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Air Resource Board of California - Diesel Nitrogen Dioxide Working Group
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Diesel exhaust oxidant potential assessed by the NO₂/NO concentration ratio, may be a major trigger of Diesel engine emission biological impact to rat lung tissue.

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The health impact of Diesel engine emissions is most frequently associated with the sole impact of particulate matter (PM) which have been classified as air toxic. In this respect, a vast majority of Diesel emission health impact studies do not consider the gaseous phase of the aerosol as a potential toxicity trigger.

The aim of the present study was to assess the respective lung toxicity contributions of PM phase and gaseous phase of Diesel engine emissions. This study was conducted in vitro on rat lung tissue in organotypic cultures exposed in a bi-phasic system to continuously sampled and diluted Diesel engine emissions with highly preserved physicochemical properties as described by Morin et al. (1999) LePrieur et al. (2000) and Bion et al. (2002).

Methods :

The engine used for these experiments is a 2l common rail direct injection turbocharged intercooled Diesel engine which is operated at various speeds, and loads, using after treatment devices and different fuels (sulphur contents) in order to modulate emission physicochemical compositions as assessed by regulated emission measurements. Soot content and size distribution were measured using an AVL415 reflectometer and SMPS respectively. CO, CO₂, NO, NO₂, HC and O₂ were measured using a Horiba Mexa 7000 analyser.

As previously described, rat lung slices organotypic cultures maintained in flow through rotating chambers were exposed in parallel to continuous flows of either clean air (control) or diluted emissions (10 to 30%).

To evaluate the respective effects of gases and PM on rat lung slices, more than 99.99% of DEPs are removed from the test atmosphere thanks to filter placed on the sampling line downstream the sampling line. This filtration which allowed to remove soot from the emissions did not modify the gaseous fraction characteristics.

Typical exposure duration to diluted emissions was 3 hours as in previously published studies. Three main biological pathways and DNA alterations were assessed in this study.

ATP and total glutathione intracellular levels were assayed from lung slice perchloric extracts using ATP Bioluminescence HSII kit (Roche Diagnostics) and according to Mistry et al. 1991 respectively.

Enzyme activity assessment : Slices were washed twice in ice cold Tris/NaCl buffer and homogenized in 0.5ml of buffer using a glas/glas Kontess tissue grinder. Superoxide dismutase (SOD EC-1.15.1.1), glutathione peroxidase (GPX EC-1.11.1.9), catalase (CAT EC-1.11.1.6) and glutathione-S-transferase (GST EC-2.5.1.18) activities were assayed according to established methods (Crapo (1978), Wendel (1981), Aebi et al. (1974) and Habig et al. (1974) respectively).

Tumor necrosis factor alpha (TNF α) concentrations were assayed in culture medium using the rat TNF α ELISA kit (Endogen)

Tissue nucleosome content were assayed in lung slices using the Rat Nucleosome ELISA kit (Amersham).

ICAM I in situ histological detection was performed on lung slice cryosections using anti-ICAM-I antibody (R&D system). Sections were observed using an Axiovert 100 epifluorescence microscope.

Results :

Regulated emissions :

Among a vast series of experimental conditions screened, four experimental conditions yielding typical pollutant patterns have been selected A, C, E and G. situations B, D, F and H being their filtered counterparts. Table 1 summarizes regulated emission measurements. Emissions appear to be representative of the latest light duty Euro3 Diesel engine generation low HC and CO outputs. Conditions A and C had similar higher soot contents than conditions E and G having also similar contents. SMPS measurements could not evidence any difference in PM size distributions according to experimental situations, with a mean aerodynamic diameter of 90-100nm.

While total NO_x did not differ to a large extent according to the experimental condition, the most striking feature was the wide range of NO₂/NO ratio variations from 0.06 up to 1.16. Although NO₂ may act per se, we have considered the NO₂/NO ratio as a marker of the emission oxidising potential and have classified experimental situation as exhibiting low, moderate and high oxidising potentials.

Table 1 : Pollutant assessment

		DIESEL ENGINE EXHAUSTS							
Pollutants	Unit	A	B	C	D	E	F	G	H
HC	ppm C	29	29	19	19	10	10	0	0
CO	ppm	137	137	0	0	0	0	0	0
NO _x	ppm	423	423	406	406	467	467	484	484
NO	ppm	399	399	300	300	277	277	224	224
NO ₂	ppm	24	24	106	106	191	191	260	260
NO ₂ /NO		0.06	0.06	0.35	0.35	0.69	0.69	1.16	1.16
Oxidising potential		+		++		+++			
Soot	FSN	1.8	NA	1.8	NA	0.7	NA	0.7	NA
	mg/m ³	44	NA	44	NA	12	NA	12	NA

Biological parameters :

As shown in table 2, conditions A,B, C, and D did not interfere with intracellular ATP levels thus pointing out to a well preserved lung tissue viability upon exposure. A moderate decrease in tissue viability was observed for conditions E and F while a marked decrease in tissue viability was evidenced for conditions G and H. A clearcut correlation could be established between tissue viability impairment and increasing emission NO₂/NO ratio.

Filtration of emissions E and F did not modify tissue viability levels thus pointing out to the predominant impact of the gaseous phase on tissue viability losses.

Intracellular GSH levels were decreased and GPx activities were increased whatever the experimental condition compared to control situation. Again, emission filtration did not alter the extent of GSH depletion nor of GPx activity increase.

GST and MnSOD activities were markedly increased under A, B, C and D situations but remained unaffected upon E, F, G and H situations. Again, emission filtration did not modify the extent of GST nor of MnSOD activity modification upon exposure.

Catalase activity was increased under E and F conditions and remained unaffected under A, B, C, D, G and H conditions

TNF alpha release in culture medium increased under A, C and D conditions, was slightly decreased under E and F conditions and markedly decreased under G and H conditions. Emission A filtration yielding the B condition withdrew TNFalpha release induction thus pointing out to the PM phase responsibility of inflammatory response induction at low NO₂/NO ratio. At moderate NO₂/NO ratio, increased TNFalpha output was evidenced which was not altered by emission filtration. Finally, at higher NO₂/NO ratios, TNFalpha output was reduced compared to the control situation. The extent of decreased output appeared to be correlated with increasing NO₂/NO ratio and consequently with cytotoxicity occurrence. It is interesting to notice that emission filtration had no impact on TNFalpha output under C, D, E, F, G and H conditions, thus pointing out to a predominant role of the gaseous phase.

DNA alterations :

Lung tissue nucleosome content increased under A, C and D conditions, and remained unaffected under E, F, G and H conditions. Emission A filtration yielding the B condition withdrew nucleosome content elevation, thus pointing out to the PM phase responsibility of inflammatory response induction at low NO₂/NO ratio.

At high NO₂/NO ratio, no alteration of nucleosome content could be evidenced suggesting the absence of apoptosis phenomenon.

Table 2 : Biological parameters assessment

	BIOLOGICAL PARAMETERS							
	Cell Viability	Oxidative stress					Inflammatory response	DNA damages
Exhausts	ATP	GSH	GST	GPx	Catalase	MnSOD	TNF alpha release	Nucleosome content
A	=	--	++	+	=	++	++	++
B	=	--	++	+	=	++	=	=
C	=	--	++	+	=	++	++	++
D	=	--	++	+	=	++	++	++
E	-	--	=	+	++	=	-	=
F	-	--	=	+	++	=	-	=
G	--	--	=	+	=	=	--	=
H	--	--	=	+	=	=	--	=

Discussion :

In this study, we clearly show that either fuel sulphur content or oxidation catalysis modulate the pro-oxidant potential of emission as reflected by the NO₂/NO ratio assessed in raw exhausts. At low NO₂/NO ratio, PM phase represents the dominant trigger of inflammatory reaction and DNA alteration, while oxidant stress is mainly induced by the gas phase of the emissions. At moderate NO₂/NO ratios, emission filtration was unable to induce a

modification of the toxicity pattern and endpoint impairment, thus showing that emission gas phase impact was taking over PM phase impact which could not be anymore evidenced by emission filtration. At high NO₂/NO ratios (E, F, G, H), cytotoxicity did occur, major oxidant stress did overwhelm tissue adaptation capacity, inflammatory reaction and apoptosis were abolished. These last conditions show high toxicity potential of emissions due to gas phase components.

We suggest that NO₂/NO ratio representing the pro-oxidant potential of emissions could be considered as a useful candidate-marker of detrimental biological impact of these emissions.

It is to date too early to state about the specific NO₂ action in these emissions which will be studied in a close future.

We wanted to draw the attention of Air Resources Board on these yet not published data that will shortly be submitted for publication in peer review international journal.

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Authors are ready to answer to further information requests from Air Resources Board if felt necessary. These results have been obtained on light duty Diesel engines. Authors do not have easy access to heavy duty engine facilities and would be prepared to collaborate with Air Resource Board for technology transfer under contractual arrangement if desired by Air Resource Board.

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