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NANOPARTICLE EMISSIONS FROM THE TRANSPORT SECTOR: HEALTH AND POLICY IMPACTS

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Contents

List of Figures

No table of figures entries found.

List of Tables

Glossary

ALI	Air-Liquid Interface
AUTH	Aristotle University of Thessaloniki
CERTH	Centre for Research and Technology
CNG	Compressed Natural Gas
CSIC	Consejo Superior de Investigaciones Científicas
IRFMN	Mario Negri Institute for Pharmacological Research
KI	Karolinska Institute
KTH	KTH Royal Institute of Technology
LDH	Lactate dehydrogenase
NP	Nanoparticle
NS	Non-significant
qRT-PCR	Quantitative reverse transcription - polymerase chain reaction
PHEV	Plug-in hybrid electric vehicle
SU	Stockholm University
WP	Work package

Executive Summary

We have analysed the toxicity of transport-related nanoparticles from 11 different outdoor locations and 17 different nanoparticles generated in laboratories representing emissions from road traffic, subway, shipping and airplanes. Three models were used to test the toxicity; air liquid interface, submerged, and zebrafish embryo models, and several endpoints have been studied, e.g. cell viability and cytotoxicity, inflammatory response, DNA damage, reactive oxygen species induction, and gene expression changes. The results from all exposure experiments will be used by WP6 to develop toxicity scores to be able to rank the toxicity of nanoparticles from the different transportation modes.

1. Introduction

The main objective of WP5 is to compare the toxic effects of nanoparticle emissions from various transport modes, focusing on markers of relevance for oxidative stress, inflammation, and genotoxicity. In order to do so, particles from different transport modes were used for toxicity testing either by direct cell exposure using an ALI system or sampled on filters for submerged cell exposure. Furthermore, zebrafishes were exposed to the organic part of the particles. In parallel, extra filters were sampled for the chemical characterization of the emissions. Even though the nPETS project focuses on nanoparticles, some of the experiments have used larger particles due to technical reasons.

So far, experiments in different labs (chassis dyno, tribology and aerosol) and outdoor environments (background, harbour, road tunnel, subway, airport) have been successfully performed. Several partners participated in WP5: KI, SU, KTH, AUTH, CERTH, IRFMN, Brembo, and CSIC. The results of WP5 have been delivered to WP6 in order to establish toxicity scores.

This report summarizes the main results of WP5 regarding the toxicity of nanoparticles from various transport modes using three different models: air-liquid interface (ALI), submerged and zebrafish embryo model.

2. Methods

Toxicity testing and cell culturing were performed according to the nPETS protocol or with minor modifications with three models: ALI, submerged, and Zebrafish embryos. For the ALI experiments we used the alveolar cell line A549, and for submerged we used either A549 or human monocyte cell line THP-1 differentiated to macrophages (referred to as THP-1 from now on).

- **ALI exposure.** Air-Liquid Interface (ALI) exposure enables direct deposition of un-changed airborne particles onto lung cell cultures to mimic human inhalation of airborne particles. Cells were placed in the exposure chambers of the ALI system. Two inserts were exposed to gaseous emissions without particles (gases), while the others were exposed to the total aerosol (gases and particles). To estimate the exposure dose in ALI, a particle counter or an SMPS system was attached prior to and after the ALI system, enabling on-line characterization of the particle concentration. Cells were exposed to aerosol for 2 h, and then incubated for 24 h prior to toxicity evaluation.
- **Submerged exposure.** Particles originating from different transportation modes were collected on filters. Thereafter the particles were removed by sonication in MilliQ water, and the mass of particles was determined by comparing the weight of the filter before and after particle removal. The particle suspensions were then diluted in cell medium and cells were exposed to different particle concentrations up to 100-200 µg/mL (approx. 30-60 µg/cm^2). Cells were, in general, exposed for 24 h. For all samples from Barcelona the organic fraction was used (extracted with dichloromethane:methanol 1:1).
- **Zebrafish embryos.** Exposure experiments followed the OECD Test Guideline 236 for zebrafish embryos. The exposure solution consisted of a ratio of 1µl extract:1mL fish water (extracted with dichloromethane:methanol 1:1). The exposure was maintained from 4 days until 5 days post-fertilization. At 5 days post-fertilization, before collecting the samples, the morphology of the larvae was observed under a microscope.

Standard protocols of Alamar blue/resazurin and MTT were used to assess cell viability (based on metabolic activity of the cells), and lactate dehydrogenase (LDH) was used for cytotoxicity testing (based on cell membrane leakage). The release of pro-inflammatory cytokines was assessed by ELISA or Multiplexing. DNA damage was assessed using comet assay. Gene expression of oxidative stress, inflammation, and metabolism of xenobiotics was analysed by qRT-PCR.

ALI is a relevant method to test the toxicity of airborne particles since it resembles the inhalation of particles into human lungs. In nPETS, we developed an ALI system for outdoor usage, including particle size selection of nanoparticles. Earlier, nearly only in-laboratory studies have been performed with ALI system, and only with the whole aerosol i.e. with all generated particle sizes (without particle size selection). Therefore, our ALI system offered a novel but high-risk approach that had not been tested before.

To perform the size selection of particles in the ALI system we initially used a PM2.5 cyclone and a nano-impactor (<450 nm) in series. At our first campaigns, we got an under pressure in the ALI system. The impactor has very small holes, and we figured that this design created the under pressure. An under pressure made it impossible to use our particle concentration measuring instruments, and even worse, the cells in our exposure system died. That forced us to remove the nano-impactor and instead test the toxicity of fine particles.

Further development of the system eventually solved the problem with the under pressure. We realized that the PM2.5 cyclone was not enough for removal of large particles (particles larger than

2.5 µm) prior to the nano-impactor. Particles passing the cyclone (< 2.5 µm) blocked the nano-impactor and created the under pressure. Upon adding a second cyclone; a PM1 cyclone, we were able to remove particles larger than 1 µm prior to the nano-impactor and succeeded in preventing the blocking of the nano-impactor. Then, less under pressure was created in the system, and the ALI system could be used for toxicity testing of the nanoparticle fraction.

Submerged and zebrafish experiments have been used for toxicity testing of all collected nanoparticles in each city, thus ensuring that we have results for nanoparticles from all transportation modes within the nPETS project. Some submerged experiments were also made with PM2.5 for comparison with ALI exposures to fine particles.

2.1 Toxicology studies

The following tables summarize the experiments involving the ALI, submerged and zebrafish experiments in laboratory conditions **(Table 1)** and outdoor environments **(Table 2)**.

In the tribometer laboratory (KTH), wear particles of 4 car brakes (FM1-FM4) and 2 rail brakes (C6, C20) were generated. FM1 and FM2 are low metallic and Cu-free brake materials. FM3 is a low metallic brake material and Cu-enriched. FM4 is non-asbestos organic (NAO). In the disk brake dynamometer (Brembo), wear particles from the stage 2 in the DGI (PM0.8-0.4) were sampled for submerged exposures. M1a is a low metallic and Cu-free brake material. M2b is identical with FM3. M3 is a NAO brake material. In the chassis dyno laboratory (AUTH), 4 different cars (petrol, hybrid-petrol-Plug-in hybrid electric vehicle (PHEV), compressed natural gas (CNG) and hybrid-petrol) generated exhausts. A combination of two different driving cycles (a mild and a dynamic one), two different start temperatures of the engine (hot/cold start of engine) and fresh/aged emissions were examined. In the aerosol lab (SU) nanoparticles from rail materials were generated using spark discharge.

Outdoor environments covered in this study include subway, traffic sites/road tunnel, airport, harbour and background sites. Toxicity of exhaust from ships and aeroplanes, as well as emissions (including non-exhaust emissions from e.g. brake wear) from vehicles and subway trains were also tested. For traffic exhaust, experiments from Barcelona and Milan were performed in an urban traffic area while in Stockholm, in a road tunnel. ALI experiments carried out in Stockholm (urban traffic and subway) used a particle concentrator.

Table 1. Summary of the laboratories generating particles used for toxicity testing

No.	Particle source	Transport mode - emission	Particle *generation*	Toxicity tested		
				ALI	*Submerged*	*Zebra fish*
1	Car brake (FM1)	Car - brake wear	Pin-on-disc - KTH	X	X	
2	Car brake (FM2)			X	X	
3	Car brake (FM3)			X	X	
4	Car brake (FM4)			X	X	
5	Rail brake (C6)	Train - brake wear		X	X	
6	Rail brake (C20)			X	X	
7	M1a	Brakes wear	Disc brake dynamometer - Brembo		X	
8	M2b (=FM3)				X	
9	M3				X	
10	Car 1 - Petrol	Car – Petrol exhaust	Chassis-dyno - AUTH	X	X	
11	Car 2 - Hybrid (petrol PHEV)	Car – Petrol exhaust		X	X	
12	Car 3 - CNG	Car – CNG exhaust		X		
13	Car 4 – Hybrid (petrol)	Car – Petrol exhaust		X		
14	Rail	Subway - rail aerosol	Spark discharge - SU		X	
15	Third rail	Subway - third rail aerosol			X	
16	Rail wheel	Subway - rail wheel aerosol			X	
17	Iron reference	Reference subway related aerosol			X	

Table 2. Summary of the outdoor environments used for toxicity testing

No.	Particle source	Transport mode - emission	*Partner responsible for filter sampling*	Toxicity tested		
				ALI	*Submerged*	*Zebrafish*
18	Background - Barcelona	Urban background	CSIC	X	X	X
19	Background Milan		IRFMN		X	
20	Background Stockholm		KTH			X
21	Harbour Barcelona	Ship exhaust	CSIC	X	X	X
22	Airport - Barcelona	Aircraft exhaust	CSIC		X	X

23	Airport – Thessaloniki		AUTH	X		
24	Airport - Milan		IRFMN		X	
25	Urban traffic - Milan	Traffic exhaust	IRFMN		X	
26	Urban traffic - Barcelona	Traffic exhaust	CSIC		X	X
27	Urban traffic - Stockholm	Traffic exhaust	KTH	X	X	X
28	Subway - Stockholm	Rail traffic emissions	KTH	X	X	X

3. Results

Tables 3-5 summarize the main results of the toxicity evaluation using ALI **(Table 3)**, submerged **(Table 4)** and Zebrafish embryos (**Table 5**) performed by the different partners of the nPETS project. All the results were compared to a negative control (incubator control for the ALI experiments). In the chassis dyno laboratory, 4 different cars (petrol, hybrid-petrol-PHEV, CNG and hybrid-petrol) generated exhausts. A combination of two different driving cycles (mRDE (mild) and combined), and hot/cold start of the engine were used for all cars. Experiments with car 4 were also performed with fresh and aged particles.

Table 3. Summary of the main toxicity results of the ALI exposures in laboratories

No.	Particle source – Size		Exposure dose (µg/cm²)	Description of the main results
1	Car brake (FM1) – PM2.5		3.15	▪ No change in the cell viability (Alamar blue) ▪ No change in cytokine levels
2	Car brake (FM2) – PM2.5		5.25	
3	Car brake (FM3) – PM2.5		4.27	
4	Car brake (FM4) – PM2.5		4.69	
5	Rail brake (C6) – PM2.5		6.97	▪ No change in cell viability (Alamar blue) ▪ No change in cytokine levels
6	Rail brake (C20) – PM2.5		6.97	
10	Car 1 – petrol – NP	mRDE hot	N.A.	▪ Decrease of cell viability (Alamar blue) and increase of cytotoxicity (LDH release), especially for a cold start ▪ No change in toxicity between the driving cycles (mRDE or combined) ▪ Increase of IL-1β and TNF-α especially for cold start
		mRDE cold		
		combined hot		
		combined cold		
11	Car 2 - Hybrid (petrol PHEV) – NP	mRDE hot	N.A.	▪ Decrease of cell viability (Alamar blue) and an increase of cytotoxicity (LDH release), especially for combined driving cycle ▪ No difference between toxicity of emissions from cold and hot start ▪ Increase of IL-1β and TNF-α
		mRDE cold		
		combined hot		
		combined cold		
12	Car 3 – CNG – NP	mRDE hot	N.A.	▪ Decrease of cell viability (Alamar blue) and increase of the cytotoxicity (LDH release), especially for combined driving cycle and cold start ▪ Increase of IL-1β and TNF-α
		mRDE cold		
		combined hot		
		combined cold		
13		mRDE hot – fresh	N.A.	▪ Decrease of cell viability (Alamar blue) and increase of cytotoxicity (LDH

	Car 4 - Hybrid (petrol) – NP	mRDE hot – aged		release), especially for cold start and aged emissions ▪ Increase of IL-1β and TNF-α ▪ Aged emissions from cold engine start induced more IL-1β than hot start
		mRDE cold – fresh		
		mRDE cold – aged		
		combined hot – fresh		
		combined hot – aged		
		combined cold – fresh		
		combined cold – aged		

Table 4. Summary of the main toxicity results of the ALI exposures in outdoor environments

No.	Particle source – Particle size – season - city	Exposure dose (μg/cm²)	Cell model / Description of the main results
18	Background – PM2.5 and NP – Winter - Barcelona	PM2.5: 0.04 ±0.016 UPF: 0.04 ± 0.014	▪ No change in cell viability (Alamar Blue) (PM2.5 and NP) No changes in marker's expressions (TIPARP, CYP1A1 1, HMOX, IL-8, SOD2, TNF-α, ATM)
21	Harbour – PM2.5 and NP – Winter - Barcelona	PM2.5: 0.09 ± 0.025 UPF: 0.05 ±0.017	▪ No change in cell cytotoxicity (LDH assay) ▪ Increase of CYP1A1 1 and ATM
23	Airport – NP – Summer - Thessaloniki	N.A.	▪ Decrease of cell viability (Alamar Blue) and increase in cytotoxicity (LDH) ▪ Increase of IL-1β and TNF-α
27	Road tunnel – PM2.5 – winter - Stockholm	1.45 ± 0.82	▪ No change in cell viability (Alamar blue) ▪ Slight increase (NS) especially in IL-1β, and IL-8 but not in IL-6
28	Subway – PM2.5 – winter - Stockholm	N.A.	▪ No change in cell viability (Alamar blue) ▪ Decrease of IL-8, TNF-α, and IL-1β
	Subway – PM2.5 and NP – summer - Stockholm	N.A.	▪ No change in cell viability (Alamar blue) (A549 and co-culture)

Table 5. Summary of the main toxicity results of submerged exposures with laboratory-generated particles

No.	Particle source – Particle size	Exposure dose (µg/mL)	Description of the main results
1	Car brake (FM1) - PM2.5	10-200	▪ No change in cell viability (A549 and THP-1 - Alamar blue) ▪ No clear change in IL-8, IL-1β, TNF-α but a slight increase of IL-6 for FM1 (THP-1)
2	Car brake (FM2) - PM2.5	10-200	
3	Car brake (FM3) - PM2.5	10-200	
4	Car brake (FM4) - PM2.5	10-200	
5	Rail brake (C6) - PM2.5	10-200	▪ No change in cell viability (A549 and THP-1 - Alamar blue) ▪ No clear change in IL-8, IL-1β, TNF-α but a slight increase in IL-6 for C6 (tested in THP-1)
6	Rail brake (C20) - PM2.5	10-200	
7	M1a – PM0.8-0.4	10-75	▪ No change in cell viability (A549 - Alamar blue) ▪ Dose-dependent decrease in cell viability (THP-1 – Alamar Blue)
8	M2b – PM0.8-0.4	10-75	▪ No change in cell viability (A549 and THP-1 - Alamar blue)
9	M3 – PM0.8-0.4	10-75	▪ No change in cell viability (A549 and THP-1 - Alamar blue)
10	Car 1 mRDE hot	0.5-5	▪ Significant decrease in cell viability (A549 - MTT, Alamar Blue) ▪ No induction of programmed cell death (Caspase 3/7) ▪ Strong induction of oxidative stress (A549 - (ROS Glo)
	Car 1 mRDE cold	1.25-5	▪ No change in cell viability (A549 – MTT, Alamar blue)
	Car 1 combined hot		
	Car 1 combined cold		
11	Car 2 mRDE hot	1.25-5	▪ No change in cell viability (A549 – MTT, Alamar blue)
14	Rail – NP	10-200	▪ Decrease of cell viability, significant for A549 but not THP-1 ▪ Increase of DNA strand breaks at 100 µg/mL (A549 and THP-1) ▪ Increase of IL-8 gene expression in THP-1 but not A549 and no change in GADD45a, HMOX1 , IL-1ß , IL-6 , and TNF-α. ▪ No change in cytokine release (THP-1, A549)
15	Third rail – NP	10-200	▪ Slight decrease of cell viability (significant for A549 but not THP-1) ▪ Increase of DNA strand breaks at 100 µg/mL in THP-1 ▪ Increase IL-8 and HMOX1 gene expression in THP-1 but not A549 and no change in GADD45a, IL-1ß, IL-6 , and TNF-α.

			▪ No change in cytokine release (IL-1β, IL-6, IL-8, TNF-α) in either THP-1 or A549
16	Rail wheel - NP	10-200	▪ Slight decrease in cell viability (significant for A549 but not THP-1) ▪ Increase of DNA strand breaks at 100 µg/mL in both A549 and THP-1 ▪ No change in gene expression of GADD45a, HMOX1, IL-1ß, IL-8, IL-6 and TNF-α in either THP-1 and A549 ▪ Increase release of IL-1β, IL-6 and TNF-α but not IL-8 in THP-1 but no change in A549
17	Iron reference - NP	10-200	▪ Slight decrease in cell viability (significant for A549 but not THP-1) ▪ Increase of DNA strand breaks (at 100 µg/mL in THP-1) ▪ Increase of IL-8 gene expression (in THP-1 but not A549) ▪ No change in cytokine release (IL-1β, IL-6, IL-8, TNF-α) in either THP-1 or A549

Table 6. Summary of the main toxicity results of submerged exposures with particles (or organic fraction) from outdoor environments

No.	Transport mode – Particle size – season – City	Exposure dose (µg/mL)	Cell model / Description of the main results
18	Background- organic fraction of NP -winter - Barcelona	20-60	▪ No change in cell viability (A549 and THP-1) ▪ No increase of ROS (A549 and THP-1) ▪ Increase of IL-8 (×1.2) and TNF-α (×2.3) in THP-1
	Background- organic fraction of NP – Summer - Barcelona	60-100	▪ No change in cell viability (A549 and THP-1) ▪ No increase of ROS (A549 and THP-1) ▪ Increase of TNF-α (×2) in THP-1
19	Background – NP – Summer - Milan	10-75	▪ No change in cell viability (A549, THP-1) ▪ Dose-dependent cytokines release (IL-1β, IL-6, IL-8, TNF-α) (THP-1) when compared to non-exposed but not when compared to blank filter exposed cells
	Background – NP – Winter - Milan	10-75	▪ No change in cell viability (A549, THP-1)
21	Harbour- organic fraction of NP - Winter - Barcelona	100-160	▪ No change in cell viability (A549 and THP-1) ▪ Increase of ROS (×1.8 in A549, ×1.4 in THP-1) ▪ Increase of IL-8 (×1.4) and TNF-α (×5.8) (THP-1)
	Harbour- organic fraction of NP -Summer - Barcelona	30-110	▪ No change in cell viability (A549 and THP-1) ▪ Increase of ROS (×1.2 in A549, ×1.2 in THP-1) ▪ Increase of IL-8 (×1.2) and TNF-α (×2.6) (THP-1)
22	Airport – organic fraction of NP – Winter - Barcelona	30-85	▪ No change in cell viability (A549 and THP-1) ▪ Increase of ROS (×1.2 in A549) (not in THP-1) ▪ Increase of IL-8 (×1.3) and TNF-α (×7) (THP-1)

	Airport – organic fraction of NP – Summer - Barcelona	40-65	▪ No change in cell viability (A549 and THP-1) ▪ Increase of ROS (×1.5 in A549) (not in THP-1) ▪ Increase of TNF-α (×2.2 in THP-1), no change of IL-8 (THP-1)
24	Civil airport – NP – Summer - Milan	10-75	▪ No change in cell viability (A549, THP-1) ▪ Dose-dependent cytokines release (IL-1β, IL-6, IL-8, TNF-α) (THP-1) when compared to non-exposed but not when compared to blank filter exposed cells
	Civil airport – NP – Winter - Milan	10-75	▪ No change in cell viability (A549, THP-1)
25	Urban traffic – NP – Summer - Milan	10-75	▪ No change in cell viability (A549, THP-1) ▪ Dose-dependent cytokines release (IL-1β, IL-6, IL-8, TNF-α) (THP-1) when compared to non-exposed but not when compared to blank filter exposed cells
	Urban traffic – NP – Winter - Milan	10-75	▪ No change in cell viability (A549) ▪ Dose dependent decrease of cell viability (THP-1)
26	Traffic site – organic fraction of NP – Winter - Barcelona	50-85	▪ No change in cell viability (A549 and THP-1) ▪ Increase of ROS (×1.6 in A549, ×1.7 in THP-1) ▪ Increase of IL-8 (×1.3) and TNF-α (×2.2) (THP-1)
	Traffic site – organic fraction of NP – Summer - Barcelona	85-150	▪ No change in cell viability (A549 and THP-1) ▪ No increase of ROS (A549 and THP-1) ▪ Slight increase (NS) of IL-8 and increase of TNF-α (×1.5) (THP-1)
27	Road tunnel NP –winter- Stockholm	10-200	▪ Decrease of cell viability (A549 but not THP-1) at 200 µg/mL ▪ Increased release of IL-8 and TNF-α (×3) at 100 µg/mL in THP-1 but not in A549 ▪ Increase of DNA strand breaks at 100 µg/mL in both A549 and THP-1
	Road tunnel - PM2.5 – winter - Stockholm	10-200	▪ Decrease in cell viability at higher doses (100-200 µg/mL) in THP-1 but not A549 ▪ Increased release of IL-6 and TNF-α in A549, dose-dependent increase in release of all cytokines (IL-6, IL-8, TNF-α and IL-1β) (×18 IL-8 at 100 µg/mL – THP-1)
28	Subway NP –Winter - Stockholm	10-100	▪ No clear effect on cell viability ▪ A slight (NS) increase of IL-8 and TNF-α (approx. ×2) at 100 µg/mL in THP1 but not in A549 ▪ Increase of DNA strand breaks at both 10 and 50 µg/mL in A549 but not in THP-1
	Subway PM2.5 summer - Stockholm	10-200	▪ Decrease in cell viability in both A549 and THP-1 ▪ Increased release of IL-6 and TNF-α in A549 and increased release of all cytokines (IL-6, IL-8, TNF-α and IL-1β) in THP-1. A slight (NS) increase at 10 µg/mL (approx. ×2 IL-8) and a clear increase at 100 µg/mL (×13 IL-8 at 100 µg/mL)

Table 7. Summary of the main toxicity results of zebrafish embryos exposures to organic fraction of particles from outdoor environments

No.	Transport mode – Particle size – Season – City	Exposure dose (µg/mL)	Description of the main results
18	Background – organic fraction of NP - Winter - Barcelona	Median: 0.91 Range: 0.38-1.17	▪ No mortality in zebrafish embryos ▪ Increase of *cyp1a1 1* expression (X1.7) ▪ No change in *hao1* expression ▪ No change in *gsta* expression
	Background – organic fraction of NP – Summer - Barcelona	Median: 1.75 Range: 0.86-1.97	▪ No mortality in zebrafish embryos ▪ Significant increase of *cyp1a1 1* expression ▪ No change in *hao1* expression ▪ No change in *gsta* expression
20	Urban traffic – organic fraction of NP – Summer/Winter - Stockholm	Median: 3.28 Range: 1.09-5.23	▪ No mortality in zebrafish embryos. ▪ Increase of *cyp1a1* expression (X2.5) ▪ Increase of *hao1* expression (X2.4) ▪ No significant difference in *gsta* expression
21	Harbour – organic fraction of NP – Winter Barcelona	Median: 2.37 Range: 1.96-3.19	▪ No mortality in zebrafish embryos ▪ Increase of *cyp1a1* expression (X5.1) ▪ No change in *hao1* expression ▪ No change in *gsta* expression
	Harbour – organic fraction of NP – Summer - Barcelona	Median: 1.07 Range: 0.63-2.21	▪ No mortality in zebrafish embryos ▪ No change in *cyp1a1* expression ▪ No change in *hao1* expression ▪ No change in *gsta* expression
22	Airport – organic fraction of NP – Winter – Barcelona	Median: 0.92 Range: 0.61-1.68	▪ Mortality in zebrafish embryos: 2/84 ▪ Increase of *cyp1a1* expression (X1.3) ▪ No change in *hao1* expression ▪ No change in *gsta* expression
	Airport – organic fraction of NP – Summer - Barcelona	Median: 0.83 Range: 0.82-1.26	▪ No mortality in zebrafish embryos ▪ Increase of *cyp1a1* expression (X1.2) ▪ No change in *hao1* expression ▪ No change in *gsta* expression
26	Urban traffic – organic fraction of NP – Winter - Barcelona	Median: 1.61 Range: 1.06-1.69	▪ No mortality in zebrafish embryos ▪ Increase of *cyp1a1* expression (X4.8) ▪ No change in *hao1* expression ▪ No change in *gsta* expression
27	Background – organic fraction of NP – Summer – Stockholm	Median: 1.10 Range: 1.10-1.57	▪ No mortality in zebrafish embryos. ▪ Increase of *cyp1a1* expression (X1.7) ▪ Increase of *hao1* expression (X2.1) ▪ No significant difference in *gsta* expression
28	Subway – organic fraction of NP – Summer/Winter - Stockholm	Median: 1.48 Range: 1.21-2.23	▪ No mortality in zebrafish embryos ▪ Increase in *cyp1a1* expression (X2.7) ▪ No change in *hao1* expression ▪ No change in *gsta* expression

Discussion

This report presents the main results regarding the toxicity evaluation of airborne nanoparticles from several transport modes as part of WP5 of the nPETS project. We investigated traffic, shipping, aviation and subway emissions using three different toxicity models: air-liquid interface, submerged, and zebrafish embryo exposure models. Emissions were generated outdoors or in laboratories (representing different parts of outdoor emissions).

Two ALI systems were successfully built in the first year of the project to make sure that the same ALI exposure method was used at all sites in Sweden (road tunnel, subway, tribology lab), Greece (chassis-dyno lab, airport), and Spain (urban background, harbour). The same, or close to the same protocol was also used for all submerged exposures. Only one laboratory preformed exposure of Zebrafish embryos, i.e. the same method was used for all experiments.

Several exposure experiments have been performed so far, covering all transportation modes, such as subway, traffic sites/road tunnel, airport, harbour and background sites, and regarding in-laboratory studies we have covered brakes, exhausts, and rail-related materials. Still, more experiments will be performed the coming year. For example, coming toxicity testing will involve brake wear (in the chassis-dyno in Brembo, November 2023), subway-related materials (in aerosol laboratory, Stockholm, spring 2024), emissions from clutches, and exhaust of different fuels (chassis dyno, Thessaloniki). All these results will be presented in the second deliverable of the toxicity testing results, due in November 2024.

Results in the lab (ALI and submerged)

A laboratory facility has a controlled environment, making it possible to look at a single exposure scenario at the time. Regarding the ALI experiments in laboratory facilities, most of the experiments in the chassis-dyno (**exhausts**) had an effect on cell viability and cytokine release, showing that gases as well as nanoparticles emitted from the vehicles negatively impacted the cells. However, the **brake wear** particles studied in the tribology laboratory showed low or no effect on the cell viability and inflammatory response at the experimental conditions.

The results from the submerged exposure of laboratory generated nanoparticles and PM2.5 showed various effects. The **brake materials** tested showed mainly low toxicity (cell viability and inflammation). The **subway related nanoparticles** generated by spark discharge showed slightly higher toxicity in the sense that some decreased cell viability was observed, but they did not in general cause substantial cytokine release (similarly to the brake wear). However, for these nanoparticles we also explored gene expression changes and several of them caused increase expression of IL-8. Furthermore, they all caused increase in DNA strand breaks. The nanoparticles sampled from the chassis-dyno lab (**exhaust**) could only be tested in lower concentrations (up to 5 µg/mL) but interestingly effects on viability and ROS could still be observed for one condition (Car 1 mRDE hot), indicating that these nanoparticles show relatively high toxic potency.

Results outdoors (ALI, submerged and zebrafish embryos)

ALI exposures in outdoor environments are challenging as particle concentrations vary a lot, and furthermore, concentrations outdoors are low compared to those in the laboratory experiments. To increase the particle concentration, a concentrator from Tampere University was used. This device increased the concentration administered to the ALI system by five times, however, the exposure dose was still low compared to the ones tested at submerged conditions. Note that some of the doses in ALI have not yet been evaluated, and are therefore missing in the table.

Concerning **traffic emissions**, experiments in Barcelona and Milan were performed in urban traffic locations while in Stockholm, they were carried out in a road tunnel. Emissions at urban traffic locations can possibly be influenced by "non-transport related" sources like industries. Similarly, emissions from ships can be possibly be influenced by emissions from road traffic.

The ALI results in the **road tunnel** showed no change in cell viability, and only a slight (NS) increase in cytokine levels. Results in the **subway** showed a low or no change in the cell viability in A549 and during the two seasons (winter/summer) and a decrease in cytokine levels (only summer). Results in the **harbour** of Barcelona showed sporadic toxicity in ALI (probably linked to when ship engines were on), likely linked to the gas fraction rather than to NPs. Results of ALI at Thessaloniki **airport** showed significant reduction of cell viability and an increase of immunological responses. For several locations (e.g. Harbour Barcelona) results showed higher cytotoxicity in winter season than in summer.

Regarding the submerged exposure, NPs were collected on filters and cells were in most cases exposed to a particle suspension (in Barcelona; submerged exposure to organic fraction). Due to challenges collecting enough mass of these small particles, the mass concentration of the stock suspensions sometimes became low. The particles were extracted from the filters with water and without any evaporation, and therefore the cell medium was diluted with water which might have affected the results in some cases. Even though we tried to overcome this by adding serum to the stock suspension, the problem seems to remain in some cases. This was evident for the exposures from Milan in which the blank control sometimes showed too much deviation from the non-exposed cells, which makes the results hard to interpret. Clearly, samples collected in Barcelona (both **harbour** and **airport**) and in Stockholm (**road tunnel** and **subway**) showed some increase in cytokine release. Regarding these particles, the ones from the subway (PM2.5) appeared to be the most cytotoxic. Interestingly, both nanoparticles from the road tunnel and subway caused increase of DNA strand breaks and for the subway nanoparticles effects were observed in the lowest dose (10 μg/mL). In contrast, the road tunnel particles seemed to be more inflammatory and furthermore, PM2.5 appeared more potent than the nanoparticles. For the road tunnel PM2.5, significant increase of IL-8 and TNF-α levels were observed in the lowest dose tested (10 μg/mL) and at the highest dose (100 μg/mL) the fold increase compared to control was ×18, which was the highest reported among all samples.

Results from zebrafish embryos experiments using **urban background, airport, harbour, urban traffic** and **subway** samples showed no or low embryo mortality. However, cyp1a1 activation (dioxin-like activity) was observed for all the transport modes except the harbour (summer). An increase of *hao1* was reported only for background (summer) and urban traffic in Stockholm and no changes of *gst* expression was observed for any transport modes.

General discussion

The use of different exposures doses, among the different models, makes it difficult to compare different experiments. On one side, although our ALI system allows an efficient deposition, ALI experiments were dependent on the particle concentration of the emission source and, in outdoor environments, this concentration can be limited. On the other side, particles suspensions used in submerged exposures, can normally reach high concentrations, but again, the mass concentration that could be collected on filters was often insufficient. Overall, for the several ALI experiments and for some submerged experiments, the concentration used was not enough to see a toxic effect. This fact also makes it difficult to compare effects in ALI vs submerged exposure systems. Furthermore, ALI was performed with A549, whereas submerged used both A549 and THP-1 cell lines. One of the analysed endpoints was cytokine release, which was always much higher in THP-1 cells. This clearly makes sense

(since they are macrophages), but it is one more thing that made the ALI and submerged methods difficult to compare.

Conclusions

Urban road traffic

NPs from urban traffic showed toxic effects in most of the tests performed, particularly at submerged conditions for which the concentrations tested were higher and THP-1 cells also was used for inflammation endpoints. The results based on the organic fraction from Barcelona suggested that winter samples induced more ROS production and release of pro-inflammatory cytokine IL-8 and TNF-α than summer samples. Results from car brake wear generated in the lab indicated relatively low toxicity suggesting that these particles are likely not a main cause of toxicity. In contrast, emissions from car exhaust caused evident toxicity. When comparing the different fuels and driving conditions, the main conclusion is that petrol produces more toxic effects on A549 cells. Both the car engine start temperature and the dynamics of the driving cycle can affect toxicity. Cold start engine and combined cycles seems to be more potent. Furthermore, aged particles appear to have a more intense effect, as far as cytotoxicity and inflammation is concerned.

Subway and rail

The subway particles showed some toxic effects, and the effects were more pronounced for PM2.5 compared to NPs. The inflammatory effect appeared to be lower when compared to traffic induced effects (road tunnel) for both NPs and PM2.5. However, the subway NPs caused DNA strand breaks and this effect was also observed for the rail-related NPs generated by spark discharge. The rail-related NPs (rail, third-rail, wheel) caused overall rather similar effects. No clear toxic effects were observed from the rail brake wear (PM2.5) but it should be noted that formation of DNA strand breaks was not analysed for these samples.

Shipping/harbour

The NPs (or organic fraction of these) collected in the harbour showed some toxic effects in both ALI, submerged and zebrafish experimental conditions. Cell viability was only compromised in some ALI experiments in which the engine of a boat near the mobile unit was turned on. In general, winter samples induced higher levels of ROS formation, release of cytokines and cyp1a1 expression than summer samples.

Airport

Airport NPs (or organic fraction of these) showed toxic effects; some effects on cell viability, but mainly on ROS production and inflammation induction. In Barcelona airport samples, the release of cytokines was much higher in winter samples, notably the release of TNF-α in THP-1, than in summer samples. When compared to traffic sites the effect appeared lower in zebrafish. However, the sample collected at airport during winter was the only one causing mortality in zebrafish.

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