Comparative mutagenicity and genotoxicity of particles and aerosols emitted by the combustion of standard vs. rapeseed methyl ester supplemented bio-diesel fuels

V. Andre, Claire Barraud, David Capron, D. Preterre, V. Keravec, Cathy Vendeville, F. Cazier, Didier Pottier, Jean-Paul Morin, François Sichel

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Highlights

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Q1 **Comparative mutagenicity and genotoxicity of particles and aerosols emitted by the combustion of standard *vs.* rapeseed methyl ester supplemented bio-diesel fuels Impact of after treatment devices: Oxidation catalyst and particulate filter**

V. André*, C. Barraud, D. Capron, D. Preterre, V. Keravec, C. Vendeville, F. Cazier, D. Pottier, J.P. Morin, F. Sichel

- Gas phase of aerosols was mutagenic in TA98 and in TA102 w/o S9mix.
- The PAHs amount on the particles was low and did not contribute to the mutagenicity.
- Mutagenicity of particles was associated with nitroaromatic profiles exclusively.
- DOC abolished the mutagenicity of particles, then retained into DPF.
- Together, DOC + DFP efficiently reduce the particulate mutagenicity.

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Impact of after treatment devices: Oxidation catalyst and particulate filter

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ABSTRACT

Diesel exhausts are partly responsible for the deleterious effects on human health associated with urban pollution, including cardiovascular diseases, asthma, COPD, and possibly lung cancer. Particulate fraction has been incriminated and thus largely investigated for its genotoxic properties, based on exposure conditions that are, however, not relevant for human risk assessment. In this paper, original and more realistic protocols were used to investigate the hazards induced by exhausts emitted by the combustion of standard (DF0) *vs.* bio-diesel fuels (DF7 and DF30) and to assess the impact of exhaust treatment devices (DOC and DPF). Mutagenicity and genotoxicity were evaluated for (1) resuspended particles ("off line" exposure that takes into account the bioavailability of adsorbed chemicals) and for (2) the whole aerosols (particles + gas phase components) under continuous flow exposure ("on line" exposure). Native particles displayed mutagenic properties associated with nitroaromatic profiles (YG1041), whereas PAHs did not seem to be involved. After DOC treatment, the mutagenicity of particles was fully abolished. In contrast, the level of particle deposition was low under continuous flow exposure, and the observed mutagenicity in TA98 and TA102 was thus attributable to the gas phase. A bactericidal effect was also observed in TA102 after DOC treatment, and a weak but significant mutagenicity persisted after DPF treatment for bio-diesel fuels. No formation of bulky DNA-adducts was observed on A549 cells exposed to diesel exhaust, even in very drastic conditions (organic extracts corresponding to 500 μg equivalent particule/mL, 48 h exposure). Taken together, these data indicate that the exhausts issued from the bio-diesel fuels supplemented with rapseed methyl ester (RME), and generated by current diesel engines equipped with after treatment devices are less mutagenic than older ones. The residual mutagenicity is linked to the gas phase and could be due to pro-oxydants, mainly for RME-supplemented fuels.

1. Introduction

Urban air pollution has deleterious effects on human health, including cardiovascular diseases, asthma, COPD, and lung cancer [1–4]. Vehicle exhausts are responsible for a large part of this urban pollution, and diesel engines are more specifically incriminated, since they produce higher amounts of inhalable particles than gasoline engines, associated with toxic gases such as NO_x and

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aldehydes. Moreover, genotoxic and carcinogenic combustion by-products such as PAHs and nitro-PAHs are adsorbed onto particles surface. Diesel exhausts carcinogenicity was recently re-evaluated by IARC, and classified as Group1, *i.e.*, carcinogenic to humans, based on "sufficient evidence that exposure is associated with an increased risk for lung cancer" [5]. This new classification is mainly based on occupational exposure to heavy-duty engines, especially among a large cohort of underground miners, *i.e.*, in a confined environment [6,7]. Even if this decision sparked a controversy [8], it has been estimated that public health could also be compromised, particularly in developing countries, considering the many industrial applications of diesel engines [5].

Policies aiming at air quality improvement in more developed countries have led to the strengthening of the legislation through the setting-up of successive standards [9]. Diesel-fuel quality, engine technologies and exhaust after treatment devices (diesel oxidation catalysis–DOC and diesel particulate filters–DPF) were thus significantly improved over the last two decades. As a consequence, toxic pollutant emissions associated with diesel exhaust gases have generally been declining as observed by Pronk et al. [10], despite the continuous increase in the use of diesel engines. In parallel to these technical evolutions, blends of fossil fuels and renewable fuels are now used, both for economic and ecological purposes. However, the introduction of these new fuels and new after treatment devices requires a comprehensive investigation of the possible current hazards linked to their use, and an accurate evaluation of their impact on human health.

Bacterial mutagenicity and DNA adducts formation have been frequently used as a paradigm of the carcinogenic potential of diesel engine emitted particles (DEP). However, data have been mainly obtained after exposure to organic extracts (OE), which are not representative of the real and current exposure of European urban populations.

Therefore, the present study is a contribution to the *in vitro* re-evaluation of the mutagenicity and genotoxicity assessment of diesel exhausts emitted by a current diesel engine fuelled with standard diesel fuel and RME-blends, using some more realistic exposure conditions. Hence, particles alone were first resuspended in an aqueous culture medium, in order to take into account the bioavailability of compounds adsorbed onto particles. Then, the whole aerosol was studied in dynamic conditions (continuous flow of particles associated with the gas phase), using various biological models (*Salmonella typhimurium*, A549 human cells and organotypic rat lung slices) exposed in biphasic conditions (air-liquid interface, ALI).

Comparisons with data obtained from classical exposure to the corresponding OE were performed in order to demonstrate the relevance of these protocols for the evaluation the genotoxic impact after inhalation of aerosols.

The genotoxicity of a standard diesel fuel and RME-blends (7% and 30% RME) was compared, and the effects of exhaust after treatment devices (DOC and DPF) on the aerosol toxicity were also investigated.

All together, these data could contribute to the health risk assessment of current diesel exhausts.

2. Materials and methods

2.1. Diesel particles (DEPs) sampling

A 2 L direct injection turbocharged intercooled diesel engine (corresponding to Euro3 standard), representative of the majority of the French car park in 2010, was placed on a test bench. It was used according to the urban section of the ARTEMIS cycle in order to mimic emissions produced in urban driving conditions. The fuels used were a reference diesel fuel (DF0) containing less than 50 ppm of sulphur (current quality in station), and two diesel fuels supplemented with rapeseed methyl ester (RME) at 7% (DF7) and 30% (DF30), respectively. DEPs were sampled directly downstream of the diesel engine or downstream of the oxidation catalyst.

2.2. Preparation of organic extracts

Organic extracts were obtained from 200 mg of DEPs, using a soxhlet apparatus and 100 mL dichloromethane (DCM) as a solvent over 24 h. The organic phase was either concentrated under a nitrogen stream for GC–MS analysis, or evaporated. The dry residue was dissolved into DMSO for mutagenic and genotoxic potential evaluations.

2.3. Chemical analysis of organic extracts

OE were analysed with GC–MS (VARIAN 1200 TQ model). The materials used for compound identification and quantification were purchased from Sigma–Aldrich (St Quentin Fallavier, France). Hydrocarbons identifications were assigned by comparing the retention times of chromatographic peaks from samples with those from standard mixtures and by comparing mass spectra with those contained in NIST and/or WILEY libraries. The estimated quantification limit (QL) was 0.1 μg/g particles.

2.4. Mutagenicity of organic extracts

The mutagenicity of OE was evaluated in overnight cultures of 3 *Salmonella typhimurium* tester strains TA98, YG1041, and TA102, without (−S9mix) and with (+S9mix) Aroclor-induced S9 addition, using the preincubation method previously described [11]. Revertant colonies were automatically counted (Ames software, Noesis, France) after 48-h incubation at 37 °C. For each sample, four concentrations (2, 10, 50, and 200 μg equivalent-particles/plate) were tested in triplicate. Toxicity was evaluated in parallel through microscopic observation of the background lawn density. Results were expressed as the mean of at least two independent experiments.

Positive controls were 2-nitrofluorene (2NF) 2.5 μg/plate (−S9mix) and 2-aminofluorene (2AF) 0.5 μg/plate (+S9mix) for TA98; 2NF 1.25 μg/plate (−S9mix) and 2AF 0.1 μg/plate (+S9mix) for YG1041 and terbutylhydroperoxyde 2 μmol/plate for strain TA102 (−S9mix).

2.5. Mutagenicity of resuspended particles ("off line" mutagenicity)

The DEPs were resuspended in either DMSO or PBS with 0.04% tween, in order to limit the formation of aggregates in this aqueous solvent. The mutagenicity of these suspensions was evaluated using the procedure described above, in a range of concentrations from 5 to 75 μg/plate for DEPs in DMSO and from 5 to 100 μg/plate for DEPs in PBS.

2.6. Mutagenicity of whole aerosols ("on line" mutagenicity)

The Ames test procedure was adapted for a 6-well plate protocol to evaluate the mutagenicity of the whole aerosols. For TA98 and YG1041 strains, a VBE medium was complemented with an Q3 Histidin + Biotin (0.5 mM) solution (2.5 mL in 100 mL), and introduced in 6-well plates (8 mL/well). Fifty micro liter (TA98) or 40 μL (YG1041) of overnight cultures were then poured onto the agar surface. For the TA102 strain, a VBE medium was first distributed into wells, then 7.5 μL of a (Histidin + Biotin) 0.5 mM solution was

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poured onto the solid agar together with 25 μL of the TA102 overnight culture.

The plates were then preincubated 4 h at 37 °C, before exposure to diluted aerosols in chambers specifically designed and connected with a continuous aerosol flow [12]. Three exhaust sampling points were studied: directly at the diesel engine output (upstream of the DOC) (P1), downstream of the DOC (P2) and downstream of both the DOC and the DPF (P3), for the 3 diesel fuels. Dose-effect relationships were investigated. So that, in each case, the native stream was firstly diluted using a fine particle sampler (FPS, Dekati Finland), prior to a secondary dilution, controlled by mass flow regulators. Finally, the exposure concentrations corresponded to 5 and 10% of the native flow, respectively. Exposure time was of 5 h, in a continuous aerosol stream as recommended by Fall et al. [13]. After 48 h incubation at 37 °C, revertant colonies were manually counted and background lawn density was checked. Each condition was evaluated in a 6-well plate, and in at least two independent experiments. Due to the additional 4-h preincubation step at 37 °C before exposure and the limited stability of S9 fraction, the effect of a S9mix addition was not investigated in these "on line" exposure conditions.

This exposure protocol was firstly validated with NO_2, a gazeous genotoxicant, in the TA98–S9mix [14], using a 5 ppm flow (2 L/mn) for 1 h. The calculated ratio (induced revertants/spontaneous revertants) was 3.8 (data not shown).

2.7. Cultures of A549 cell line

A549 cells were cultured in a DMEM medium (Life Sciences) supplemented with 10% foetal bovine serum. Forty-eight hours before exposure, A549 cells were seeded either on flasks (for the "off line" protocol) or on PTFE membrane inserts (porosity 1 μm) for the "on line" protocol. For the latter, an ALI exposure was performed: the apical medium was removed just prior to exposure in continuous flow exposure chambers as described in Papaioannou et al. [15].

2.8. Organotypic cultures obtained from rat lung tissue

Rat lung slices were prepared as described by Le Prieur et al. [16] and further developed by Bion et al. [17]. The freshly prepared lung slices (350 μg protein) were positioned onto the titanium grid of a teflon rolling insert (Vitron) and placed into vials with opened caps (Wheaton). The vials contained 1.5 ml of DMEM/Ham F12 (50:50) medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 10^{-7} mol/L hydrocortisone, 5 ng/ml sodium selenite, 50 UI/ml penicillin, 50 μg/ml streptomycin, 4 mmol/L glutamine, and 1 g/l glucose. The vials were then placed horizontally on a roller at 37 °C in a humidified atmosphere of 5% CO_2, 95% air.

2.9. Bulky DNA-adducts post labelling

Q4 Bulky adducts formation was analysed on the DNA extracted:

(i) from A549 cells, exposed either "on line" to whole aerosols at 5% and 10% of the native flow (3 h), or "off line" to resuspended particles at 100 and 250 μg particles/mL (3 h and 24 h) or to OE at 100, 250, and 750 μg equiv. particles/mL (3 h, 24 h and 48 h),
Q5 (ii) from rat lung organotypic slices exposed 3 h *ex vivo* in "on line" conditions (Morin et al. [12]) to whole aerosols at 5% and 10% of the native flow.

For "off line" exposures, PM samples were resuspended directly in a cell culture medium to achieve the various concentrations. A549 cells were cultured on 6-well plates (Falcon 353046, Dutscher SAS, Brumath, France) in DMEM medium supplemented by 10% FBS, at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded 24 h before exposure to either particles, the whole aerosol, OE, B[*a*]P (10 μM) used as PAH control or to 1-NP (15 μM) used as nitro-PAH control. After exposure, cells were washed twice in cold PBS, scratched and centrifuged. Then, 1.2 ml extraction buffer (Tris–HCl, EDTA pH 7.4) was added to cellular pellets, together with SDS 10%, RNAse T1 and RNAse A (30 mn at 37 °C), followed by proteinase K (30 mn at 37 °C). Proteins were eliminated through phenol extraction followed by phenol:SEVAG (1/1) and SEVAG (chloroform/isoamyl alcohol 24/1). DNA was precipitated in cold ethanol, washed with ethanol, dried, and dissolved into milliQ water.

Organotypic slices were exposed in a biphasic air/liquid system [17] to the continuous flow of aerosol diluted at 10% and 5% of the raw exhaust flow for 3 h. Slices were then immediately frozen in liquid nitrogen and kept at −80 °C until DNA extraction. Slices were firstly disrupted in a 0.32 M sucrose solution using a TissueLyser device (Qiagen GmbH, Hilden, Germany). The homogenate was then centrifuged 10 min at 2500 rpm and 4 °C. The pellet was resuspended in 1.2 ml extraction buffer and then processed as described above.

DNA purity was checked through the determination of UV spectra between 228 nm and 300 nm (expected ratios: $1.8 < A^{260}/A^{280} < 1.95$ and $A^{260}/A^{230} > 2.3$). The concentration was deduced from A^{260}. DNA solutions were frozen at −80 °C until use.

DNA adduct measurements were performed using a ^{32}P post-labelling protocol adapted from Reddy and Randerath [18]. Briefly, 5 μg of DNA were digested by micrococcal nuclease (Sigma–Aldrich) and spleen phosphodiesterase (Calbiochem, VWR International France, Fontenay-Sous-Bois, France) for 4 h at 37 °C. Enrichment of adducts was obtained through nuclease P1 (Sigma–Aldrich) treatment for 30 min at 37 °C. Adduct labelling step was performed over 30 min at 37 °C by adding 10 units of T4 polynucleotide kinase (Fermentas, Saint-Rémy les Chevreuses, France) with 25 μCi ^{32}P-γATP (PerkinElmer Life Sciences) per sample. Separation of adducts was achieved through thin layer chromatography (TLC) on 12 cm × 12 cm PEI-cellulose plates (Macherey-Nagel, Hoerdt, France). Four migration solvents were used successively: 1 M sodium phosphate, pH 6.8 (D1, overnight); 8.5 M urea with 4.5 M lithium formate, pH 3.5 (D2, approximately 3 h); 8.5 M urea with 0.5 M Tris–HCl and 1.6 M lithium chloride, pH 8 (D3, approximately 3 h); 1.7 M sodium phosphate, pH 5.0 (D4, overnight). Autoradiograms were obtained after exposure of TLC-plates to Kodak Biomax films with intensifying screens for about 24 h at −80 °C. Quantification was expressed as the relative adduct levels (RALs) according to the following relation: $RAL = 110.7\ 10^{-8}\ cpm_{adducts}/cpm_{BPDE}$, where $cpm_{adducts}$ was the radioactivity measured from excised spots using a scintillation counter (Cerenkov mode), and cpm_{BPDE} was the radioactivity measured from the positive control (5 μg of DNA from calf thymus modified by Benzo[*a*]Pyrene Diol Epoxyde (BPDE), kindly provided by Dr F. Beland), corresponding to a known RAL (110.7 adducts for 10^8 normal nucleotides [19]). For each series of analyses, two negative controls (DNA extracted from a plasmid and DNA extracted from unexposed cells) were added.

2.10. Statistical analysis

Statistical analysis was performed on raw data (revertants/plate), using a one-tailed Dunnett test and ANOVA, on Sas glm procedure. Significant responses ($p < 0.05$) are in bold and the corresponding mutagenicity was calculated from the linear part of dose-response curve for Tables 2–4. For Table 5, since particles deposition was not quantified during "on line" exposures, only raw data (revertants/well) are displayed.

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Table 1
HAP content (μg/m³ exhaust) adsorbed on particles sampled from the 3 fuels, either downstream of the diesel engine or after the oxidation catalyst.

Molecules	W/o oxidation catalyst			With oxidation catalyst		
	DF0	DF7	DF30	DF0	DF7	DF30
Naphtalene	0.147	0.048	0.177	0.140	0.297	0.313
Phenanthrene	0.271	0.411	0.287	0.049	0.057	0.097
Anthracene	0.027	0.026	0.041	nd	0.06	0.010
Fluoranthene	0.076	0.069	0.076	nd	nd	0.021
Pyrene	0.130	0.154	0.147	nd	nd	0.012
Total	0.652	0.709	0.728	0.193	0.368	0.452

DF0: standard diesel fuel, DF7 and DF30: diesel fuels supplemented with 7% and 30% of rapeseed methyl ester, respectively. nd: not detected.

3. Results

3.1. Chemical analysis

Particles emitted by the combustion of the 3 diesel fuels were analysed for their PAH content (Table 1). The particles sampled downstream of the diesel engine (native particles) displayed a higher total PAHs content than the particles sampled downstream of the DOC (oxidised particles). The nature of the fuels had no effect on the total PAHs content adsorbed onto native particles. In contrast, oxidised particles emitted by the combustion of RME-supplemented fuels had about a two-fold higher total PAHs content than those emitted by DF0, which increased together with the RME content.

No PAH with a molecular weight higher than 202 g/mole was found. On native particles, the most abundant PAH was the Phenanthrene > Naphtalene ≥ Pyrene > Fluoranthene > Anthracene. All these PAHs were strongly reduced or eliminated on oxidised particles, except naphtalene which remained unchanged for DF0 but which was 1.8- to 6-fold increased for the RME-supplemented diesel fuels.

Other compounds detected (data not shown) included linear alcanes (C11–C31), methyl- and dimethyl-naphatalene, biphenyl, 9H-fluorenone, 1H-phenalenone, and 2-naphtalenecarboxaldehyde.

3.2. Mutagenicity of the organic extracts

The mutagenic data showed a systematic dose-mutagenicity relationship. Organic extracts obtained from native particles were mutagenic in TA98 (Table 2). Upon addition of S9mix, the mutagenicity was increased for DF0, but response was unchanged or slightly decreased for DF7 and DF30, respectively. In YG1041, the mutagenicity was strongly reinforced compared to TA98, leading to bactericidal effects at the highest concentrations. Upon addition of S9mix, the revertant numbers were reduced, but the bactericidal effect was not abolished at the highest dose tested. Calculated mutagenicities indicated that DF0 tended to be the more mutagenic, but extracts from RME-supplemented fuels were more bactericidal.

Organic extracts obtained from particles sampled downstream of the oxidation catalyst were not mutagenic in TA98, either without or with S9mix, except for DF30 which displayed a borderline mutagenicity. In YG1041, significant mutagenicities were reported, which were reduced upon addition of S9mix.

No mutagenicity was observed in TA102 whatever the conditions (native/oxidised particles, with/without S9mix).

3.3. Mutagenicity of the particles

Under "off line" exposure conditions, suspensions of particles in DMSO were firstly tested (Table 3) and mutagenicity was observed only for native particles (P1). In TA98, a weak response was obtained at the highest concentration without S9mix only. In YG1041, mutagenicity was reinforced again, particularly without S9mix, but significant mutagenicities were also obtained after addition of S9mix. RME-supplemented diesel fuels were more mutagenic than the DF0.

When resuspended in PBS + 0.04% tween, native particles displayed globally a mutagenic pattern (Table 4) comparable to that of particles resuspended in DMSO (Table 3).

Whatever the solvent used, particle suspensions were not mutagenic in TA102 (Tables 3 and 4).

3.4. Mutagenicity of the whole aerosols

Whole aerosols ("on line" exposure) emitted by the combustion of DF0 and DF7 fuels were mutagenic in TA98 when sampled downstream of the diesel engine (P1) (Table 5). A dose-effect trend was observed for DF30 aerosol, without reaching however the significant threshold. Downstream of the DOC (P2), only the DF0 aerosol remained mutagenic. Mutagenicity was fully abolished downstream of the DPF (P3).

In YG1041, no mutagenicity was observed, whatever the fuel type and the sampling point considered.

The aerosol emitted by DF0 combustion was not mutagenic in TA102. Recurrent bactericidal effects were observed with DF7 aerosol sampled in P1 or P2, and with DF30 in P1, which prevented the precise evaluation of the mutagenicity. Besides, dose–mutagenic responses were observed in P2 for DF30 and in P3 for DF7 and DF30 aerosols.

3.5. Formation of Bulky DNA-adducts

Bulky DNA-adducts were efficiently generated in A549 cells after 24 h exposure to B[a]P 10 μM and, to a lesser extent, to 1-NP 15 μM used as positive controls (Fig. 1). For BaP, one spot was observed, located on the central part of the cellulose sheet, in a position comparable to that of the BPDE-DNA adduct used as internal standard in each experiment. The spot formed with 1-NP displayed a lower intensity and was located nearer the origin.

None of the other experimental conditions performed on A549 cells and on organotypic rat lung slices led to the formation of DNA adducts. In more details, A549 cells were exposed to OE prepared from the various particles, and tested at 100 and 250 μg equiv. particles/mL during 3 h, 24 h, or 72 h or to 500 μg equiv. particles/mL for 48 h. Cytotoxicity was observed after 72 h exposure to 750 μg equiv. particles/mL. A549 cells were also exposed "off line" to particles resuspended in culture medium, at concentrations of 100 or 250 μg/mL for 3 h and 24 h. At last, A549 cells and organotypic lung slices were exposed "on line" to the whole aerosols for 3 h only, due to the drying of the cultures in the continuous aerosol flow.

4. Discussion

This paper compares in an original way, the mutagenicity and the genotoxicity of diesel exhausts according to three exposure

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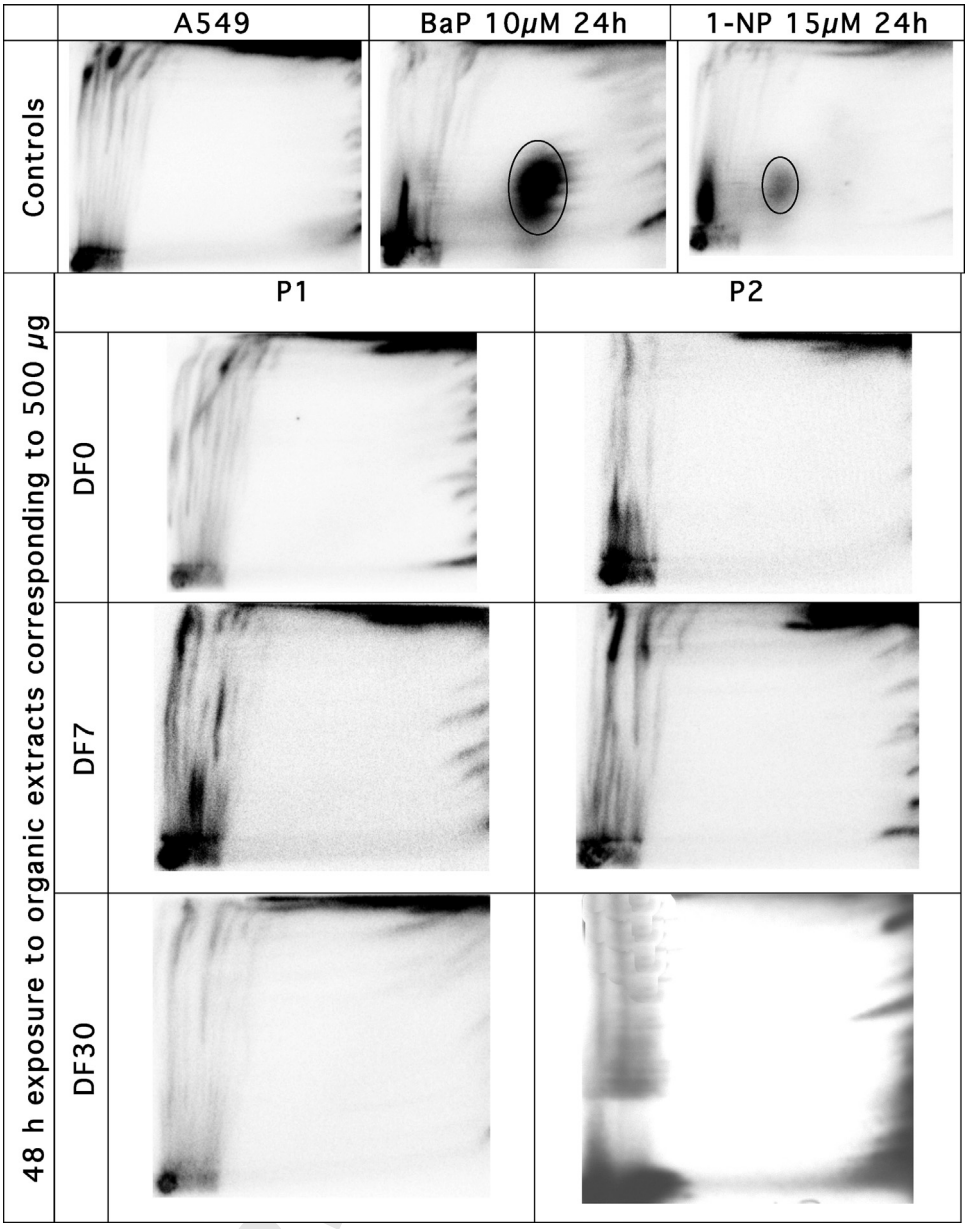

Fig. 1. Representative DNA adduct patterns obtained from A549 cells exposed 48 h to organic extracts (OE) prepared from diesel particles.
(P1) = OE prepared from particles collected downstream of the engine.
(P2) = OE prepared from particles collected downstream of the oxidation catalyst.
DF0 = standard diesel fuel, DF7 and DF30 = diesel fuels supplemented with 7% or 30% EMC, respectively.
Negative controls = DNA isolated from non exposed A549 cells.
Positive control = BaP 10 μM for PAHs and 1-nitropyrene (1-NP) 15 μM for nitro-PAHs.

conditions: the most often studied exposure to organic extracts (OE), the "off line" exposure to particles resuspended in an experimental aqueous medium and the "on line" exposure to a continuous aerosol flow. The former is mainly used for mechanistic interpretations, but corresponds to artificial situations as previously underlined by Borm et al. [20]. In contrast, the "off line" protocol is a first optimisation that integrates the notion of bioavailability of the adsorbed chemicals whereas the "on line" conditions are thought to be more realistic for the evaluation of the genotoxic impact after inhalation of the whole aerosol, and thus could be more relevant for a contribution to the health risk assessment of current diesel exhausts.

Interestingly, our results show that the mutagenic pattern of particles evaluated through "off line" exposure conditions clearly contrasts with that observed with the whole aerosols tested under "on line" conditions.

The main known contributors to the mutagenicity of DEP are the nitro-PAHs compounds adsorbed onto the particle surface. This is also suggested by the highest mutagenicity obtained in nitrocompounds-sensitive strain YG1041–S9mix, and the decreased response after S9mix addition. The involvement of non substituted-PAHs to the mutagenicity is very limited, since in TA98 + S9mix, no increase of the response was observed, but, on the contrary, a decrease in most cases. Thus, the borderline mutagenicity reported on TA98 strain could be mainly attributed again to nitro-PAHs, after a limited bioactivation in relation with the constitutive expression of the nitroreductase enzymes.

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Table 2
Mutagenicity of the organic extracts obtained from particles sampled either downstream of the diesel engine (P1) or downstream of the oxidation catalyst (P2). Extracts were tested at 2, 10, 50, and 200 μg equivalent particles/plate, either without (-S9) or with S9mix (+S9).

TA98	Fuel	DF0					DF7					DF30				
	μg/plate	0	2	10	50	200	0	2	10	50	200	0	2	10	50	200
P1	−S9	38 ± 6	41 ± 7	71 ± 2	108 ± 4	294 ± 15	37 ± 2	77 ± 23	66 ± 9	147 ± 17	448 ± 21	37 ± 3	36 ± 11	64 ± 9	136 ± 27	571 ± 41
		1.25 revertants/μg					2 revertants/μg					2.7 revertants/μg				
	+S9	54 ± 3	66 ± 8	100 ± 10	306 ± 23	334 ± 36	46 ± 13	62 ± 1	67 ± 16	153 ± 27	425 ± 20	100 ± 3	120 ± 10	130 ± 4	173 ± 14	511 ± 33
		5 revertants/μg					1.9 revertants/μg					2 revertants/μg				
P2	−S9	31 ± 17	34 ± 6	33 ± 5	36 ± 3	28 ± 4	50 ± 5	57 ± 13	54 ± 15	56 ± 5	59 ± 6	37 ± 1	32 ± 1	39 ± 8	46 ± 11	98 ± 21
		Not mutagenic					Not mutagenic					0.3 revertants/μg				
	+S9	36 ± 4	29 ± 8	34 ± 5	36 ± 5	29 ± 10	60 ± 9	59 ± 11	63 ± 12	65 ± 1	60 ± 13	58 ± 9	62 ± 7	56 ± 12	72 ± 4	106 ± 3
		Not mutagenic					Not mutagenic					0.2 revertants/μg				

YG1041	Fuel	DF0					DF7					DF30				
	μg/plate	0	2	10	50	200	0	2	10	50	200	0	2	10	50	200
P1	−S9	61 ± 10	154 ± 1	545 ± 1	1217 ± 6	(T)	68 ± 6	146 ± 17	463 ± 76	(T)	(T)	59 ± 11	68 ± 10	333 ± 57	(T)	(T)
		48.5 revertants/μg					39.5 revertants/μg					29 revertants/μg				
	+S9	73 ± 6	141 ± 20	354 ± 20	631 ± 23	(T)	65 ± 15	84 ± 10	188 ± 62	473 ± 45	(T)	112 ± 20	133 ± 13	192 ± 19	535 ± 47	(T)
		27.7 revertants/μg					12.5 revertants/μg					8.4 revertants/μg				
P2	−S9	83 ± 10	103 ± 11	105 ± 11	147 ± 8	284 ± 11	73 ± 11	92 ± 1	122 ± 12	184 ± 51	378 ± 33	61 ± 4	63 ± 5	177 ± 15	519 ± 70	(T)
		0.9 revertants/μg					1.3 revertants/μg					9.2 revertants/μg				
	+S9	86 ± 11	86 ± 2	88 ± 8	102 ± 6	177 ± 23	50 ± 3	64 ± 3	61 ± 6	72 ± 8	124 ± 15	96 ± 7	94 ± 12	108 ± 12	153 ± 11	438 ± 42
		0.5 revertants/μg					0.3 revertants/μg					1.7 revertants/μg				

TA102	Fuel	DF0					DF7					DF30				
	μg/plate	0	2	10	50	200	0	2	10	50	200	0	2	10	50	200
P1	−S9	419 ± 17	391 ± 11	369 ± 13	350 ± 21	390 ± 32	494 ± 61	459 ± 82	453 ± 58	425 ± 21	511 ± 100	480 ± 30	431 ± 11	426 ± 28	450 ± 28	458 ± 87
		Not mutagenic					Not mutagenic					Not mutagenic				
	+ S9	420 ± 17	436 ± 24	468 ± 52	483 ± 22	501 ± 40	482 ± 35	526 ± 47	481 ± 2	531 ± 15	553 ± 44	547 ± 27	517 ± 32	555 ± 34	650 ± 34	549 ± 62
		Not mutagenic					Not mutagenic					Not mutagenic				
P2	−S9	486 ± 21	443 ± 31	457 ± 18	436 ± 20	452 ± 12	332 ± 20	310 ± 12	324 ± 24	338 ± 20	331 ± 7	506 ± 10	503 ± 52	474 ± 17	498 ± 58	487 ± 21
		Not mutagenic					Not mutagenic					Not mutagenic				
	+S9	505 ± 10	476 ± 46	550 ± 44	497 ± 38	501 ± 22	322 ± 6	326 ± 17	346 ± 23	357 ± 12	358 ± 8	486 ± 2	587 ± 2	545 ± 11	505 ± 48	538 ± 17
		Not mutagenic					Not mutagenic					Not mutagenic				

DF0: standard diesel fuel, DF7 and DF30: diesel fuels supplemented with 7% and 30% of rapeseed methyl ester, respectively. Significant responses (revertants/plate) are in bold and the corresponding mutagenicity (revertants/μg particles) was calculated from the linear part of the dose-response curve. (T): toxic/bactericidal effect.

The significant and comparable mutagenic responses observed for particles resuspended in organic or in aqueous solvent indicate that adsorbed chemicals are partly water soluble and thus bioavailable enough to exert their mutagenic properties. In this respect, since nitroaromatic compounds are more polar and more mutagenic than their corresponding non substituted PAHs congeners, they appear as the major contributors to the observed mutagenicity. Involvement of PAHs and nitro-PAHs in the mutagenicity of diesel exhausts was previously reported in studies conducted with organic extracts (OE) [21–26]. In the present study, mutagenic patterns were comparable for particles tested "off line" and for their corresponding OE, but responses were notably reinforced for the latter. However, even for OE, the contribution of PAHs was still very limited, and could be evoked only for DF0 extract. In a review, Hesterberg et al. [27] compared the chemical composition of particles issued from new technology diesel exhaust gases (NTDE) with particles issued from traditional diesel exhaust (TDE). They report profound modifications of the chemical composition, and notably an average 60–97% reduction of the PAHs content in particles issued from the combustion of current diesel fuels. These modifications are generally attributed to the lower sulphur content in NTDE, associated with optimized engine technologies and after treatment devices [25,28]. Nitro-PAHs content is also modified in particles issued from NTDE, and generally reduced, but some potential nitration reactions associated with after treatment technologies are also reported, mainly depending on the oxidation potential of DPF [29,30]. Artifactual formation of nitro-HAPs on the sampling devices, due to the necessity of extensive collection time to obtain enough particle mass, is also mentioned [27]. In the present study, the "off line" mutagenicity was fully abolished for particles sampled downstream of the DOC, and dramatically reduced for the corresponding OE. The residual activity was observed only in the nitro-compounds sensitive strain, and at the highest doses tested. Thus, the DOC appears efficient for the reduction of the main chemicals of mutagenic concern, and artifactual formation of nitro-PAHs seems very limited, if any. Analytical data also show that the total amount of unsubstituted PAHs is efficiently reduced for DF0, and to a lesser extent for B7 and B30. Consequently, the substrates for this artifactual PAHs nitration reaction are considerably reduced. Naphtalene is the only PAH for which the concentration increases downstream of the DOC for the RME-fuels only, in accordance with a previous report [31]. But since nitro-naphtalenes are weakly mutagenic, their potential artifactual nitration, if any, does not contribute significantly to the particles mutagenicity.

The compared mutagenicities between DF0 and RME-supplemented fuels show that particles emitted by combustion of biodiesels appear slightly more mutagenic, but no clear relationships with the RME content can be established. RME-particles were generally reported to be less mutagenic than those issued from diesel fuel [32–35]. Nevertheless, a contrasted conclusion was also reported by Bünger et al. [36] and Westphal et al. [37], in accordance with the present study. It could be hypothesised that in more recent studies, diesel fuels with improved quality ("NTDE like") are used, which are less mutagenic than the traditional ones, leading to the observed lower mutagenicity for diesel fuel compared to RME blends.

In contrast to the mutagenic pattern described above for particles, the mutagenicity of the whole aerosols is essentially linked to their gas phase. Actually, no significant mutagenicity can be

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Table 3
Mutagenicity of the particles resuspended in DMSO ("off line" exposure). Particles were sampled either downstream of the diesel engine (P1) or downstream of the oxidation catalyst (P2). They were tested at 5, 25, and 75 μg particles/plate, either without (−S9) or with S9mix (+S9).

TA98	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	75	0	5	25	75	0	5	25	75
P1	−S9	20 ± 5	25 ± 3	24 ± 1	43 ± 3	20 ± 8	22 ± 4	29 ± 9	44 ± 8	14 ± 6	14 ± 4	27 ± 6	27 ± 4
		0.3 revertants/μg				0.3 revertants/μg				0.5 revertants/μg			
	+S9	31 ± 9	38 ± 5	33 ± 4	35 ± 3	23 ± 1	21 ± 4	21 ± 8	35 ± 1	16 ± 1	20 ± 6	18 ± 6	27 ± 7
		Not mutagenic				Not mutagenic				Not mutagenic			
P2	−S9	20 ± 9	30 ± 10	24 ± 6	21 ± 2	21 ± 4	24 ± 2	17 ± 2	23 ± 1	13 ± 2	10 ± 3	13 ± 2	14 ± 4
		Not mutagenic				Not mutagenic				Not mutagenic			
	+S9	19 ± 4	26 ± 3	23 ± 5	19 ± 1	23 ± 1	17 ± 2	15 ± 2	18 ± 3	15 ± 3	21 ± 3	18 ± 4	14 ± 3
		Not mutagenic				Not mutagenic				Not mutagenic			

YG1041	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	75	0	5	25	75	0	5	25	75
P1	−S9	72 ± 5	112 ± 8	224 ± 54	462 ± 44	36 ± 9	118 ± 6	315 ± 42	(T)	69 ± 2	141 ± 19	311 ± 12	567 ± 43
		5.1 revertants/μg				10.8 revertants/μg				9.3 revertants/μg			
	+S9	72 ± 7	81 ± 12	113 ± 5	227 ± 28	75 ± 10	106 ± 15	202 ± 18	468 ± 2	59 ± 11	79 ± 11	194 ± 7	389 ± 30
		2.1 revertants/μg				5.2 revertants/μg				5.5 revertants/μg			
P2	−S9	79 ± 1	61 ± 4	78 ± 3	60 ± 7	92 ± 4	98 ± 3	95 ± 16	88 ± 14	70 ± 2	62 ± 14	85 ± 3	84 ± 16
		Not mutagenic				Not mutagenic				Not mutagenic			
	+S9	147 ± 33	149 ± 7	152 ± 21	158 ± 24	77 ± 8	64 ± 9	62 ± 19	68 ± 6	76 ± 11	65 ± 7	68 ± 12	88 ± 5
		Not mutagenic				Not mutagenic				Not mutagenic			

TA102	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	75	0	5	25	75	0	5	25	75
P1	−S9	478 ± 8	529 ± 38	493 ± 7	471 ± 29	444 ± 15	422 ± 8	355 ± 24	473 ± 38	351 ± 15	365 ± 17	354 ± 13	364 ± 7
		Not mutagenic				Not mutagenic				Not mutagenic			
	+S9	406 ± 68	496 ± 8	495 ± 4	468 ± 19	367 ± 13	416 ± 24	413 ± 24	414 ± 13	408 ± 16	427 ± 38	405 ± 23	405 ± 21
		Not mutagenic				Not mutagenic				Not mutagenic			
P2	−S9	482 ± 27	533 ± 22	503 ± 19	493 ± 24	499 ± 21	476 ± 21	463 ± 18	489 ± 34	383 ± 18	366 ± 61	414 ± 24	384 ± 12
		Not mutagenic				Not mutagenic				Not mutagenic			
	+S9	451 ± 12	453 ± 22	423 ± 25	462 ± 31	462 ± 13	448 ± 40	454 ± 2	464 ± 20	447 ± 12	461 ± 9	443 ± 27	482 ± 14
		Not mutagenic				Not mutagenic				Not mutagenic			

DF0: standard diesel fuel, DF7 and DF30: diesel fuels supplemented with 7% and 30% of rapeseed methyl ester respectively. Significant responses (revertants/plate) are in bold and the corresponding mutagenicity (revertants/μg particles) was calculated from the linear part of the dose-response curve. (T): toxic/bactericidal effect.

observed on YG1041, indicating that the major mutagenic nitro-compounds issued from particles are not bioavailable. Hence, it can be assumed that under a continuous flow of aerosols, particles are probably deposited in limited quantities, and thus do not allow the release of sufficient amounts of mutagenic chemicals. In parallel, significant and dose-dependant responses are obtained only in TA98–S9mix, and thus, can be attributed to volatile or semi-volatile compounds in the gas phase, that cannot be taken into

Table 4
Mutagenicity of the particles resuspended in PBS ("off line" exposure). Particles were sampled downstream of the diesel engine (P1). They were tested at 5, 25 and 100 μg particles/plate, either without (−S9) or with S9mix (+S9).

TA98	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	100	0	5	25	100	0	5	25	100
P1	−S9	20 ± 1	29 ± 9	33 ± 4	39 ± 6	23 ± 3	27 ± 4	28 ± 4	39 ± 6	13 ± 3	25 ± 6	29 ± 1	55 ± 12
		0.1 revertants/μg				Not mutagenic				0.3 revertants/μg			
	+S9	21 ± 2	24 ± 2	26 ± 2	42 ± 13	18 ± 1	25 ± 4	21 ± 3	30 ± 4	23 ± 6	28 ± 6	40 ± 13	59 ± 10
		0.2 revertants/μg				Not mutagenic				0.7 revertants/μg			

YG1041	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	100	0	5	25	100	0	5	25	100
P1	−S9	58 ± 1	125 ± 5	163 ± 51	371 ± 30	79 ± 7	138 ± 3	354 ± 20	696 ± 95	60 ± 15	76 ± 18	234 ± 31	533 ± 33
		2.6 revertants/μg				10.9 revertants/μg				7.2 revertants/μg			
	+S9	51 ± 4	56 ± 6	116 ± 24	320 ± 22	56 ± 7	82 ± 3	176 ± 8	534 ± 52	50 ± 1	65 ± 2	122 ± 14	339 ± 10
		2.7 revertants/μg				4.8 revertants/μg				2.9 revertants/μg			

TA102	Fuel	DF0				DF7				DF30			
	μg/plate	0	5	25	100	0	5	25	100	0	5	25	100
P1	−S9	409 ± 27	445 ± 13	530 ± 30	494 ± 29	391 ± 5	354 ± 20	343 ± 11	344 ± 17	397 ± 37	390 ± 4	393 ± 2	430 ± 8
		Not mutagenic				Not mutagenic				Not mutagenic			
	+S9	454 ± 41	400 ± 15	419 ± 27	466 ± 12	332 ± 16	381 ± 28	413 ± 22	360 ± 18	464 ± 13	443 ± 29	440 ± 22	437 ± 22
		Not mutagenic				Not mutagenic				Not mutagenic			

DF0: standard diesel fuel, DF7 and DF30: diesel fuels supplemented with 7% and 30% of rapeseed methyl ester, respectively. Significant responses (revertants/plate) are in bold and the corresponding mutagenicity (revertants/μg particles) was calculated from the linear part of the dose–response curve. (T): toxic/bactericidal effect.

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Table 5
Mutagenicity (revertants/well) of the whole aerosols ("on line" exposure). Aerosols were sampled downstream of the diesel engine (P1), downstream of the oxidation catalyst (P2) and downstream of the particulate filter (P3). They were tested at 5% and 10% of the native flow, without S9mix (−S9).

TA98	Fuel	DF0			DF7			DF30		
	% native flow	0	5%	10%	0	5%	10%	0	5%	10%
−S9mix	P1	16 ± 3	35 ± 6	51 ± 12	15 ± 3	31 ± 5	41 ± 6	25 ± 8	38 ± 14	47 ± 11
	P2	16 ± 3	30 ± 8	39 ± 5	14 ± 4	26 ± 11	16 ± 4	22 ± 6	30 ± 7	35 ± 13
	P3	17 ± 1	14 ± 2	19 ± 2	19 ± 5	21 ± 4	38 ± 6	29 ± 10	31 ± 6	31 ± 12
YG1041	Fuel	DF0			DF7			DF30		
	% native flow	0	5%	10%	0	5%	10%	0	5%	10%
−S9mix	P1	44 ± 2	51 ± 10	52 ± 10	38 ± 9	53 ± 11	58 ± 12	48 ± 6	56 ± 12	65 ± 5
	P2	44 ± 2	43 ± 2	66 ± 5	39 ± 8	53 ± 9	52 ± 10	48 ± 9	53 ± 13	61 ± 13
	P3	42 ± 2	43 ± 6	50 ± 3	49 ± 9	55 ± 8	56 ± 8	49 ± 10	62 ± 7	62 ± 6
TA102	Fuel	DF0			DF7			DF30		
	% native flow	0	5%	10%	0	5%	10%	0	5%	10%
−S9mix	P1	94 ± 20	90 ± 4	93 ± 12	112 ± 31	97 ± 17	(T)	63 ± 14	80 ± 13	(T)
	P2	71 ± 3	63 ± 6	77 ± 8	148 ± 22	(T)	(T)	74 ± 6	93 ± 26	116 ± 20
	P3	94 ± 20	78 ± 17	90 ± 6	77 ± 6	94 ± 18	106 ± 10	52 ± 7	57 ± 6	82 ± 12

DF0: standard diesel fuel, DF7 and DF30: diesel fuels supplemented with 7% and 30% of rapeseed methyl ester respectively. Significant responses are in bold. (T): toxic/bactericidal effect.

account through the "off line" exposure protocol. It is reported that, from diesel fuel combustion, most of the PAHs are emitted in the gas phase [31,38]. However, the direct mutagenicty observed here under "on line" conditions cannot be ascribed to this class of pollutants, since assays were performed without S9mix only. For the validation of the "on line" protocol, NO_2 was used according to Aufderheide et al. [14] and this gas was found mutagenic in TA98–S9mix. Since NO_2 is one of the major components produced in the gaseous phase during the combustion of diesel fuels, it could contribute to the responses obtained with the whole aerosols. Another discrepancy between "off line" and "on line" mutagenic patterns was observed in TA102 in which low but significant mutagenicity was obtained, with RME-fuel exhausts after treated with DOC and DPF devices (P3). Besides, a recurrent toxicity was obtained with DF7 and DF30 in P1 and in P2 for DF7 only, that could hide a mutagenic response in these conditions. Compared with the complete absence of mutagenicity obtained in "off line" conditions, it appears that the gas phase may also lead to a mutagenicity associated with a pro-oxydant pattern. In this respect, NO_2 and/or NO_x may be involved again. Besides, numerous carbonyl compounds are generated, such as formaldehyde, acetaldehyde, acrolein, or crotonaldeide [37] and could also contribute to the mutagenicity and/or the bactericidal effect.

Consequences of after treatment technologies on the mutagenicity of the various diesel fuels can also be evaluated from the "on line" exposure protocol. Hence, DOC clearly reduces the direct mutagenicity of whole aerosols in TA98 (P2), even if a significant activity persists for DF0 only. In TA102, the toxicity of DF7 was reinforced. Additional after treatment with DPF abolished the mutagenicity for DF0, whereas a borderline response was still observable for RME-blends in TA102 and in TA98 for DF7 only. Thus, combined after treatments (DOC + DPF) led to the release of a non mutagenic exhaust for DF0. However, for RME-diesel fuels, a weak mutagenicity was still apparent, presumably in relation with a pro-oxydant mechanism, with a higher impact for DF7 compared to DF30.

Few papers only deal with the mutagenic effects of the gas phase produced by diesel fuel and pure or blend RME [35–37]. Moreover, in such cases, authors investigated some condensates that were firstly concentrated, and it could be assumed that chemicals concentration was thus by far higher than in real exposure conditions or even than in the present "on line" protocol. Nevertheless, Westphal et al. [35] did not find any clear difference between mutagenicity of pure RME- or 5% RME blend-condensate and diesel fuel condensate in TA98. In all cases, mutagenic activities were reduced upon S9mix addition. But in a more recent study, the same authors reported that pure RME condensates were about 4-fold more mutagenic, in TA98 and TA100, than those extracted from diesel fuel, with again a significant reduction after S9mix addition [37]. In contrast, for Bünger et al. [36], mutagenic responses of a pure RME condensate were globally lower than with a diesel fuel condensate in these two *Salmonella* strains. In the present study, diesel fuels were only supplemented with RME, but gas phase issued from blends did not appear globally more mutagenic than those issued from DF0.

The impact of after treatment devices on the gas phase mutagenicity is poorly documented. Westphal et al. [35] compared the mutagenicity of condensates without or with DOC for different fuels and biodiesels in TA98. In all cases, mutagenicity was fully abolished with DOC after treatment.

Besides the mutagenicity determined in a prokaryote model, genotoxic properties of diesel exhausts have also been evaluated in eukaryote models, both from OE, resuspended particles and whole aerosols. No formation of bulky DNA-adducts was observed, even if A549 cells were fully able to metabolise nitroaromatics and PAHs as checked with exposures to 1-NP and BaP, respectively. These data confirm those concerning mutagenicity discussed above, and notably, the very limited role of PAHs, in contrast with most of the previous studies. For example, Topinka et al. [39] recently showed a pattern of adducts including a DRZ in an acellular model (DNA calf thymus) exposed 24 h to OE issued from particles obtained with diesel, biodiesel, and rapseed oil. They reported higher adduct levels upon S9mix addition, indicating the involvement of PAHs. Additionally, unidentified direct acting compound(s) led to a strong spot without exogenous activation system. These authors also reported that genotoxicity was mainly dependent on the type of engine (bus, truck, and tractor engines were compared) and on the test cycle applied (ESC *i.e.*, European Steady State Cycle and WHSC *i.e.*, World Harmonized Steady State Test Cycle, were compared) rather than on the fuel type. But it must be stressed that only heavy-duty engines were used in this paper, and without any after

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treatment devices, in contrast to the experiments performed in our study.

Under the "on line" conditions used in the present study, time exposure was necessarily reduced to 3 h due to the alteration of the cell monolayer, induced by the continuous aerosol stream. Using a comparable organotypic lung slices model, exposed with continuous rotation in and out a medium containing BaP 10 μM, Harrigan et al. [40] observed DNA add ucts formation after 24 h exposure, but not after only 4 h. Thus, the reduced exposure time applied with the "on line" protocol was clearly too short to allow the deposit of a relevant amount of particles and a sufficient contact duration with slices.

Bioavailability is also a key parameter after exposure to resuspended particles or to aerosols. Hesterberg et al. [27] stated that "whole diesel exhaust is not genotoxic to cells in culture due to the minimal bioavailability of the mutagenic compounds, *e.g.*, PAHs and nitro-PAHs, in diesel exhaust particles in lung fluids". In a study specifically designed for the evaluation of PAH bioavailability from particles, Borm et al. [20] did not report any leaching of PAHs from carbon black and diesel particles to aqueous medium, even when surfactant was added. They detected DNA adduct formation, including a DRZ, on A549 cells after 24 h exposure to some of the OE only, but not after exposure to the particles. From their extrapolations, they concluded that a minimal concentration of 0.01 μM of soluble BaP would be necessary to induce detectable DNA adducts. But the particle concentrations required for such amounts of soluble BaP are toxic under *in vitro* exposure conditions and are totally irrelevant for *in vivo* deposition processes. Based on a study performed in dogs, Gerde et al. [41] reported that BaP, used as surrogate PAH, was deposited at 80% in the alveolar region, quickly desorbed from soot and passed into the blood without major biotransformation, whereas the remaining 20% were deposited in the conducting airways then slowly desorbed with intense metabolism. Besides, no specific DNA-adducts were found in rat lungs after 13-week exposure to carbon black particles, and the authors concluded that PAHs played no significant role in the induction of tumors lung in rats, but ascribed the mechanism to chronic inflammation processes and associated cell proliferation [20].

Nitroaromatics are more polar than PAHs, and could partially desorbe from particles as objectivated by the mutagenic activity found in *S. typhimurium* YG1041, a strain that overexpresses enzymes involved in the metabolism of nitrocompounds. Nitroreduction capabilities of eukaryote cells are, however, by far lower compared to YG1041, and thus do not allow an efficient bioactivation of nitrocompounds, a necessary step for DNA adduct formation.

In our laboratory, an *in vivo* study is currently ongoing in rats, in order to objectivate the deposition of particles in lung tissue after chronic inhalation of whole aerosols, and to re-evaluate the genotoxicity of current diesel exhaust gases in the target tissue.

5. Conclusions

The mechanistic study performed "off line" on raw particles shows that PAHs are poorly represented in current engine exhaust and do not contribute to the mutagenic profile. In contrast, highly mutagenic nitroaromatics are bioavailable enough to induce significant mutagenicity in the specifically designed prokaryote model. However, this mutagenicity is abolished after DOC after treatment. DNA adducts formation was not observed on A549 cells, even under drastic exposure conditions. Together, these data indicate that the genotoxicity of particles issued from current diesel fuels is notably reduced compared with previous ones, and underline the efficiency of the DOC devices.

The "on line" exposure model takes into account the particles and the gas phase of the exhaust simultaneously, at concentrations far lower than in OE, in dynamic (continuous flow) and biphasic (air–liquid interface) conditions that globally lead to more realistic exposure conditions. Current diesel fuel and RME-blends were compared, on a bench equipped with a light-duty diesel engine and after treatment devices that allowed a study of exhaust in a current European urban environment. Using this approach, we found that particles are minor contributors to the genotoxicity of whole diesel exhaust gases since they are firstly efficiently cleaned of adsorbed pollutants through the DOC as described above, then efficiently retained onto the DPF.

In contrast, the gas phase appears mutagenic downstream of the diesel engine. After combined (DOC + DPF)-treatments, a weak but significant mutagenicity is still apparent for RME-diesel fuels, potentially derived from a pro-oxidant mechanism. No apparent genotoxicity was observed on eukaryote models, but reduced exposure time appears as a limiting factor.

Finally, the present re-evaluation of the diesel exhaust properties determined *in vitro* indicates a limited genotoxicity for recent light-duty engines equipped with efficient after treatment devices. In such conditions, the efforts to come must be focused on the gas phase toxicity, particularly for RME-diesel fuels. The "on line" exposure protocol appears useful for studying the effects of the whole exhaust, but it must be improved to allow extended exposure on eukaryote cellular models.

Conflict of interest

The authors declare that there are no conflicts of interest.

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