments was to evaluate the potential of exhaust from internal combustion engines to form secondary aerosols in the atmosphere. This was investigated by utilizing oxidation flow reactors for the exhaust sample flows, incorporated into the measurement setups with appropriate exhaust sampling and dilution systems and instruments that provided measurement data for the concentrations of SecA precursor gases and aerosol particles. Based on the experience and knowledge collected in the project, the SecA forming potential of exhaust can be measured repeatably and so that e.g. the effects of vehicle driving conditions and vehicle technologies on SecA formation potential can be quantified. The SecA measurement technology point of view of the project is included in the separate reports, Deliverable **D2.2** and **D2.3**.

The robust measurement system for quantifying aged PM emissions and gaseous precursors forming secondary aerosols suggested by PAREMPI is presented in PAREMPI **D2.3** report.

3.2 Description of PAREMPI measurement campaigns

Based on the initial literature database and unpublished data from PAREMPI partner organizations, gaps in the knowledge were identified to conduct complementary measurements of PAREMPI (confidential **D2.1** report). For each sector (light-duty, heavy-duty, marine and aviation) the current fleet and fuels in use were analyzed. Common measurement methodology appliable to all sectors and all campaigns was designed, and sector-specific measurement approaches were also done. The results from experimental campaigns aimed to supplement and build on existing knowledge. These measurements and the initial results are described in public PAREMPI **D2.2** report.

Five intensive measurement campaigns were conducted in the PAREMPI project (***Figure 2*** and ***Table 1***). Two of the campaigns focused on exhaust from light-duty passenger cars and heavy-duty trucks, two were done in laboratory setups to mimic emission from marine engine exhaust and aircraft engine exhaust, and one campaign investigated brake dust emissions. The experimental campaigns aimed to gain valuable insights into fresh and aged particulate emissions as well as gaseous precursor emissions from the transportation sectors, addressing significant knowledge gaps in these areas. A detailed description of measurements can be found from the PAREMPI deliverable **D2.2.** and toxicity experiments were linked with robust systems for emission characterizations, described in deliverable report **D2.3** of the project.

Table 1 includes a summary of vehicles, technologies and fuels used in the PAREMPI exhaust measurement campaigns. Shortly, in the **LD campaign** to reflect the EU light-duty vehicle fleet, a diverse selection of cars was tested, including plug-in hybrids, non-hybrids, gasoline, diesel, Euro 6, and older models (Vehicles: PHEV-G-EU6d (V1), PHEV-D-EU6d (V2),G-EU6d (V3), D-EU6d (V4), G-EU6b (V5), D-EU4 (V6), CNG-G-EU6d (V7).) All vehicles had direct injection engines and relatively low mileage, representing popular global or local brands. Fuels used met EN228 (gasoline) and EN590 (diesel) standards, with winter-grade diesel used at –9 °C. A bi-fuel CNG vehicle primarily ran on Finnish pipeline CNG but used EN228 gasoline during start-up and heavy loads, as per manufacturer specifications. The RDEsim test cycle was developed to simulate real-driving emissions (RDE) under extended and repeatable conditions in a laboratory setting. Based on the Euro 7 proposal COM (2022) 586, the cycle was designed to be applicable across various ambient temperatures and vehicle types. A reference on-road RDE test, known as the "golden PEMS RDE test," was conducted first and this cycle was then replicated on a chassis dynamometer for all tested light-duty vehicles. Emission measurements were carried out at BOSMAL's Emission

Laboratory using a 4WD AVL Zoellner 48” dynamometer and a WEISS WK 643 climatic chamber, with tests performed at –9 °C, +23 °C, and +35 °C. The Aged PM results of the campaign have been described in the publication by Simon et al., (2025). Several other publications about the results are currently being prepared.

LD campaign-specific toxicological setup used in PAREMPI toxicological measurement: Exposures to car exhaust were performed next to the chassis dynamometer test cell at BOSMAL. The exposure chamber was connected via Tygon tubing and the dilution ration was: avg. DR (23°C) = 49; avg. DR (-9°C) = 58. Exposure time was 72 min per day (each sample was exposed twice, in total 144 min exposure/sample).

Table 1. Summary of vehicles, technologies and fuels used in the measurement campaigns.

Exp.	Code	Technology	Fuel Type
LD	PHEV-G (V1)	PHEV, Gasoline, Euro 6d	EN228 Gasoline*
LD	PHEV-D (V2)	PHEV, Diesel, Euro 6d	EN590 Diesel (summer/winter grade)
LD	EU6d-G (V3)	Gasoline, Euro 6d	EN228 Gasoline*
LD	EU6d-D (V4)	Diesel, Euro 6d	EN590 Diesel (summer/winter grade)*
LD	EU6b-G (V5)	Gasoline, Euro 6b	EN228 Gasoline*
LD	EU4-D (V6)	Diesel, Euro 4	EN590 Diesel (summer/winter grade)*
LD	EU6d-CNG (V7)	Bi-fuel CNG-Gasoline, Euro 6d	Finnish pipeline CNG (primary), EN228 Gasoline (secondary)
HD	EU VIE (Truck 1)	Euro VI E	EN590 Fossil Diesel (no FAME)*, EN15940 Renewable Diesel*
HD	EU VIA (Truck 2)	Euro VI A	EN590 Fossil Diesel (no FAME)*, EN15940 Renewable Diesel
Marine	DMB, DMB+S, DMB+ash, DMB+NH_3, EN590, HVO	Diesel Generator (no aftertreatment)	DMB*, DMB high-sulfur, DMB ash-forming component*, NH_3 injection in exhaust*, EN590 Diesel, HVO
Aviation	J1-J7	Cast combustion system and oil generator	Jet A-1 (typical market fuel)*, Jet A-1 (higher S and naphthalene, lower aromatics)*, two HEFA-SPK blends, F-35, FT-SPK (Fischer-Tropsch Synthetic Paraffinic Kerosene)*
Other		Permeation oven experiments	

** on-line toxicity measurements*

For the **HD campaign,** two trucks were selected for the winter-time heavy-duty truck emission measurement campaign of the project. The newer truck conformed to Euro VI E emission standard and had the odometer reading of 65465 km, and the older truck conformed to Euro VI A standard and had odometer reading of 1207725 km. Two types of fuels were utilized in the heavy-duty measurements: fossil diesel fuel (F), which complies with the EN590 standard, and renewable diesel (R), which meets the EN 15940 standard. Notably, the fossil diesel fuel did not contain any fatty acid methyl esters (FAME). The instrumentation was placed on the sea container, placed on the trailer behind the truck. Electricity and pressurized air were produced with instruments placed on the trailer. On-road experiments with two trucks covered various distances (80-500km) and routes in middle and northern Finland in winter conditions.

HD campaign-specific toxicological setup used in PAREMPI toxicological measurement: The ALI system was mounted inside a trailer towed by HD trucks, enabling mobile, real-driving exposure sampling in Tampere. The exposure chamber was connected via Tygon tubing and the dilution was

set for Truck 1 (DRavg=48) and for Truck 2 (DRavg=65). The exposure time was according to the route Tampere-Jämsä-Tampere (avg. 180 minutes for Truck 1 measurements and 240+ minutes for Truck 2 with defect).

In the experimental campaign focusing on **marine exhaust**, the exhaust was generated in the laboratory facilities to mimic the characteristics of marine engine exhaust. Simulation of different marine exhaust compositions were conducted by using a diesel generator from which exhaust aftertreatment system was removed prior to the measurements and marine fuels. Fuels included ISO 8217 grade DMB, DMB with doping agents or injection of chemicals to produce initial exhaust obtained with fuels with high sulfur, ash, or ammonia content, and other fuels covered EN590 and HVO. Ammonia was fed to the exhaust after the diesel generator, using in-house-developed system based on water-ammonia mixture and evaporation method to achieve three ammonia concentrations in the exhaust.

The CAST generator was used in **aviation emission studies** due to its low fuel consumption (a few ml/h) compared to full-sized aircraft engines and thus better possibilities to conduct experiments with various fuels and fuel mixtures. In the aviation fuel experiment, several different fuels were tested, representing commercial fossil jet fuels and sustainable aviation fuels (SAF) containing synthetic paraffinic kerosene (SPK) as a synthetic blending component (SBC). Toxicological testing was performed on selected aviation fuels representing both conventional and alternative compositions. Specifically, experiments included J1-F1 (representative fossil Jet A-1 fuel), J2-F2 (fossil Jet A-1 with modified composition), and J3-F1-SPK50 (a semi-synthetic blend containing a HEFA-SPK component). Both fresh exhaust and secondary aerosol (SecA) were evaluated for J1-F1 and J3-F1-SPK50, while J2-F2 was assessed under fresh exhaust conditions. This selection allowed comparison between conventional and alternative fuel compositions under realistic exposure scenarios.

Marine and Aviation campaign-specific toxicological setup used in PAREMPI toxicological measurement: Conducted at VTT using a diesel generator with marine fuel to simulate exhaust formation in a marine engine, and CAST to simulate exhaust formation of aircraft. The exposure chamber was connected to a PAM reactor via Tygon tubing. Exposures were performed on both complex 3D models and simpler BEAS-2B cells. The dilution ratios in marine simulation were DRavgMucilAir=108 and DRavgBEAS=101, in aviation simulation DRavgMucilAir=530 and DRavgBEAS=960. The 3D models were exposed with the PAM UV lights turned off (120 min), whereas in the BEAS-2B (90 min) experiments the PAM UV-lights were alternated on and off (fresh and aged). Ozone was removed from the sample prior to BEAS-2B exposures using an ambient-temperature catalytic stripper, because ozone is generated in the OFR and is harmful to the cells even at very low levels. The ambient temperature catalytic stripper had only modest effects on the particles and VOCs.

3.3 ALI Exposure System

Toxicological exposures were performed using a portable air–liquid interface (ALI) exposure system developed and validated for field-deployable applications. This system is described in robust measurement system report PAREMPI **D2.3 (*Figure 3*)**. The system consists of airtight exposure boxes (17 × 13 × 9 cm), each accommodating up to eight 6 mm membrane inserts arranged in a standard 24-well plate configuration. Within each box, the aerosol sample is symmetrically distributed across each cell insert, ensuring uniform exposure conditions for all cell cultures. The

sealed design enables controlled delivery of either diluted exhaust or filtered control air, while minimising contamination, humidy losses and temperature shocks during handling.

The exposure boxes are placed inside a compact incubator (40 × 35 × 45 cm inner dimensions) that provides stable physiological conditions during exposure. Both sample and control air are conditioned prior entering the exposure boxes. The aerosol flow is heated to 37 °C, humidified to relative humidity above 85 %, and supplemented with 5 % CO_2 to maintain optimal culture conditions. The entire system is self-contained and transportable, with power consumption and weight compatible with mobile deployment.

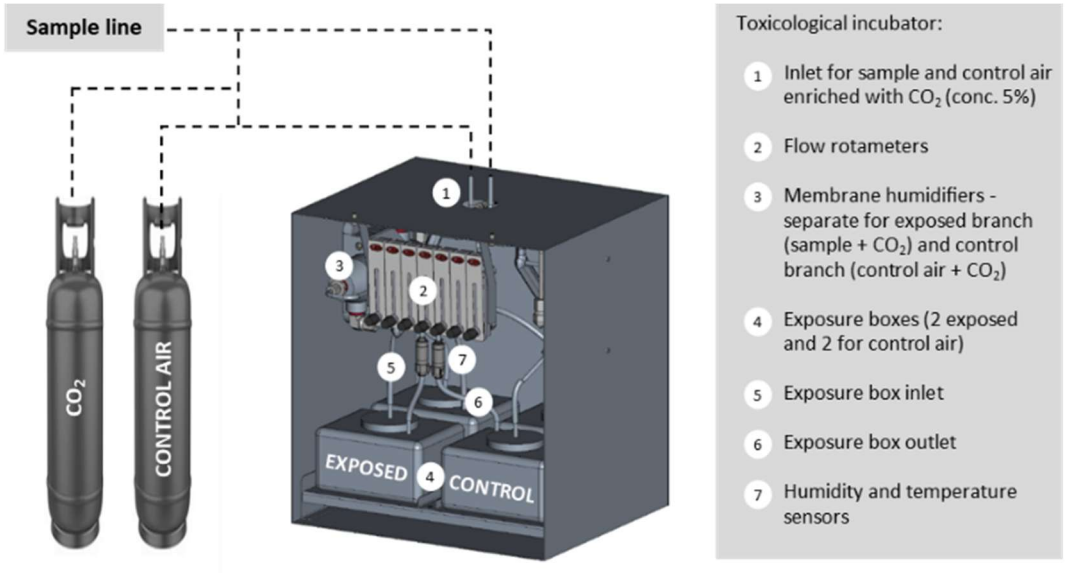

***Figure 3**. Toxicological incubator used during PAREMPI campaigns. Detailed information is presented in Vojtisek-Lom et al., (2025).*

Importantly, no artificial particle deposition enhancement techniques (such as electrostatic charging or thermophoresis) were applied, in order to preserve representativeness of combined gas and particle exposures. Particle losses in the conditioning system and deposition characteristics were experimentally characterized during system validation, confirming reproducible operation across campaigns. The system has been successfully deployed in laboratory environments as well as in fully mobile configurations, including installation in a trailer and direct operation on a moving heavy-duty vehicle, representing the first integration of an ALI exposure chamber within an on-board emissions monitoring context. This configuration enables direct toxicological assessment of diluted exhaust under real-world operating conditions, bridging conventional emission measurements with biologically relevant exposure.

3.4 Air–Liquid Interface (ALI) Models

The primary toxicological model applied within PAREMPI was a differentiated three-dimensional human airway epithelium (MucilAir™) maintained at the air–liquid interface (ALI) (***Figure 4***). This model consists of primary human bronchial epithelial cells cultured on permeable inserts and differentiated into a pseudostratified epithelium consisting of basal cells, ciliated cells and mucus-

producing goblet cells. Under ALI conditions, the tissue develops functional characteristics of the human airway, including tight junction formation, measurable barrier integrity, mucus production and coordinated ciliary activity. These features make MucilAir™ particularly suitable for assessing tissue-level responses to complex aerosol mixtures and for evaluating effects on epithelial structure and barrier function in human respiratory system.

MucilAir™ was selected as the model of choice because it provides a physiologically relevant representation of the human bronchial epithelium and allows evaluation of responses beyond single-cell stress reactions. The differentiated architecture enables assessment of coordinated inflammatory signaling, barrier disruption and morphological alterations, which are relevant endpoints in the context of inhalation exposure. Maintaining the tissue under ALI conditions ensures direct interaction of the apical surface with both particulate and gaseous components of diluted exhaust and aged aerosol, thereby preserving exposure realism.

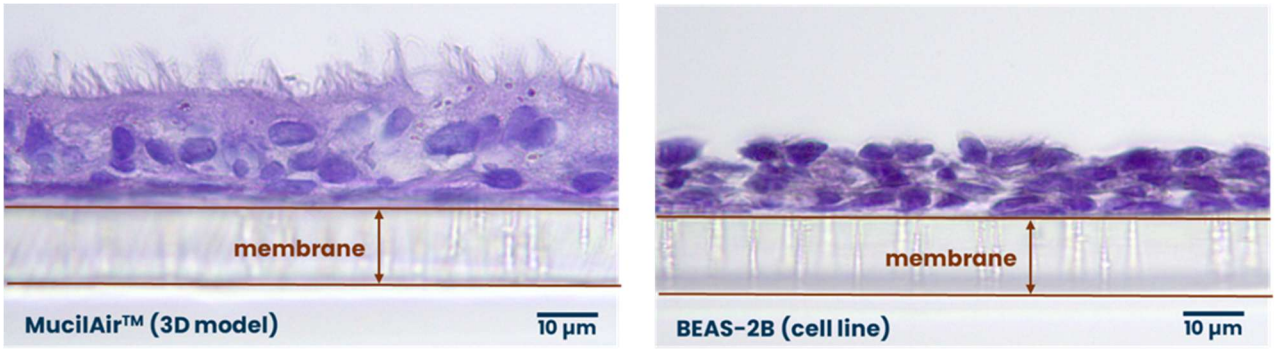

Figure 4. *Representative histological staining of MucilAir™ (left) and BEAS-2B (right) cells cultured on Transwell® inserts under air–liquid interface (ALI) conditions. BEAS-2B cells form a relatively thin epithelial layer on the porous membrane, whereas the fully differentiated MucilAir™ 3D airway epithelium consists of basal, ciliated and goblet cells arranged in a pseudostratified structure with functional barrier properties. The position of the membrane is indicated in both images. Scale bar: 10 µm.*

In selected campaigns, particularly in the marine and aviation experiments, the bronchial epithelial cell line BEAS-2B was included as a complementary model (**Figure 4**). BEAS-2B cells, derived from normal human bronchial epithelium and widely used in inhalation toxicology, are characterised by higher sensitivity to oxidative and genotoxic stress. Their inclusion was motivated by the specific exposure conditions associated with secondary aerosol (SecA) experiments, where dilution prior to and within the oxidation flow reactor (OFR), as well as particle losses during ageing, may reduce delivered particle mass to the exposure system. Under such conditions, a more responsive model can enhance detection of subtle biological effects. The BEAS-2B system therefore supported mechanistic interpretation and sensitivity assessment, while MucilAir™ remained the reference model for physiologically relevant evaluation.

The combined use of a differentiated primary airway tissue as the reference model and a more sensitive epithelial cell line under selected conditions ensured both physiological relevance and analytical sensitivity across campaigns. In both cases, the inserts were transferred in sterile conditions into the exposure boxes while maintaining sterility and stable physiological conditions (***Figure 5***). Culture medium was supplied only to the basal compartment during exposure, preserving the ALI setup with the apical surface directly exposed to the aerosol stream. This approach allowed consistent comparison of fresh and aged emission effects between transport sectors while accounting for differences in aerosol concentration and composition introduced by

dilution and ageing procedures. By prioritising a human-relevant 3D tissue model and complementing it with a responsive mechanistic system, when necessary, the experimental design balances translational relevance with robustness, thereby strengthening the interpretability of the toxicological findings within the broader PAREMPI framework.

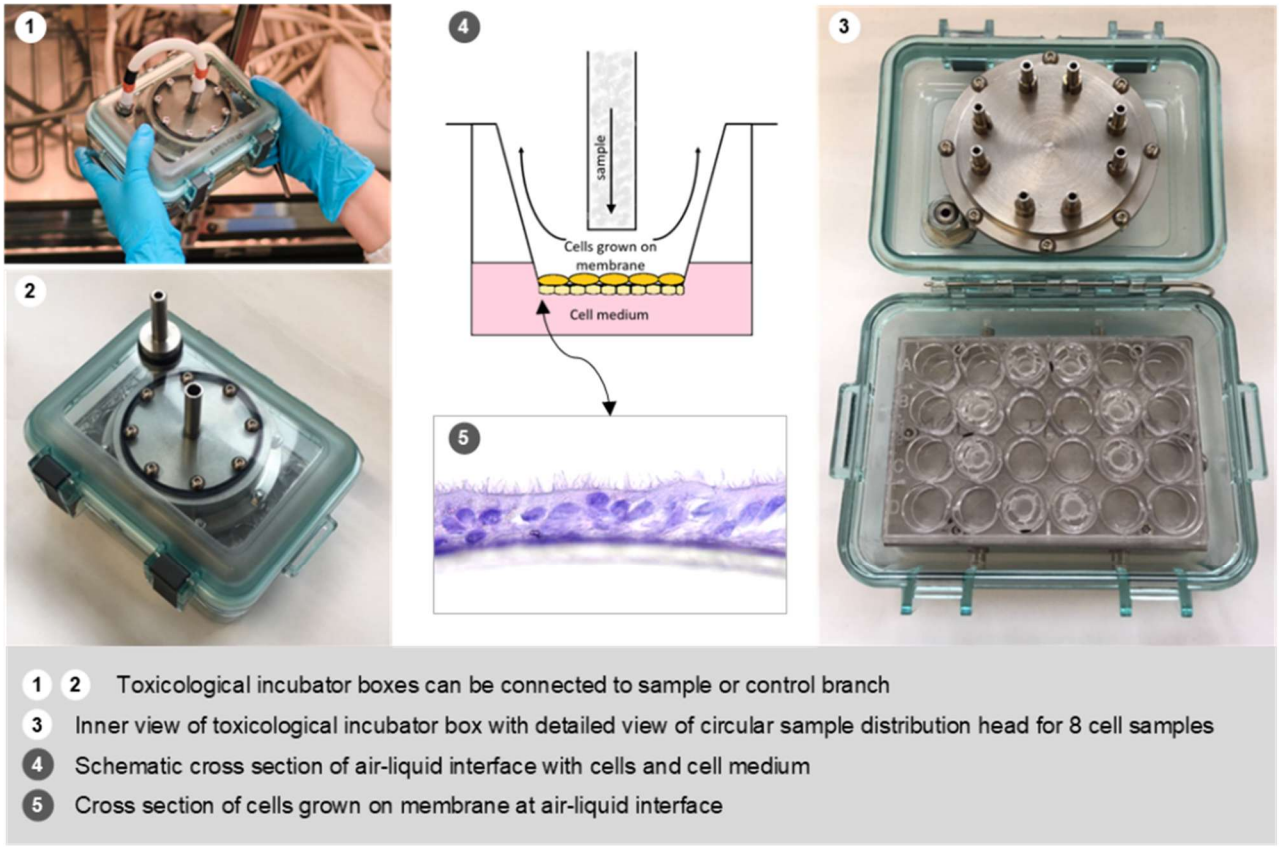

***Figure 5**. Air–liquid interface (ALI) exposure setup used for toxicological experiments. Toxicological incubator boxes can be connected to either the sample or control branch (1–2). The inner view of the exposure box shows a circular distribution head delivering the aerosol stream uniformly to multiple cell inserts (3). Cells are cultured on porous membranes with culture medium supplied from the basal side while the apical surface is exposed to the aerosol flow (4). Cross section of differentiated epithelial cells grown on a membrane under ALI conditions (5).*

3.5 Toxicity Endpoints and Assays

A panel of complementary endpoints was selected to assess structural integrity, cytotoxicity, oxidative stress, genotoxicity and transcriptomic changes following exposure to fresh exhaust (PM_{fresh}) and aged aerosol (PM_{aged}). The selected assays cover different levels of biological responses, from functional tissue integrity to molecular effects, enabling a comprehensive evaluation of potential cellular damage. Structural and cytotoxicity markers provide information on epithelial barrier integrity and cell viability, while oxidative stress and genotoxicity endpoints detect

early cellular damage associated with reactive species and DNA alterations. In addition, transcriptomic analyses allow identification of changes in gene expression that may not be detectable by cytotoxicity assays. Such molecular responses can reveal early biological effects and activation of specific cellular pathways, potentially indicating mechanisms that may contribute to longer-term health impacts. Together, these complementary approaches enabled evaluation of both acute cellular stress responses and more specific effects, providing a robust assessment of the biological effects induced by transport-related emissions.

3.5.1 Tissue barrier

Transepithelial electrical resistance (TEER) was used as a quantitative, non-destructive measure of epithelial barrier integrity. TEER measurements were performed using an EVOM2 ohm meter (World Precision Instruments, Sarasota, FL, USA) in combination with an STX2 electrode (Rossner et al. 2019). This method assesses tight junction dynamics by measuring electrical resistance across the epithelial layer, with higher resistance values indicating intact barrier function. Measurements were conducted under aseptic conditions prior to each exposure. Preheated culture medium was added to the apical compartment before measurement, and the electrode was equilibrated in sterile phosphate-buffered saline (PBS) and preheated medium prior to use. Triplicate measurements were obtained for each insert, and resistance values were corrected for blank membrane resistance and surface area according to standard calculations:

$$TEER\ (\Omega \cdot cm^2) = (R_{tissue} - R_{blank}) \times surface\ area$$

where the blank membrane resistance was 100 Ω and the epithelial surface area was 0.33 cm². TEER monitoring enabled early detection of potential barrier disruption following exposure.

3.5.2 Cytotoxicity Markers

Cytotoxicity was assessed by quantifying the release of intracellular enzymes into the culture medium following membrane damage. Lactate dehydrogenase (LDH) activity was measured using the Roche Cytotoxicity Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions (Rossner et al. 2019). LDH is a stable cytosolic enzyme released upon loss of membrane integrity (rupture or leakage), and its extracellular activity serves as a marker of acute cytotoxicity. In parallel, adenylate kinase (AK) release was determined using the Adenylate Kinase Cytotoxicity Assay Kit (Abcam, Cambridge, UK). AK release reflects membrane disruption and cell death, providing a sensitive complementary measure to LDH. Absorbance at 490 nm for LDH detection and luminescence for the AK assay were measured using a SpectraMax® M5e microplate reader (Molecular Devices, San Jose, CA, USA). Results were expressed as percentage cytotoxicity relative to the positive control (1% v/v Triton X-100 exposure for 1 h at 37 °C). The combined use of LDH and AK assays enhances robustness by confirming membrane damage through two independent detection principles.

3.5.3 Oxidative Stress Markers

Oxidative damage was evaluated by quantifying 15-F2t-isoprostane, a stable and well-established biomarker of lipid peroxidation (Hanzalova et al. 2010). Concentrations were measured using a commercially available ELISA kit (Cayman Chemicals Company, Ann Arbor, MI, USA) according to

the manufacturer's instructions. Absorbance was detected at 405 nm using a SpectraMax® iD3 reader (Molecular Devices, San Jose, CA, USA). Concentrations were calculated from a standard calibration curve with a detection range of 0.8–500 pg/mL. Measurement of isoprostanes provides a quantitative assessment of oxidative stress-mediated lipid damage induced by exposure in either exposed or control cells.

3.5.4 Genotoxicity Markers

DNA damage was assessed using a modified alkaline Comet assay capable of detecting both DNA strand breaks and DNA oxidative damage (Novotna et al. 2007). The assay was performed with and without treatment with the bacterial repair enzyme formamidopyrimidine DNA glycosylase (Fpg; New England Biolabs, Ipswich, MA, USA) to distinguish total DNA damage from oxidative DNA lesions. Slides were analysed using the Metafer MF-1273 automated image analysis system (MetaSystems, Heidelberg, Germany). DNA damage was expressed as the percentage of DNA in the comet tail (tail DNA%). Oxidative DNA damage was calculated as the difference between Fpg-treated and buffer-treated samples.

In selected experiments, phosphorylation of histone H2AX (γH2AX) was also evaluated as an additional marker of DNA double-strand breaks.

3.5.5 Toxicogenomics

Transcriptomic analysis was performed to evaluate global gene expression changes induced by exposure (Mussalo et al. 2024). Total RNA was isolated from exposed cells using the AllPrep DNA/RNA Micro Kit (Qiagen, USA) according to the manufacturer's instructions. RNA quality and quantity were assessed prior to library preparation. mRNA libraries were prepared using the NEBNext® Ultra™ II Directional RNA Library Prep Kit with Beads together with NEBNext Multiplex Oligos for Illumina (New England Biolabs, USA). Sequencing was carried out on an Illumina NovaSeq 6000 platform using the NovaSeq 6000 S1 Reagent Kit v1.5 (200 cycles) (Illumina, San Diego, USA).

Raw sequencing data (FASTQ files) were processed using the Galaxy web-based platform. Gene-level read counts were generated for each sample and analysed in RStudio using the DESeq2 package to identify differentially expressed genes (DEGs) between exposed and control samples. Subsequent interpretation focused on identifying biological pathways and cellular processes affected by exposure. Transcriptomic profiling therefore provides complex mechanistic insight into molecular responses that may not be detectable using conventional toxicity endpoints.

3.5.6 Microscopy

Fluorescence microscopy was used to visualise intracellular membranes and cell nuclei after exposure. Samples were fixed with frozen methanol at −20 °C for 10 min. Intracellular membranes were stained with 3,3-diethyloxacarbocyanine iodide (DiOC6(3); 1 µg/mL in PBS, pH 7.4; Invitrogen) for 30 min at room temperature. DiOC6(3) is a lipophilic fluorescent dye that accumulates in intracellular membranes, enabling visualisation of cellular morphology and membrane integrity. Cell nuclei were subsequently stained with propidium iodide (PI; 5 µg/mL in PBS, 5 min; Sigma-Aldrich), which intercalates into DNA and allows identification of nuclei and

assessment of nuclear morphology. After staining, samples were washed with PBS. Images were acquired using an Olympus IX70 fluorescence microscope equipped with an Olympus DP80 camera and a 20× objective. DiOC6(3) fluorescence was detected using a FITC filter set (excitation ~484 nm, emission ~501 nm), and PI fluorescence using a TRITC filter set (excitation ~535 nm, emission ~617 nm).

4. Toxicological Findings Summary

4.1 Light-duty campaign

In light-duty campaign on a chassis dynamometer in BOSMAL, Poland clear differences in toxicological results were seen between different transport sectors, fuels and driving conditions. In the LD campaign five cars ((Vehicles: PHEV-G-EU6d (V1), G-EU6d (V3), D-EU6d (V4), G-EU6b (V5), D-EU4 (V6)) were exposed to real driving emissions (RDE) conditions at temperatures of +23°C and -9°C, along with clean air controls and blanks, totaling ten distinct RDE scenarios. Cells underwent repeated RDE simulations (two exposures of 72 minutes each) per vehicle and were harvested 24 hours after the second exposure. Samples collected included 114 cell samples (15 sets, 8 samples/set), 342 medium samples (before exposure, 24h after exposure, 48h after exposure), and 224 apical medium samples (before exposure, 48h after exposure).

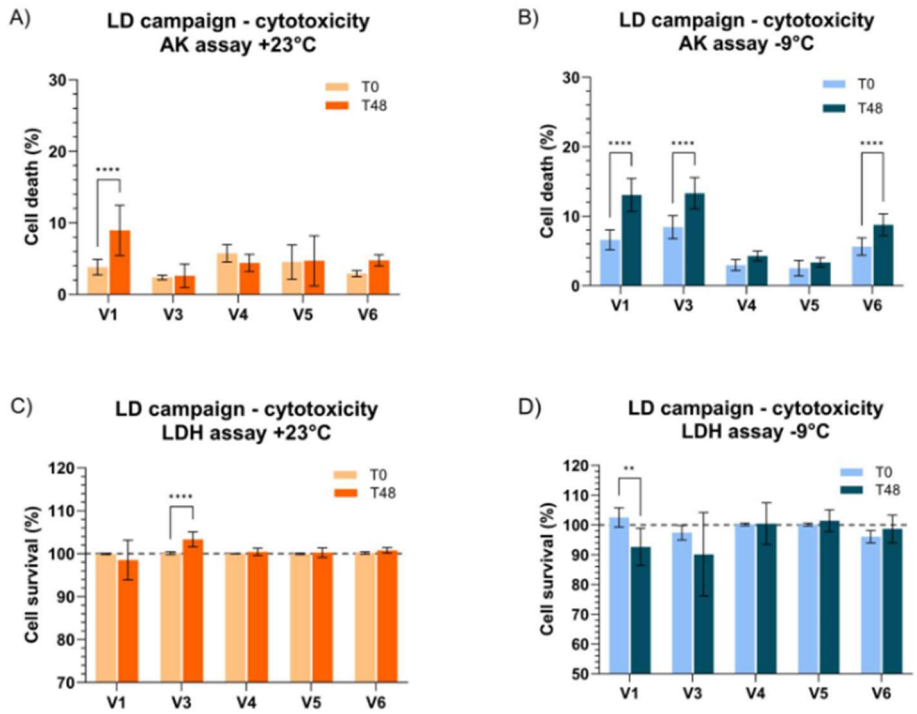

***Figure 6**. The cytotoxicity results from Adenylate Kinase assay (AK assay) are represented at 23 and -9°C at A) and B); Lactate dehydrogenase assay (LDH assay) at 23 and -9°are presented at C) and D).*

Cytotoxicity assays (LDH and AK) revealed variability in cellular responses to emissions from different vehicles (V1–V6), though overall cytotoxicity remained within acceptable biological limits. At +23°C, the AK assay indicated a significant increase in cell death for PHEV-G (V1) at T48 compared to T0, while the LDH assay showed significantly reduced cell survival in EU6d-G (V3) over the same period. Under cold exposure conditions (-9°C), the AK assay demonstrated elevated cell death in PHEV-G (V1), EU6d-G (V3), and EU4-D (V6) at T48. The LDH assay further confirmed a significant reduction in cell survival for PHEV-G (V1) at T48. These findings highlight temperature- and vehicle-dependent differences in cytotoxic profiles, with more pronounced effects evident under colder conditions, as shown in ***Figure 6***.

Transepithelial Electrical resistance (TEER) measurements indicated maintained tissue integrity, consistently above acceptable thresholds at both temperatures. At +23°C, significant TEER decreases occurred in EU6b-G (V5), EU4-D (V6), and controls at 48h after exposure versus before exposure. At -9°C, significant TEER increases were observed in EU4-D (V6), blank samples, PHEV-G (V1), EU6d-G (V3), and controls at 48h after exposure compared to before exposure, indicating preserved tissue integrity (***Figure 7***). Oxidative damage, measured by isoprostane levels, was generally lower in treatment groups compared to controls, especially significant in V6. DNA damage, assessed by Comet assay and histone H2AX phosphorylation, revealed substantial oxidative DNA damage at +23°C, notably in samples EU6d-G (V3) and EU6d-D (V4), while minimal damage occurred at -9°C. Although histone H2AX levels were elevated in some samples, differences were not statistically significant. Transcriptomic analysis revealed temperature as the primary driver of transcriptomic changes in exposed cells. Over 2,000 genes were differentially expressed between -9°C and +23°C exposures, highlighting a strong temperature-dependent response. Remarkably, 72 of these genes were shared between PHEV-G (V1 EURO6 plug-in hybrid gasoline) and EU4-D (V6 EURO4 diesel vehicle without DPF), despite their contrasting emission profiles. This indicates that cold conditions had a more pronounced impact on gene expression than vehicle type within the short exposure period used. While extended testing might uncover clearer technology-specific effects, our findings emphasize the importance of comparing responses across temperature conditions—even brief exposures revealed substantial differences linked to ambient temperature.

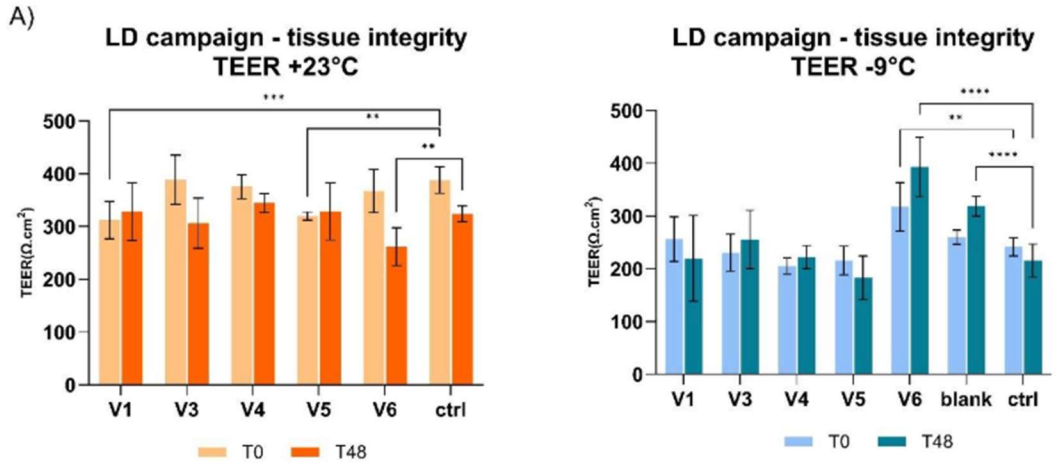

Figure 7*. The tissue integrity results from Transepithelial Electrical resistance (TEER) at +23°C (A) and -9°C (B)*

4.2 Heavy-duty campaign

In the heavy-duty (HD) campaign, the emission and toxicity measurements for two trucks using EN590 and HVO fuels were conducted in field under winter conditions in Finland. Cytotoxicity (AK assay, **Figure 8 left**) results showed significantly higher cell death for EN590 and defective conditions in Truck 1 at 24h after exposure compared to prior exposure, indicating cytotoxic impacts. Tissue integrity (TEER assay, **Figure 8 right**) generally remained intact across conditions, with significantly increased TEER values observed under EN590 cold-start scenarios. DNA damage assessment indicated significant oxidative DNA damage for Truck2 with defective EN590, whereas

histone H2AX phosphorylation was notably elevated during the HVO cold-start condition in Truck 1. Oxidative stress, measured via isoprostane levels, revealed no significant differences between treated and control samples, suggesting no lipid peroxidation in exposed cells throughout the HD campaign.

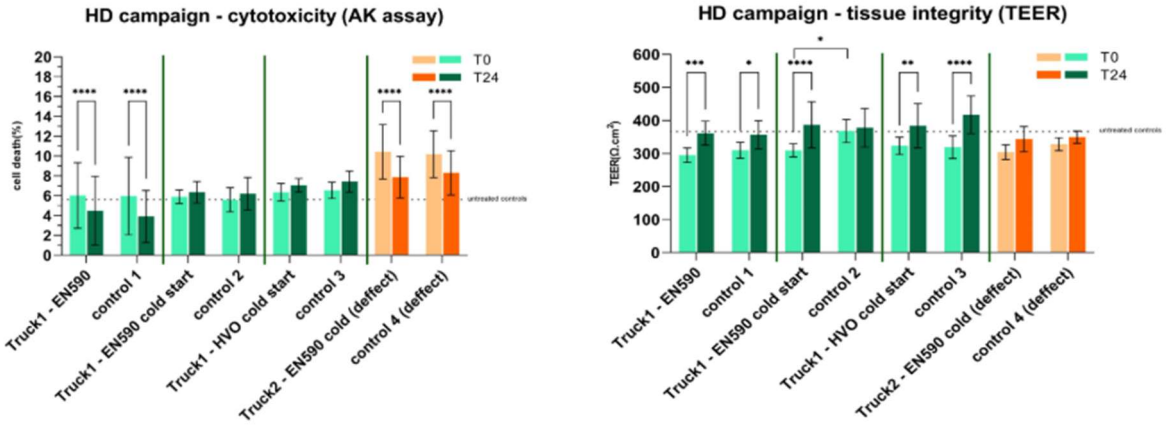

***Figure 8**. Left: The cytotoxicity results from Adenylate Kinase assay (AK assay) during HD campaign, Tampere, Finland. Right: The tissue integrity results from Transepithelial Electrical resistance (TEER) during HD campaign.*

Compared to LD, the HD campaign showed even lower transcriptomic responses. In Truck1, only 85 genes (Truck 1 EN590), respectively 59 genes (Truck 1 EN590 cold) were significantly affected by EN590 fuel emissions compared to only 5 genes using HVO fuel (Truck 1 HVO cold). Truck 2 (Truck 2 EN590 cold) showed only 2 affected genes. The differences between the first test of Truck 1 (85 genes) and second test (60 genes) with EN590 show a similar level of gene expression change, which is related to the same type of truck, but they differ in the initial conditions at startup. The effect is most pronounced with this type of fuel. Most of the genes are highly upregulated genes and mostly known as developmental regulators, mitochondrial RNAs, transcription factors, or pseudogenes, which are not typical markers of xenobiotic, inflammatory, or oxidative stress responses. The 5 genes affected by HVO fuel are not known to have any direct or indirect function in the synthesis, degradation, or microbial production. Only two genes were slightly upregulated in Truck 2 test which was compromised by truck defect. It can be expected that these effects at the level of gene expression changes are negligible.

4.3 Aviation and Marine campaign

In the aviation and marine campaign, simulated in laboratory at VTT, Finland, both simpler (BEAS-2B) and more complex (MucilAir™ 3D nasal tissue) *in vitro* models were used to evaluate the toxicological effects of emissions from combustion of various fuel. As part of a broader toxicological assessment—including genotoxicity, oxidative stress, and gene expression analyses—the LDH assay was used to assess cytotoxicity. BEAS-2B cells were included to enable testing of a wider range of samples, as their ease of handling allowed for higher throughput. This approach significantly expanded the dataset and involved extensive experimental effort.

4.3.1 Aviation campaign

BEAS-2B cells were exposed to either fresh jet fuel samples or secondary aerosol, J1-F1 (fresh sample, SecA sample, fresh sample with lube), J2-F2 (fresh sample) and J3-F1-SPK50 (fresh sample, SecA sample). Cytotoxicity remained very low (<5%) across most conditions, with the highest response (~4.5% cell death) observed for J1-F1 LUBE at 24 h (***Figure 9***). For comparison, untreated control cells showed a baseline cytotoxicity of approximately 2%, indicating only mild increases upon exposure. More complex model MucilAir™, tested with J1-F1 and J3-F1-SPK50, showed minimal cytotoxicity (<5%) at both time points. Overall, while slightly elevated responses were observed in the BEAS-2B model—particularly with lubricant-containing emissions—the 3D tissue model demonstrated minimal acute cytotoxic effects under the tested conditions.

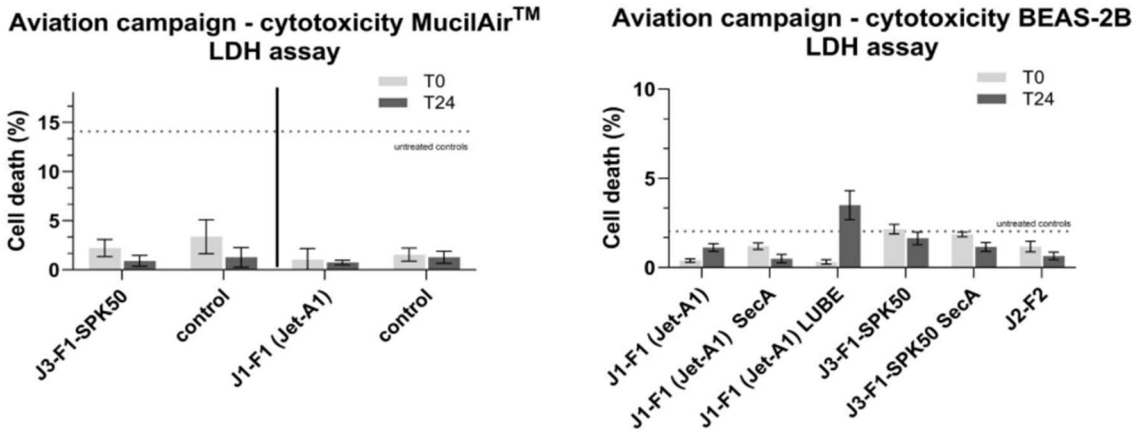

Figure 9. *The cytotoxicity results from Lactate dehydrogenase assay (LDH assay) during Aviation campaign. Left: 3D tissue model MucilAir™. Right: BEAS-2B cell line.*

Transcriptomic profiling (NGS RNA-seq) of the MucilAir™ 3D airway model following exposure to aviation emissions revealed distinct gene expression responses depending on the jet fuel type. In the comparison between J3-F1-SPK50 and control, we identified 6704 differentially expressed genes — including 5,379 upregulated and 1,325 downregulated. In contrast, exposure to J1-F1 (Jet-A1) resulted in a smaller transcriptional response and only 20 differentially expressed genes were identified (14 upregulated, 6 downregulated). Notably, four genes were commonly differentially expressed under both fuel conditions: CNPPD1 (a putative phosphodiesterase-like gene), FBLN5 (an extracellular matrix protein involved in elastic fibre formation), R3HCC1 (an RNA-binding regulator), and USH1C (a scaffold protein important for cell-cell junction integrity). Despite these changes, parallel toxicity assays (viability, oxidative stress and tissue integrity) showed minimal to no acute damage under our exposure conditions. It suggests low acute toxicological potential for both fuels at the tested doses with common cellular defence and maintenance mechanisms. J3-F1-SPK50 caused adaptive/early response pathways without translating into acute injury. This means that while the exposure was enough to activate biological pathways that typically respond to stress or xenobiotics, it was not strong or dangerous enough to result in detectable damage or toxicity right away. However, because transcriptomic shifts could still matter for longer or repeated exposures and for sensitive subpopulations (people with pre-existing illnesses or weaker immune systems), it would be important to carry out broader testing across different timepoints, concentrations, and cell models to gain deeper insight. Additionally, some

uncertainty remains regarding the chemical composition and stability of the exhaust composition during sampling, which could influence the interpretation of transcriptomic responses. Combustion of alternative fuel blends in the CAST burner may not fully reproduce the combustion characteristics of operational aircraft engines, potentially affecting the chemical composition of the generated exhaust. Variability in exhaust flow or chemical content—especially for complex emissions like aviation exhaust—may result in fluctuations in cellular exposure, potentially masking or exaggerating biological effects. Therefore, conclusions should be considering these limitations. Finally, it is important to emphasize that different fuel blends have distinct and often variable chemical profiles. These differences can make it impossible to predict health impacts based solely on fuel type or composition. As such, experimental toxicity testing remains essential for evaluating the safety of aviation fuels.

4.3.2 Marine campaign

BEAS-2B cells were exposed to various simulated marine exhaust components, including diesel marine blend (DMB), its secondary aerosol (DMB SecA), DMB combined with ammonia (DMB + NH_3), secondary aerosol formed from DMB and ammonia (DMB + NH_3 SecA), and DMB combined with ash. Organics dominated SecA for DMB and nitrates and ammonium dominated SecA for DMB + NH_3. Cytotoxicity remained low across all conditions (<5%) and both cell models, with the highest response (~4%) observed for DMB + NH_3 at 24 h (***Figure 10***). Untreated control cells showed a baseline cytotoxicity of approximately 2%, suggesting only mild increases upon exposure. In the more complex MucilAir™ model, DMB and DMB + NH_3 exposures resulted in slightly elevated cytotoxicity (up to ~6% at T0), but overall levels remained modest and well below the 15% toxicity threshold. Together, the results indicate low acute cytotoxicity across both models, with slightly higher sensitivity observed in response to combined DMB and ammonia exposure.

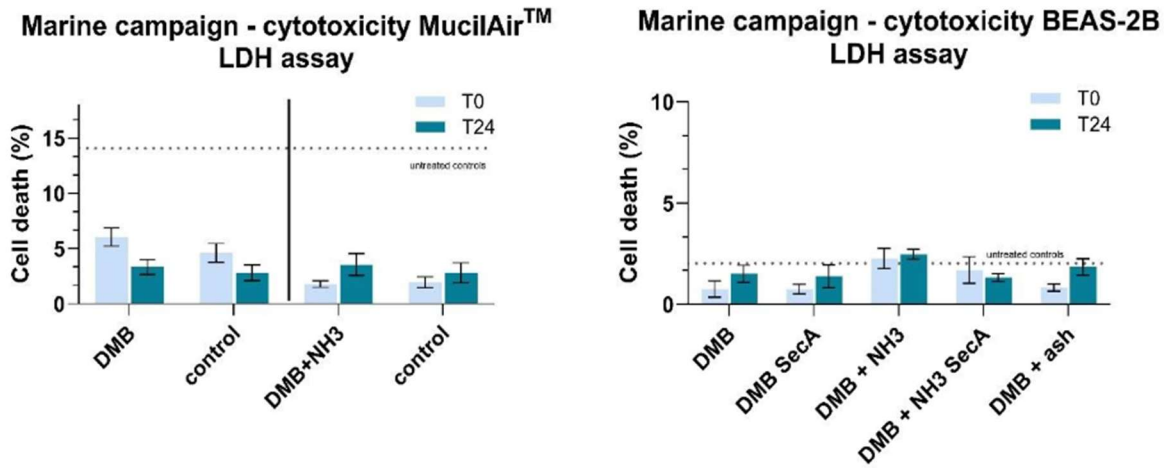

Figure 10. The cytotoxicity results from Lactate dehydrogenase assay (LDH assay) during Marine campaign. Left: 3D tissue model MucilAir™. Right: BEAS-2B cell line.

Transcriptomic profiling (NGS RNA-seq) of the MucilAir™ 3D airway model following exposure to simulated marine emissions showed distinct gene expression patterns depending on the emission composition (***Figure 11, left***). Exposure to DMB alone led to the expression of 17,334 genes, with 32 genes identified as differentially expressed—16 upregulated and 16 downregulated—indicating a moderate transcriptional response. In contrast, DMB combined with ammonia (DMB + NH_3)

triggered a substantially broader response, with 27,917 genes expressed and 488 differentially expressed, of which only 4 were upregulated and 484 downregulated. This profile points to a strong overall suppression of gene expression. Two genes—MT-RNR1 (mitochondrial 12S rRNA) and MT-RNR2 (mitochondrial 16S rRNA and Humanin precursor)—were commonly altered under both exposure conditions, suggesting mitochondrial involvement in cellular stress or toxicity pathways. These findings highlight both the scale and directional bias of the response as major differentiating features between DMB alone and DMB + NH_3 in MucilAir^{TM}.

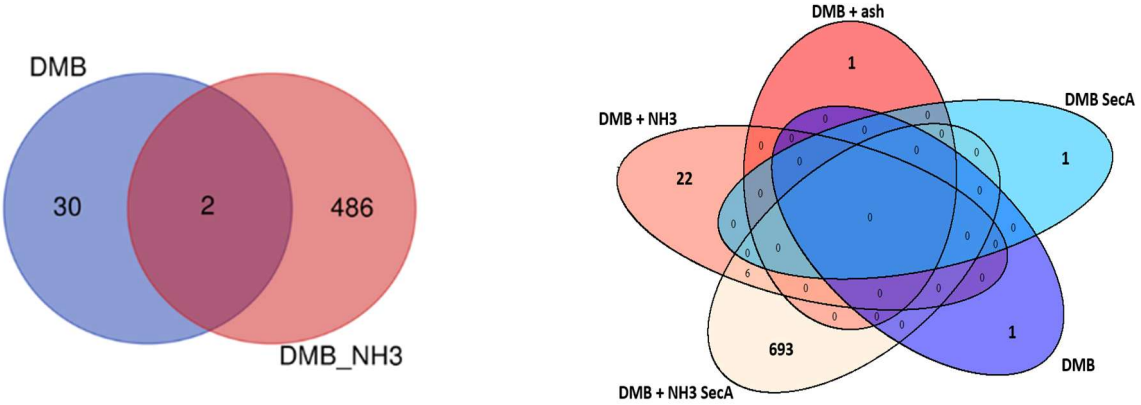

***Figure 11**. Venn diagram of shared DEGs for MucilAir^{TM}(left) and BEAS-2B (right) during Marine campaign, VTT, Finland.*

Exposure of BEAS-2B cells to emissions from marine engine fuel DMB generally induced only a weak transcriptional response, with minimal numbers of DEGs detected (***Figure 11, right***). Interestingly, the addition of ammonia (NH_3) to DMB fuel markedly amplified the biological impact, producing 28 DEGs in the primary aerosol and a much stronger response in the secondary aerosol (DMB + NH_3 SecA) exposure with 699 DEGs), indicating that the introduction of NH_3 into marine fuel combustion products drives a coordinated cellular stress and damage-response in BEAS-2B.

Fluorescence microscopy was used to assess cellular morphology and membrane integrity after exposure to particulate matter collected on filters from different exhaust conditions. In ***Figure 12,*** selected microscopic pictures of BEAS-2B cells are shown after exposure for 24 h to PM from DMB, DMB+NH3 and DMB+S. Control cells formed a dense and well-spread monolayer with typical elongated morphology and uniform intracellular membrane staining with DiOC6(3), while propidium iodide staining revealed nuclei with regular morphology. Following exposure, concentration-dependent morphological changes were observed across all tested conditions. At the highest applied filter fraction (1.25%), a pronounced reduction in cell density and marked morphological alterations were visible, including cell rounding, shrinkage and loss of the characteristic elongated shape. Similar effects were observed for particles collected under DMB, DMB + NH_3 and DMB + S conditions. At intermediate filter fractions (0.625–0.313%), partial recovery of cell density and morphology was observed, although an increased number of rounded or irregularly shaped cells remained visible compared with the control samples. At lower filter fractions (0.156–0.039%), cell morphology and confluency approached those of control cultures, with relatively uniform membrane staining and preserved nuclear morphology.

Notably, cells exposed to particles from the DMB + NH_3 condition showed more pronounced reductions in cell density compared with DMB or DMB + S at comparable filter fractions, with visible effects still present at 0.156% of the filter extract. In contrast, cells exposed to DMB + S PM showed relatively preserved morphology already at 0.156%. This observation is more pronounced as the filters used for extraction contained different particulate loads corresponding to the sampled raw

exhaust volumes (14.08 L for DMB, 12.44 L for DMB + NH_3 and 15.16 L for DMB + S). The stronger effects observed for DMB + NH_3 occurred despite the lower raw exhaust volumes represented by the applied fractions, suggesting that observed response reflect different PM composition.

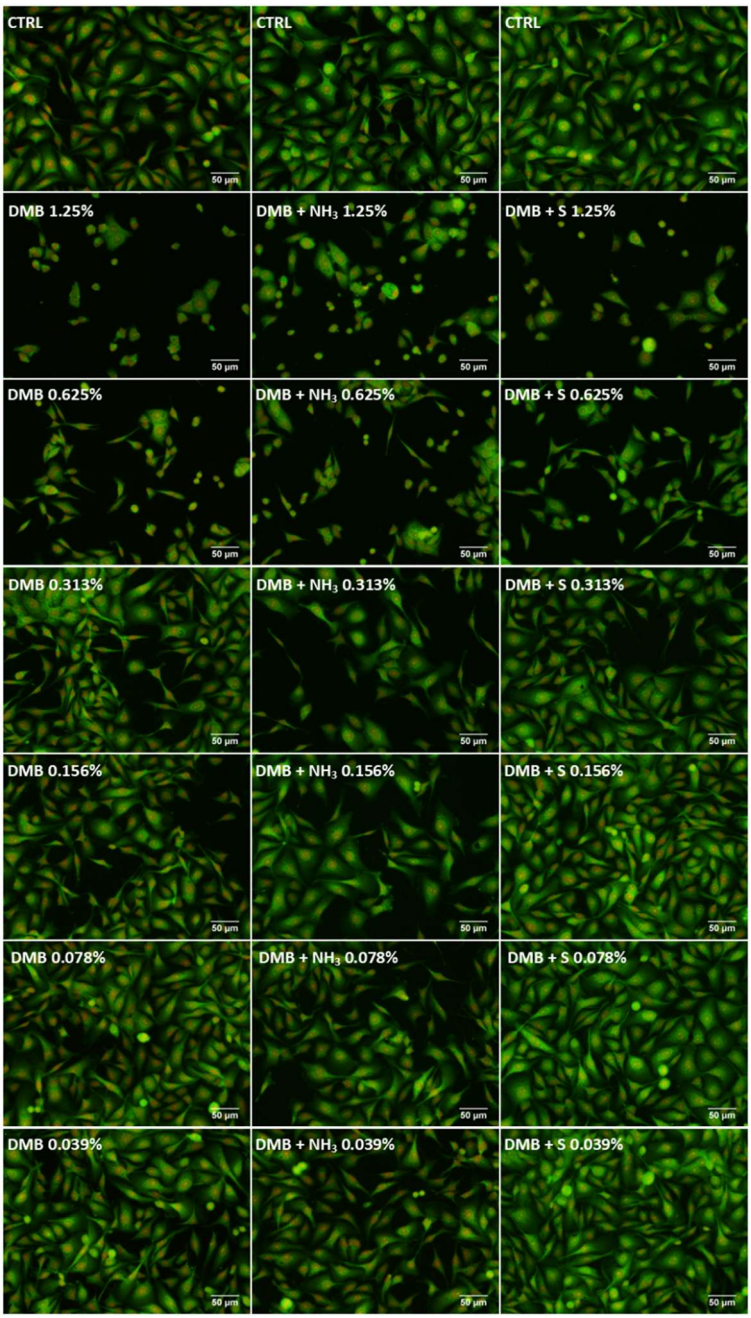

***Figure 12**. Representative microscopic pictures of BEAS-2B cells exposed to collected PM from raw exhaust from DMB, DMB+NH3 and DMB+S on Fluorophore filters during Marine campaign, VTT, Finland. PM was extracted from filters via sonication in methanol, followed by methanol evaporation and re-suspension in dimethyl sulfoxide. The applied filter fractions corresponded to the following raw exhaust-equivalent volumes: DMB (1.25% = 0.176 L; 0.625% = 0.088 L; 0.313% = 0.044 L; 0.156% = 0.022 L; 0.078% = 0.011 L; 0.039% = 0.0055 L), DMB + NH_3 (1.25% = 0.156 L; 0.625% = 0.078 L; 0.313% = 0.039 L; 0.156% = 0.019 L; 0.078% = 0.0097 L; 0.039% = 0.0049 L), and DMB + S (1.25% = 0.190 L; 0.625% = 0.095 L; 0.313% = 0.047 L; 0.156% = 0.024 L; 0.078% = 0.0118 L; 0.039% = 0.0059 L).*

5. Robustness and Limitations

5.1 Methodological Robustness

The toxicological assessment conducted within PAREMPI was integrated into a harmonised and validated measurement framework to ensure reproducibility across transport sectors and experimental campaigns. The robust measurement system for quantification of fresh and aged particulate matter, including precursor gases and OFR ageing, is described in detail in Deliverable **D2.3**. The integration of the ALI exposure system within this framework ensured that toxicological experiments were performed under controlled and well-characterised emission conditions.

Robustness was supported by several methodological features. First, exposures were conducted using standardised dilution procedures and defined ageing conditions across campaigns, enabling comparability between fresh exhaust and aged aerosol. Second, the portable ALI exposure system was validated for both stationary laboratory and mobile field applications, including deployment during real-driving heavy-duty measurements. Particle losses, aerosol conditioning parameters and environmental conditions within the exposure chamber were characterised during system validation, contributing to reproducible exposure scenarios.

Biological robustness was strengthened through the use of complementary *in vitro* models and multiple endpoints assessing barrier integrity, cytotoxicity, oxidative stress, genotoxicity and transcriptomic responses. The combined use of biochemical assays and gene expression profiling reduces the likelihood that conclusions are driven by a single methodological artefact. Furthermore, technical replicates and internal negative and positive controls were included in all campaigns to verify assay performance and sensitivity.

Despite these measures, biological systems inherently exhibit variability. Differences in donor-derived primary tissues, cell passage number, and environmental conditions during extended field campaigns may contribute to inter-experimental variation. However, the consistent exposure design and harmonised protocols across campaigns support the overall robustness of observed response patterns.

5.2 Influence of Environmental and Operational Factors

The toxicological outcomes observed in PAREMPI must be interpreted in the context of environmental and operational factors. Engine load, driving cycle, ambient temperature and fuel composition were shown to affect both primary emissions and secondary aerosol formation potential, as documented in Deliverables **D2.2** and **D2.3**. The integration of direct air–liquid interface (ALI) exposure into complex emission measurement campaigns was proved technically feasible and provided relevant data without compromising measurement integrity. However, dilution requirements and particle losses associated with oxidation flow reactor (OFR) ageing can substantially influence the particle dose delivered to the cells. The combined use of a differentiated 3D airway epithelium as the primary model together with a more responsive epithelial cell line under selected conditions proved important for detecting subtle responses, particularly under conditions

of higher dilution required for OFR operation and relatively short exposure durations, which may limit the effective delivered dose.

Environmental conditions also influenced observed responses. Temperature effects were evident in several campaigns, where cold-start or low ambient temperature conditions resulted in distinct emission profiles and, in some cases, altered cellular responses. In certain cases, these influences appeared to affect biological outcomes more strongly than differences attributable solely to vehicle technology or fuel type, highlighting the importance of testing under diverse operational scenarios.

The results further demonstrated the value of combining complementary endpoints. While acute cytotoxicity assays often indicated minimal effects, transcriptomic analyses revealed adaptive or stress-related responses not detectable through standard measurements alone. Importantly, such molecular changes do not necessarily indicate acute toxicity but may represent early adaptive processes requiring careful interpretation when linking mechanistic findings to health-relevant conclusions.

Engine operation under transient conditions inherently leads to variability in exhaust concentrations, reflecting real-world driving behavior. Consequently, exact repeatability of emission profiles between individual experiments may be limited. This was addressed through repeated measurements and multiple experimental runs, allowing robust interpretation.

Additional uncertainty applies to the aviation campaign, where emissions were generated using a CAST burner to simulate aircraft exhaust. Combustion in such experimental systems may not fully reproduce conditions in operational aircraft engines, particularly when testing alternative fuel blends with substantially different chemical compositions. This may affect the chemical profile and stability of the generated exhaust and contribute to variability between experimental runs. Consequently, repeatability of emission characteristics may be more challenging to achieve, and further validation of combustion conditions for emerging aviation fuels is required before drawing broader conclusions regarding their toxicological implications.

From an ethical perspective, the use of advanced human *in vitro* respiratory models aligns with the principles of the 3Rs (Replacement, Reduction and Refinement) by avoiding animal experimentation while providing physiologically relevant data. All procedures involving human-derived tissues were conducted in accordance with supplier guidelines and applicable ethical standards.

6. Conclusions

This deliverable presents a synthesis of toxicological findings obtained during the PAREMPI measurement campaigns, summarizing the biological effects of fresh exhaust and photochemically aged aerosol across light-duty, heavy-duty, marine and aviation sectors. By integrating air–liquid interface exposure systems within a harmonised emission measurement framework, the project provides a unique dataset linking physicochemical characterisation of emissions with biologically relevant cellular responses.

Across campaigns, acute cytotoxic responses were generally low under the tested exposure conditions, indicating that short-term exposures at environmentally relevant dilution levels did not induce overt epithelial damage. Nevertheless, measurable biological responses were detected at the molecular and transcriptomic levels, demonstrating that absence of acute cytotoxicity does not necessarily equate to absence of biological activity. Secondary aerosol formation altered emission composition and, under selected conditions, modified response profiles, highlighting the importance of evaluating both fresh and aged emissions in toxicological studies.

6.1 Recommendations for Future Research

The results obtained within PAREMPI highlight several areas requiring further investigation. Future studies should extend toxicological assessment beyond short-term exposures to include longer or repeated exposure scenarios. Such approaches would help determine whether the adaptive molecular responses observed in transcriptomic analyses translate into longer-term functional or health-relevant effects.

Improved characterization of the relationship between emission properties, delivered particle dose and biological response is also needed. Although the integrated measurement framework applied in PAREMPI enables simultaneous evaluation of emissions and toxicological responses, uncertainties related to particle losses during dilution and ageing processes may influence the effective exposure dose. Further refinement of exposure characterization, including better estimation of deposited particle mass under air–liquid interface conditions, would strengthen interpretation of toxicity results.

Additional research should also focus on the toxicological implications of secondary aerosol formation from transport emissions. As atmospheric ageing can substantially alter particle composition and properties, systematic evaluation of both fresh and aged emissions across different fuels, engine technologies and operating conditions remains important.

Finally, the increasing use of alternative and sustainable fuels highlights the need for continued experimental evaluation of their emission and toxicity profiles. Differences in chemical composition and combustion behavior may result in emission characteristics not fully captured by current testing frameworks, particularly in experimental combustion systems used to simulate aviation exhaust. Further validation and repeatability testing will therefore be important when assessing emerging fuel blends.

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