

# In vitro and in vivo investigations to obtain validated toxicity data of graphene nanoplatelets

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## INTRODUCTION

PLATOX (FP7-SIINN project) proposes a tiered approach to address the existing toxicological data gaps for graphene family nanoplatelets (GFN). GFN are part of the group of carbon based synthetic nanomaterials that are currently subject of studies to accelerate their toxicological characterization. Based on the existing toxicological information, a very high toxic potential is not expected, however, the characterization is presently incomplete and should be expanded to facilitate a proper risk assessment. The project has five WPs (**Figure 1**) which cover the activities on GFN characterization (WP1), *in vitro* and *in vivo* experimental work (WP2 and WP3), data analysis and evaluation (WP4) and project management (WP5).

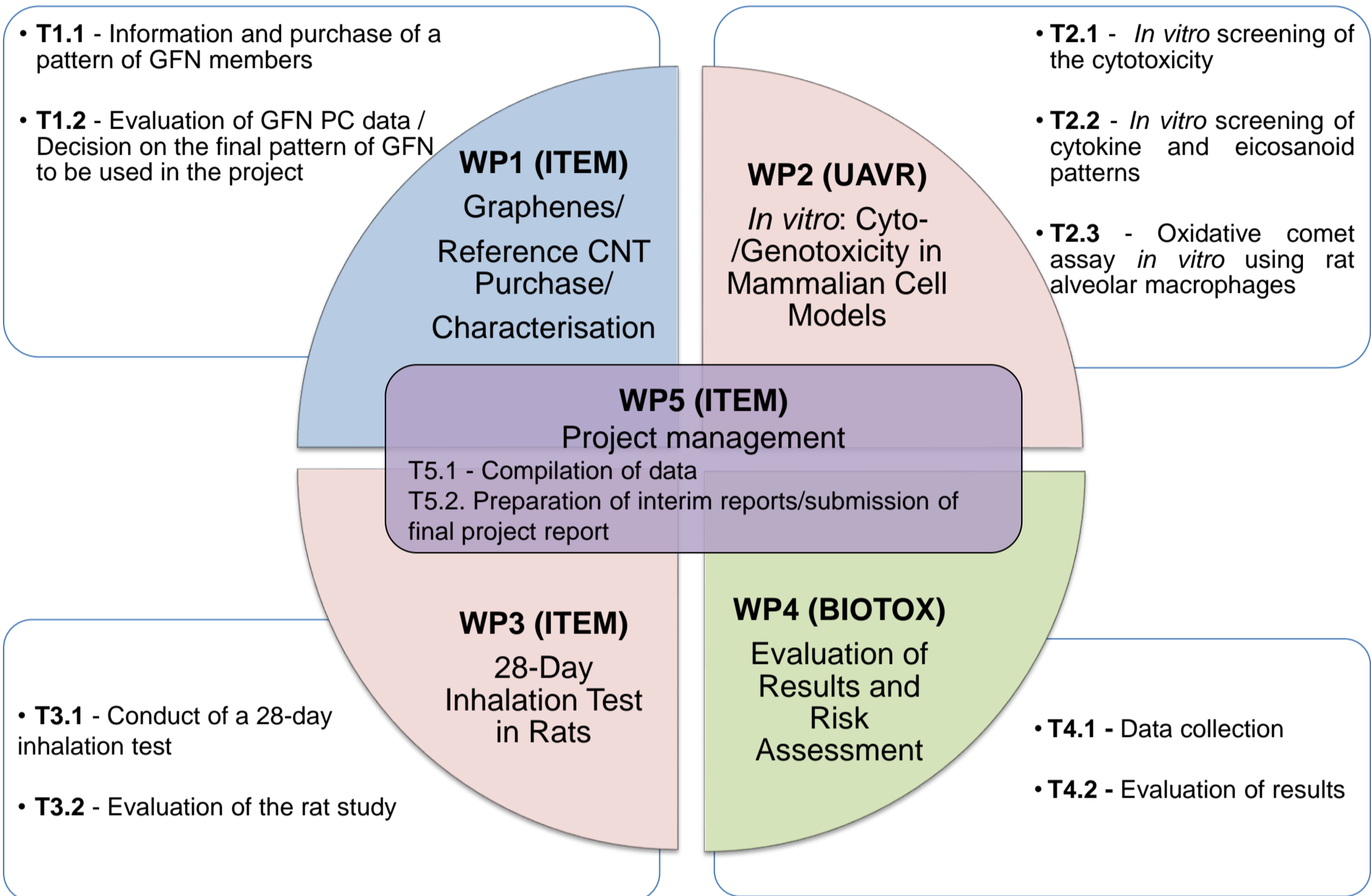


Figure 1. Representation of project structure: Work packages and related tasks

## MATERIALS AND METHODS

**Test items:** Six different commercially available GFN (P1-P6) and pristine research graphite nanoplatelets (P7) were selected for *in vitro* testing (**Table 1**). Carbon-black nanoparticles are used as spherical reference material.

Table 1. Physico-chemical properties of the investigated GFNs

No.	GFN	Diameter (µm)	Thickness (nm)	BET surface area (m <sup>2</sup> /g)	SEM image	Preparation/Properties
P1	Single layer graphene powder	~5	2 – 10	278 (400-1000)*		Thermal exfoliation reduction + Hydrogen reduction
P2	Single layer graphene (graphene factory series)	0,5 – 5	2 – 10	620 (650-750)*		1-5 atomic layer graphene nanosheets
P3	Carboxyl graphene	1 – 5	0.8 – 1.2	1.5		1) Modified Hummer's method to make graphene oxide 2) Convert –OH and C-O-C into –COOH. Carboxyl ratio: 5%
P4	Graphene nanoplatelets	~5	2 – 10	15 (20-40)*		Stacks of multi-layer graphene, with a high aspect ratio, width to thickness
P5	Single layer graphene oxide powder (S method)	1-15	0.8-1.2	5.2 (5-10)*		Stauden-maier method; oxygen content: 35%
P6	Graphite oxide powder	0,5 – 5	1 – 3	2.7		Modified Hummer's method; oxygen content: 35%
P7	Reference pristine graphene nanoplatelets (GR1)	2	3	195 (70)*		No XPS (low defects by RAMAN) all C1s carbons; 8±0,5 atomic layer graphene
P8	Reference carbon black particles (Printex 90)	14	-	317 (337)*		Specified as >99% pure carbon black, PAH=0,039 ppm

\*range given by the supplier

**Dispersion of GFN and exposure:** Materials were dispersed in serum-containing cell culture medium, 3 x 5 min of ultrasonication on ice, 1 min breaks, Bandelin Sonoplus HD2070 (70 W) with sonotrode VS70T (Ø 13 mm), 90% cycles (9/10), amplitude 80 µm<sub>ss</sub>. Cells were incubated with the test items at different concentrations of up to 50 µg/cm<sup>2</sup> for 24h or 48h.

**Test systems:** RAW 246.7 murine blood macrophage cell line (University of Aveiro), primary rat alveolar macrophages (Fraunhofer ITEM), MRC-5 human lung fibroblasts (both laboratories).

**Endpoints and assays:** Membrane damage (LDH assay), cell viability by measuring the metabolic capacity (AlamarBlue® assay), proliferation (relative increase in cell count, RICC), cytokine release (ELISA), unstimulated/stimulated eicosanoid pattern (highly sensitive and specific competitive EIA), direct genotoxicity by measuring DNA-strand breaks and oxidative DNA-damage (hOGG1-modified alkaline comet assay), cell cycle dynamics/ploidy level (flow cytometry).

## OBJECTIVES

- Selection of GFN with different chemistry suggesting a varying toxic outcome;
- In vitro* testing: cytotoxicity, direct genotoxicity cytokine and eicosanoid release in order to identify the candidates with the highest and lowest biological activity;
- Validation of *in vitro* results with an *in vivo* 28-day inhalation test (on two selected GFN, most and least active);
- To evaluate available information on physicochemical properties of the tested GFN, and compare it with the toxicological *in vitro* and *in vivo* results and exposure data;
- Perform risk assessment and derivation of DNEL for the two selected GFN according to current regulatory procedures. The workflow proposed for the risk assessment (**Figure 2**) integrates the physico-chemical characterization, *in vitro* and *in vivo* methodologies.

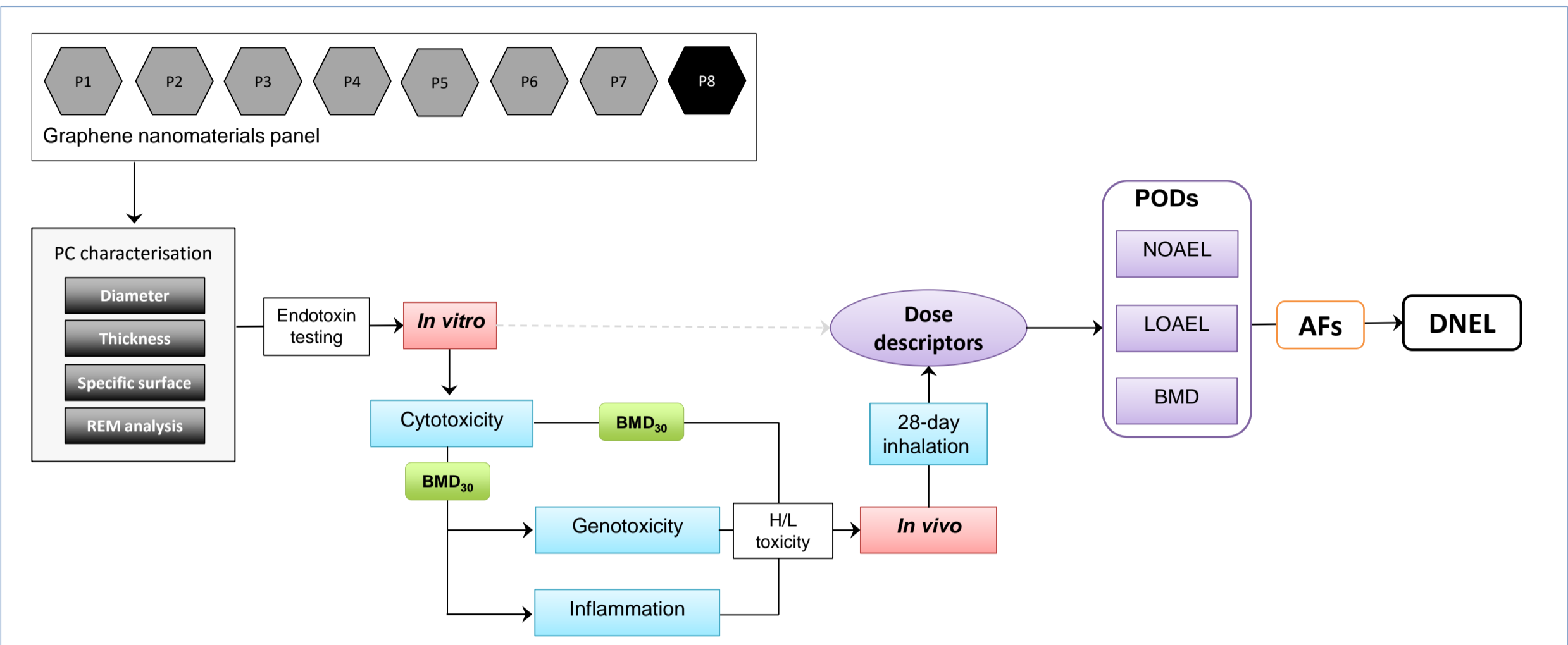


Figure 2. Workflow for risk assessment

## PRELIMINARY IN VITRO RESULTS

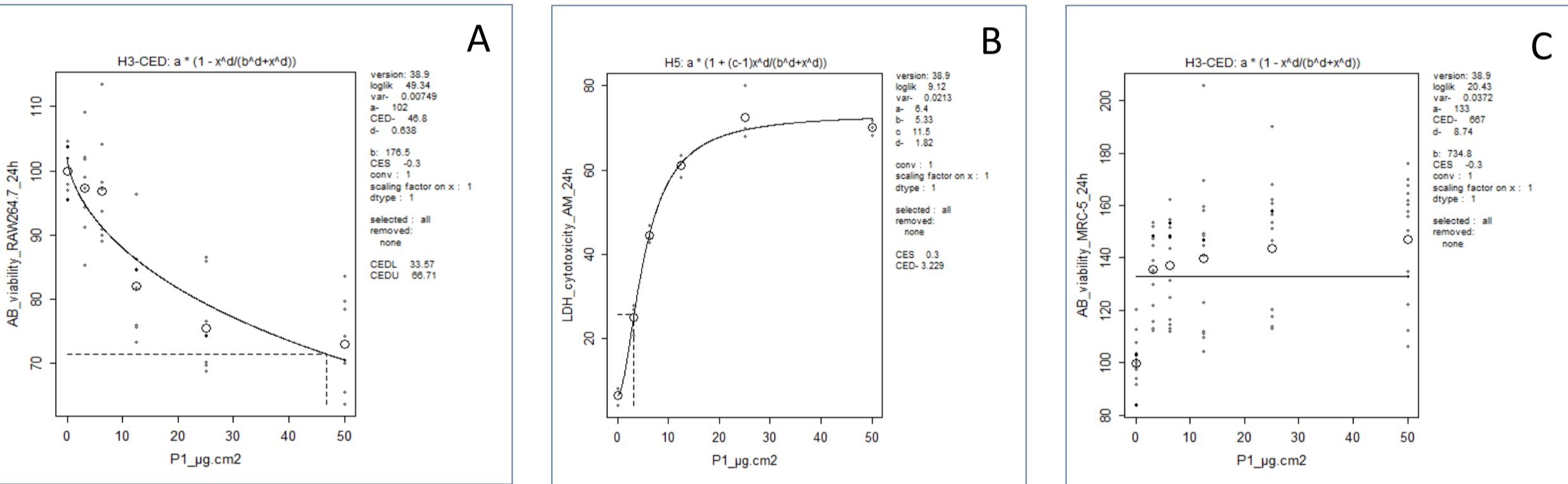


Figure 3. Cytotoxicity results after 24 h of exposure to P1 (single layer graphene) for RAW264.7 cells (A), primary rat alveolar macrophages (B) and MRC-5 cells (C). BMD30 calculation was performed with PROAST 38.9 package.

Test system	Time point	P1 (GN1P0005)	P2 (GN1PF010)	P3 (GNCP0005)	P4 (GNNP0051)	P5 (GNOS0010)	P6 (GTOP0002)	P7 (GR1)	P8 (Printex 90)	Assay	Laboratory
RAW 264.7	24h	High toxicity	High toxicity	High toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Alamar Blue	UAVR
	48h	High toxicity	High toxicity	High toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	LDH	UAVR
Primary rat alveolar macrophages	24h	High toxicity	High toxicity	High toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Alamar Blue	ITEM
	48h	High toxicity	High toxicity	High toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	Low toxicity	LDH	ITEM

Figure 4. Preliminary toxicity ranking based on cytotoxicity results and comparison of BMD30 for all GFN

## DISCUSSIONS AND CONCLUSIONS

- Fully characterized GFN are used in this study in order to perform an integrated risk assessment based on *in vitro* and *in vivo* studies.
- The preliminary *in vitro* results (cytotoxicity) show that P1, P2 and P3 are the most toxic GFN, while P4, P5 and P6 are less toxic; a higher sensitivity of macrophages was observed, as compared to lung fibroblasts, for which the BMD30 values were always >50 µg/cm<sup>2</sup>. However, the results on macrophages show no correlation between the two assays applied (**Figure 3 and 4**).
- Non-toxic concentrations of GFN will be used for evaluation of additional *in vitro* parameters (e.g. genotoxicity, cytokines release, etc.). The selection of the GFN for *in vivo* validation will thus be based on a complex *in vitro* test battery. Only two GFN (most and least active) will finally be selected for a 28-days inhalation study, in order to reduce the overall number of animals used in the project.
- In summary, the expected outcome of the project will be a toxicological ranking of the tested GFN species, providing an improved basis for risk assessment of these nanomaterials.

## Project partners



## Acknowledgements



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