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Chemical characterization of fine and ultrafine PM, direct and indirect genotoxicity of PM and their organic extracts on pulmonary cells

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ABSTRACT

Particulate matter in ambient air constitutes a complex mixture of fine and ultrafine particles composed of various chemical compounds including metals, ions, and organics. A multidisciplinary approach was developed by studying physico-chemical characteristics and mechanisms involved in the toxicity of particulate atmospheric pollution. $PM_{0.3–2.5}$ and $PM_{2.5}$ including ultrafine particles were sampled in Dunkerque, a French industrialized seaside city. PM samples were characterized from a chemical and toxicological point of view. Physico-chemical characterization evidenced that $PM_{2.5}$ comes from several sources: natural ones, such as soil resuspension and marine sea-salt emissions, as well as anthropogenic ones, such as shipping traffic, road traffic, and industrial activities. Human BEAS-2B lung cells were exposed to $PM_{0.3–2.5}$, or to the Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and $PM_{2.5}$. These exposures induced several mechanisms of action implied in the genotoxicity, such as oxidative DNA adducts and DNA Damage Response. The toxicity of PM-EOM was higher for the sample including the ultrafine fraction ($PM_{2.5}$) containing also higher concentrations of polycyclic aromatic hydrocarbons. These results evidenced the major role of organic compounds in the toxicity of PM.

Introduction

The causal relationship between levels of air pollution and mortality and morbidity, particularly for pulmonary and cardiovascular causes, has recently been reinforced by the classification of air pollution and particulate matter (PM) itself as carcinogenic to humans by the International Agency for Research on Cancer (Loomis et al., 2013). However, due to both the complexity of the physico-chemical nature of atmospheric pollutants, on the one hand, and the mechanistic nature of tumor processes, on the

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other hand, the mechanisms of action involved remain partially elucidated. PM constitutes a major component of air pollution. Despite intensive research activity, there are still many major knowledge gaps over its sources, atmospheric pathways and health effects (Gualtieri et al., 2017). Particles enter the airway and lung by inhalation, depending on their diameter. Fine PM, with an aerodynamic diameter less than 2.5 μm ($PM_{2.5}$) can penetrate deeply into the alveoli and reach the systemic circulation. It can cause respiratory diseases, such as asthma, chronic obstructive pulmonary disease and lung cancer. PM-induced toxicity is mainly triggered by several mechanisms in the framework of oxidative stress, inflammation, genotoxic, and epigenetic changes (Kim et al., 2017).

Since the well-known review by Harrison and Yin (2000), several studies investigated whether the toxicity of PM resides in some particular fraction of the particles as defined by chemical composition or size (Harrison and Yin, 2000). For example, Kroll et al. were interested in the influence of the size of PM on different toxicity mechanisms. They concluded that the smaller the PM, the higher the Reactive Oxygen Species (ROS) production. This could be explained by the significantly larger number of particles corresponding to the smallest PM *versus* larger size fractions, allowing more potential contact and surface reactivity. Therefore, the particle number may be most effective in inducing oxidative stress *in vitro* (Kroll et al., 2013). To assess the contribution of chemical components in cellular responses to PM, chemical fractionation of PM is more and more used (Heo et al., 2015; Oh et al., 2011). Depending on the family of PM components of interest, particle extraction could be performed using aqueous media, organic solvents or combination of them in order to obtain various fractions more or less polar.

Using a multidisciplinary approach involving physico-chemical characterization and *in vitro* toxicity assays, the present work aimed to strengthen scientific knowledge on the pulmonary toxicity of fine and ultrafine fractions of PM sampled on a multi-influenced site. Two atmospheric PM samples have been considered: $PM_{0.3–2.5}$ corresponding to the fine particles excluding ultrafine one, and $PM_{2.5}$ integrating both the fine and ultrafine particles. Our work then focused on the genetic pulmonary toxicity of $PM_{0.3–2.5}$ in its entirety, as well as the organic fractions of $PM_{0.3–2.5}$ and $PM_{2.5}$. After a thorough physico-chemical characterization, the toxicity of PM and of their organic fractions was investigated in a model of a non-tumor human bronchial cell line (BEAS-2B cell line). The explored mechanisms of action of PM were the biotransformation of organic compounds and genotoxic modifications.

1. Materials and methods

1.1. PM sampling

The sampling site was located in the city center of Dunkerque (latitude: 51°2′10″N; longitude: 2°22′46″E), a heavy industrialized sea-side city counting about 210,000 inhabitants with its suburbs and located on the southern coast of the North-Sea. This site is impacted by various PM sources related to urban activities such as the motor vehicle traffic arising from the urban road network and motorways, industrial activities from the nearby industrial park (*e.g.*, iron and aluminum metallurgy, oil refinery, chemical and pharmaceutical industry) and also marine activities with natural sea spray, and shipping emissions (Kfoury et al., 2016).

$PM_{0.3–2.5}$ sampling was performed during 5 months in spring–summer 2011 using a high volume (68 m^3/hr) five-stages plus back up cascade impactor (model 235 TFIA-2, Staplex®, USA), using a protocol previously validated (Cazier et al., 2016). Briefly, impaction plates were mounted without any filter except on the first stage in order to retain particles with the highest diameters. The impaction system was changed every 7 days, and two devices were used in parallel in order to collect sufficient masses of $PM_{0.3–2.5}$ and therefore to be able to study both physico-chemical characteristics and toxicological endpoints. After sampling, impaction plates were dried under a laminar flow hood during 48 hr, and then $PM_{0.3–2.5}$ were recovered from the collection plates and immediately stored at −20°C.

$PM_{2.5}$ was collected in parallel using the high volume sampler DA-80 (DA80, DIGITEL®, Switzerland) equipped with a $PM_{2.5}$ cut-off diameter impactor head and working at a 30 m^3/hr flow rate (Kfoury et al., 2016). The high purity quartz microfiber filters (grade QM-A, Whatman®, GE Healthcare Life Sciences, United Kingdom) used for sampling were heated up to 450°C during 8 hr beforehand in order to eliminate the presence of carbon-containing impurities. DA80 sampler was configured to automatically change the filter every 14 days or when the pump was not able to maintain the 30 m^3/hr air flow rate (overloading of the filter with particles) to minimize the number of collected filters during the whole period. After sampling, all filters were dried under a laminar flow hood during 24 hr and then stored in a freezer (−20°C) until their use for chemical analysis and toxicity tests.

1.2. Characterization of collected PM samples and their fractions

Both $PM_{0.3–2.5}$ and $PM_{2.5}$ samples were submitted to an extensive physical and chemical characterization. $PM_{0.3–2.5}$ size distribution was performed using the scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM–EDX; Leo 438 VP microscope and IXRF analysis system; LEO Electron Microscopy Ltd., Carl Zeiss, United Kingdom). Water-soluble Fractions (WFs) were obtained by lixiviation of $PM_{0.3–2.5}$ and portions of $PM_{2.5}$ filters in ultra-pure water, followed by 30 min of sonication. Metals and ionic species were then quantified in PM and WFs by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, iCAP 6000 Series, Thermo Scientific, UK), by ICP-Mass Spectrometry (ICP-MS, Varian® 820-MS, Varian, USA) and by Ion Chromatography (IC, Dionex® DX 100, Thermo Scientific, UK) respectively. Polycyclic aromatic hydrocarbons (PAHs) were quantified after Soxhlet extraction with dichloromethane by Gas Chromatography–Mass Spectrometry (GC–MS, 1200 TQ, Varian, USA). PolyChlorinated Dibenzo-p-Dioxins (PCDD) and -Furans (PCDF), and Dioxin-Like PolyChlorinated Biphenyls (DLPCB) were analyzed by High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS, HP6890, Agilent Technologies, France/Autoconcept, Mass Spectrometry International, UK). The analytical procedures used for this study have been published elsewhere (Billet et al., 2007, 2008; Cazier et al., 2016; Ledoux et al., 2006).

1.3. Sample preparation for toxicological studies

To explain the role of organic and inorganic component of PM, Extractable Organic Matters (EOMs) were carried out by Soxhlet extraction from $PM_{0.3–2.5}$ and $PM_{2.5}$ filters using dichloromethane at 40°C for 16 hr. Dichloromethane was then evaporated under a nitrogen flow before reconstitution of the organic extract in dimethylsulfoxide (DMSO).

BEAS-2B cells were directly exposed to EOMs diluted in cell culture medium. When using particulate samples, $PM_{0.3–2.5}$ were suspended in cell culture medium to obtain the final concentration and sonicated for 5 min immediately before exposure, in order to maximize particle dispersion.

1.4. Cell culture, cytotoxicity and cell proliferation assay

BEAS-2B cell line was obtained from European Collection of Cell Cultures (ECACC, Wiltshire, UK), and was originally derived from normal bronchial epithelial human cells obtained from autopsy of non-cancerous individuals. BEAS-2B cells were cultured in complete Bronchial Epithelial cell Growth Medium (BEGM) from Lonza (Vervier, Belgium) and kept in a humidified atmosphere at 37°C and 5% CO_2. All the BEAS-2B cells used in this study derived from an original cell culture.

Prior conducting toxicological studies, the cytotoxicity of $PM_{0.3–2.5}$ sample, and EOMs of $PM_{0.3–2.5}$ and $PM_{2.5}$ was evaluated using cytotoxicity and proliferation assays. Lactate Dehydrogenase (LDH) release was measured in cell-free supernatant, while Mitochondrial Succinate Dehydrogenase (MDH) activity was measured in BEAS-2B cells exposed to $PM_{0.3–2.5}$ and EOMs of $PM_{0.3–2.5}$ and $PM_{2.5}$ after 24, 48 and 72 hr (Landkocz et al., 2017). Concerning the toxicological studies, culture medium was removed and replaced by fresh medium with or without $PM_{0.3–2.5}$ and EOMs of $PM_{0.3–2.5}$ and $PM_{2.5}$ during 6, 24, 48, or 72 hr (Table 1). Cells were exposed to $PM_{0.3–2.5}$ samples at a concentration of 3 or 15 $\mu g/cm^2$, selected to overlap the 10% inhibitory concentration according to the dose–response relationship previously determined by Landkocz et al. (2017). The dose of exposure to EOMs corresponds to the highest used PM-equivalent concentration (15 $\mu g/cm^2$). At the end of the exposure time (*i.e.*, 6, 24, 48, and 72 hr), cells were washed twice in ice-cold PBS, then separated for flow cytometry analysis, or immediately frozen at −80°C until studying other parameters (Table 1) .

1.5. Gene expression determination

Total RNA extraction from exposed and unexposed cells were carried out using TaqMan® Gene expression Cell-to-Ct kit (Ambion, Invitrogen-Life Technologies), following manufacturer's instructions. cDNA synthesis from gDNA-free mRNA was performed using high capacity cDNA RT (Applied Biosystems, Fisher scientific, France). cDNA coding for cytochromes P450 (CYP) 1A1, CYP1B1, NAD(P)H quinone deshydrogenase 1 (NQO1), 8-oxoguanine DNA glycosylase 1 (OGG1) and 18S rRNA (endogenous control) were then quantified using the TaqMan® Gene Expression Assay (Applied Biosystems) using specific probe/primers sets (respectively Hs00153120_m1, Hs00164383_m1, Hs02512143_s1, Hs01114116_g1, and Hs99999901_s1). Amplification was realized through 40 cycles, using the 7500 Fast Real-Time PCR System (Applied Biosystems). After PCR, the cycle threshold (Ct) was determined with Sequence Detection 7500 Software v2.0.3 (Applied Biosystems) and relative quantification (RQ) between exposed and untreated cells (UC) was achieved for each exposure time using the following calculation: $RQ = 2^{-\Delta\Delta Ct}$, (where $\Delta\Delta Ct = \Delta Ct_{exposed} - \Delta Ct_{unexposed}$ and $\Delta Ct = Ct_{target\ gene} - Ct_{18S}$) as described by Livak and Schmittgen (Livak and Schmittgen, 2001). Statistical significance was obtained when RQ was below 0.5 or above 2.0.

1.6. Genotoxicity

PM-induced genotoxic effects were monitored in cells, at different levels, using 5 complementary methods: enzyme-linked immunosorbent assay (ELISA) detection of an oxidized DNA nucleotide, ^{32}P post-labeling for bulky DNA adducts assay, Histone 2A.X phosphorylation (γ-H2A.X) quantification, telomerase activity and telomeres length measurement.

1.6.1. Oxidative DNA Damage

DNA adduct 8-OHdG concentrations were studied in cell lysates using a commercially available enzyme immunoassay, according to the original method (Toyokuni et al., 1997) with minor modifications (Garcon et al., 2006). Briefly, DNA was extracted using Nucleospin DNA column (Macherey-Nagel, France) and quantified by spectrophotometry. After thermal denaturation, DNA was digested by nuclease P1 (overnight, 37°C) and dephosphorylated by alkaline phosphatase (2 hr, 37°C). 8-OHdG adducts were then quantified using the 8-OHdG ELISA kit (Gentaur, Belgium), according to the manufacturer's instructions.

1.6.2. DNA bulky adducts

After a 72 hr exposure time, DNA was extracted, precipitated in cold ethanol, dried and dissolved in ultrapure water. DNA purity was checked by spectrophotometry. DNA adduct detection was performed using a ^{32}P post-labeling protocol as described previously (André et al., 2011). Briefly, after DNA digestion, enrichment of adducts was obtained by nuclease P1. The adduct labeling step was performed by T4 polynucleotide kinase with ^{32}P-γATP. Separation of adducts was achieved by thin layer chromatography (TLC) on PEI-cellulose plates, using successively 4 migration solvents (sodium phosphate, urea with lithium formate, urea with Tris–HCl and lithium chloride, and sodium phosphate). Autoradiograms were obtained after exposure of TLC-plates to Kodak Biomax films, before the measurement of the radioactivity of excised spots using a scintillation counter (Cerenkov mode). For each series of analyses, one positive control (calf thymus DNA modified by Benzo(*a*)Pyrene Diol Epoxide, BPDE) and two negative controls (DNA issued from a plasmid and DNA issued from unexposed cells) were added.

1.6.3. DNA Damage Response (DDR)

After 72 hr of exposure with or without $PM_{0.3–2.5}$ and EOMs of $PM_{0.3–2.5}$ and $PM_{2.5}$, cell pellets were suspended in cold PBS (4°C), and 37% formaldehyde solution was added at a final concentration of 2% (*v*/*v*). Cells were fixed for 20 min at room temperature, chilled on ice for 1 min and centrifuged (Lepers et al., 2014). Cell pellets were then suspended in ice-cold methanol (90%), incubated on ice for 30 min to allow cell permeabilization, and stored at −20°C until use. Flow collect

γ-H2A.X detection kit (Merck-Millipore, Germany) was used to quantify phosphorylation of H2A.X variant from histone H2A, as a marker of recognition, signaling and repair of Double-Strain Breaks (DSB) in cells, according to the manufacturer method. Alexa 488-anti-γ-H2A.X antibody and Alexa 488-mouse IgG isotype control were used. At least 10,000 individual cells were taken into account for analysis. γ-H2A.X was quantified as the differences in Median Fluorescence Intensity (ΔMFI) between antibody-labeled cells and isotype control-labeled cells for each sample. Induction ratio was calculated between exposed cells and control.

1.6.4. Telomerase activity and telomere length measurement

Telomerase activity was assessed using TRAPEZE RT Telomerase detection kit (Merck-Millipore, Germany), following manufacturer's instructions. Briefly, real-time PCR is used to quantify telomere-repeats amplified by telomerases in a first incubation stage. PCR was carried out using 7500 Fast Real-Time PCR System (Applied Biosystems, Life technologies, France). Bicinchoninic acid reagent kit was used to determine total protein content in cellular lysates. For evaluation of telomere length, DNA was extracted using the same procedure described above (RNA and DNA Nucleospin kit, Macherey Nagel, France). The size of telomeres was measured by qPCR (7500 Fast Real-Time PCR System) with primers for telomeres and 36B4 reference gene on DNA sample (Cawthon, 2009) and Fast Sybrgreen amplification mix (Applied Biosystems). After PCR, cycle threshold (Ct) was determined with Sequence Detection Software v2.0.3 (Applied Biosystems). The ratio of the length of samples was calculated by the technique of ΔΔCt, where $\Delta\Delta Ct = \Delta Ct_{exposed} - \Delta Ct_{unexposed}$ and $\Delta Ct = Ct_{telomere} - Ct_{36B4}$). Results for telomerase activity or telomere length were expressed as median value [Q1;Q3] of induction ratio between exposed cells versus control from 4 independent experiments.

1.7. Statistical analyses

Results were expressed as median and 1st and 3rd quartile [Q1;Q3] of 4 independent experiments. For each exposure time and concentration of PM, or EOMs exposed cells data were compared to those obtained for unexposed cells ($^{*}p < 0.05$) using Mann–Whitney U test. Comparison between highest concentration of PM and EOMs was also performed using Mann–Whitney U test ($^{\#}p < 0.05$). Statistical analyses were performed on SPSS software (SPSS 20.0, IBM, France) and Graphpad Prism 7.00 for Windows (GraphPad Software, USA).

2. Results

2.1. Inorganic and ionic composition of PM samples

By weighing of the collected particles on filters, the mean $PM_{2.5}$ concentration was estimated to be 14 μg/m^3 during the whole sampling period. This mean value appeared below the $PM_{2.5}$ annual limit values (LV) of 25 μg/m^3 (European Directive 2008/50/CE) but also higher than the WHO $PM_{2.5}$ guidelines (10 μg/m^3) even if concentration values higher than 25 μg/m^3 were observed many times, especially in March. The proportion of $PM_{0.3–2.5}$ in the $PM_{2.5}$ sample was estimated to be 58%. Inorganic components of $PM_{0.3–2.5}$ and $PM_{2.5}$ have been characterized (Fig. 1). In the $PM_{0.3–2.5}$ sample, the main elements counting for PM mass were Fe (3.1%), Na (2.0%), Ca (1.5%) and Al (1.5%) whose ambient concentration exceeded 100 ng/m^3. Concentration values for other detected elements, including Mn, Zn, Pb, Ni and Cr known for their toxicity (Fortoul et al., 2015) were below 50 ng/m^{-3}. The comparison between the composition of particles and its water soluble fraction illustrates the high solubility of Ca, Na, Mg, K and Sr. Elements as Mn, Ba Pb and Ni were found to have a solubility ratio in water ≥10% while Fe, Al and most of trace elements remained mainly in the particulate state in an aqueous medium.

2.2. Organic composition of PM samples

2.2.1. Polycyclic aromatic hydrocarbons

The total concentration of the 16 measured PAHs were found to be 0.67 ng/m^3 in $PM_{0.3–2.5}$ and 7.7 ng/m^3 in $PM_{2.5}$ during the sampling period. This observation reveals that PAHs were much more distributed in the ultrafine fraction of PM and could be likely related to the contribution of combustion sources (Kawanaka et al., 2009; Liu et al., 2015). The PAH concentration value in $PM_{2.5}$ appeared to be in the range 0.6–17 ng/m^3 as previously reported in the northern France region (Crenn et al., 2017). The PAH profile pattern (Fig. 2) showed that the more abundant compounds were BbF, BkF, Chr, BghiP and InPy corresponding to 64% and 77% of total PAHs in $PM_{0.3–2.5}$ and $PM_{2.5}$ respectively.

2.2.2. Dioxins and dioxin-like compounds

The concentration of the 17 toxic polychlorinated dibenzo-p-dioxins (PCDDs)/polychlorinated dibenzofurans (PCDFs) congeners, expressed in toxic equivalent unit (TEQ) as defined by WHO (Van den Berg et al., 2006), was estimated to be 144.5 fg TEQ/m^3 (Table 2). This value may appear high when compared to the values reported in French cities, such as Grenoble in 2006–2007 (20 to 80 fg TEQ/m^3), Toulouse in 2004 (36 to 50 fg TEQ/m^3) or Halluin also located in the northern of France (52 fg TEQ/m^3). However, it remained below the concentrations recorded in Ile-de-France (200 fg TEQ/m^3), in Cologne (Germany) (240 fg TEQ/m^3), and in Manchester (United-Kingdom)(410 fg TEQ/m^3) (Lohmann and Jones, 1998).

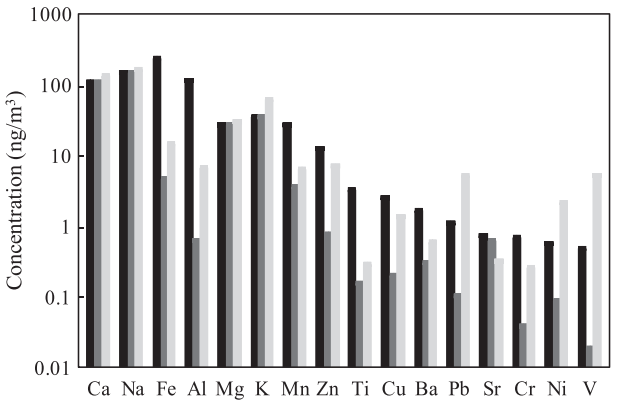

Fig. 1 – Inorganic elements detected in collected $PM_{0.3–2.5}$ (black), and in water-soluble fraction of $PM_{0.3–2.5}$ (dim grey) and of $PM_{2.5}$ (light grey). PM: particulate matter.

Table 1 – Conditions of exposure and measured toxicological endpoints.

Endpoint	Method	6 hr	24 hr	48 hr	72 hr
Gene expression	RT-qPCR	X	X	X	
DNA oxidation adduct (8-OHdG)	ELISA			X	
Bulky DNA adducts	^{32}P post-labeling				X
DNA Damage Response (γH2A.X)	Flow cytometry				X
Telomerase activity	Trapeze-PCR				X
Telomeres length	qPCR				X

RT: reverse transcription; qPCR: quantitative polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay.

2.3. Biotransformation of PM-organic compounds

Some of the organic constituents of the collected particles are known to be procarcinogens, requiring activation by Xenobiotic Metabolism Enzymes (XME) to exert their toxicity, and in particular to react with cellular macromolecules. The gene expression of CYP1A1, CYP1B1 and NQO1 involved in the metabolic activation of organic compounds was quantified (Fig. 3). CYP1A1 gene expression was induced after exposure to $PM_{0.3–2.5}$ in a dose-dependent manner (Fig. 3a). This gene induction decreased according to the time of exposure from 6 to 48 hr. DMSO control showed no induction for the three measured genes. CYP1A1 gene induction appears to be higher when cells are exposed during 72 hr to the total collected $PM_{0.3–2.5}$, compared to its organic extract. Since CYP1B1 induction follows the activation of AhR, the induction profile of this gene is quite similar than the one of CYP1A1, with a high gene induction after 6 hr of exposure in a dose-dependent manner (Fig. 3b). However, the relative intensity is lower with for CYP1B1. As the CYPs, NQO1 was induced mainly at shortest times of exposure, in a dose-dependent manner (Fig. 3c).

Table 2 – Concentrations (μg/g) and international-toxic equivalent quantities (I-TEQ; fg/m^3) of polychlorinated dibenzo-*p*-dioxins (PCDD) and -furans (PCDF), and dioxin-like POLYCHLOROBIPHENYLS (DL-PCB) detected in the air pollution $PM_{0.3–2.5}$.

Dioxins, furans and DL-PCB (μg/g)	$PM_{0.3–2.5}$	I-TEQ (fg/m^3)
1,2,3,4,6,7,8 HpCDD	0.4379	50.2
OCDD	1.2373	4.3
2,3,4,6,7,8 HxCDF	0.0577	66.2
1,2,3,4,6,7,8 HpCDF	0.2004	23.0
2,3,7,8 TCDF	0.2417	0.8
PCB 118	7.562	2.6
PCB 105	3.495	1.2
Σ Dioxins (μg/g)	1.6752	54.5
Σ Furans (μg/g)	0.4998	90.0
Σ DL-PCB (μg/g)	11.057	3.8
Σ I-TEQ (fg/m^3)		148.3

I-TEQ were calculated using International-Toxic Equivalent Factors (I-TEF) defined by WHO (Van den Berg et al., 2006). Others dioxins (*i.e.*, 2,3,7,8 TCDD, 1,2,3,7,8 PeCDD, 1,2,3,4,7,8 HxCDD, 1,2,3,6,7,8 HxCDD and 1,2,3,7,8,9 HxCDD), furans (*i.e.*, 2,3,7,8 TCDF, 1,2,3,7,8 PeCDF, 2,3,4,7,8 PeCDF, 1,2,3,4,7,8 HxCDF, 1,2,3,6,7,8 HxCDF, 1,2,3,7,8,9 HxCDF and 1,2,3,4,7,8,9 HpCDF) and DL-PCB congeners (*i.e.*, 77, 81, 123, 114, 126, 156, 157, 167, 169 and 189) were under the detection limit.

2.4. Genotoxicity

2.4.1. Oxidative DNA alteration

PM induces oxidative stress, a well-known cause of various diseases, in the lung by producing ROS which damages DNA and leads to apoptosis and other symptoms. In our experimental study, exposure to PM and to the EOM of the two PM samples produced an oxidation of DNA. 8-OHdG was indeed measured as significantly increased after a 48 hr time of exposure (Fig. 4a). This increase was dose-dependent and higher when cells were exposed to PM with ultrafine fraction.

Oxidative DNA adducts, such as 8-OHdG, can be removed by base excision repair performed by the DNA glycosylase OGG1 (Fig. 4b). The gene expression of this repair enzyme was induced only after 6 hr of exposure. This increase was measured after exposure to the highest $PM_{0.3–2.5}$ concentration, and to the organic fractions of both PM samples. However, the significance concerned only the $PM_{0.3–2.5}$ and the EOM of $PM_{2.5}$.

2.4.2. DNA bulky adducts

Exposure of DNA calf thymus to BPDE produced one DNA bulky adduct spot measurable on TLC sheets. This positive control validated the used post-labeling protocol. However, no spot was observable after exposure of BEAS-2B to $PM_{0.3–2.5}$, or to EOM of $PM_{0.3–2.5}$ and $PM_{2.5}$ during 72 hr (data not shown).

2.4.3. DDR

H2A.X phosphorylation, which has been proven to appear on serine 139 upon DDR, was measured (Rogakou et al., 1998). To improve the sensitivity of the detection of histone H2A.X phosphorylation, flow cytometry analysis was developed (Lepers et al., 2014). Exposure of the cells to total particles or EOM of both $PM_{0.3–2.5}$ and $PM_{2.5}$ resulted in high levels of phosphorylation relative to the non-exposed cells (control and solvent control) (Fig. 5).

2.4.4. Indirect genotoxicity: telomerase activity and telomere length

There was a significant decrease in telomerase activity after 72 hr of exposure of BEAS-2B cells to $PM_{0.3–2.5}$ (3 and 15 μg/cm^2) and to the EOMs (Fig. 6). The length of the telomeres was then measured in this study. Although there was a tendency to decrease telomere length in these samples, significant length reduction was observed only in the case of exposure to the highest PM concentration, and to the EOM of $PM_{2.5}$ (Fig. 6).

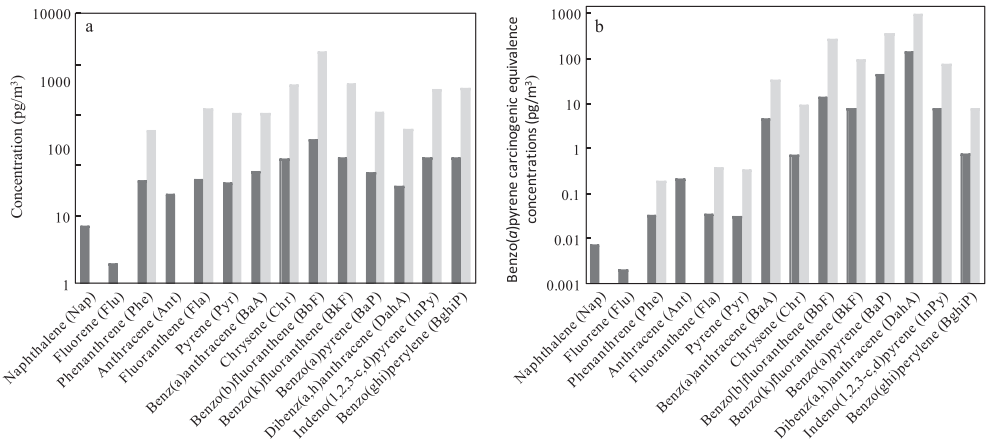

Fig. 2 – Polycyclic aromatic hydrocarbons detected in collected PM: $PM_{0.3–2.5}$ (dark grey) and $PM_{2.5}$ (light grey). Concentrations are expressed (a) in pg/m³, and (b) in pg of Benzo(a)pyrene carcinogenic equivalence concentrations (BaP-CEC)/m³. PM: particulate matter.

3. Discussion

Interactions between organic and inorganic compounds can occur even after the inhalation of PM in the human body. Additive, antagonist, and synergistic effects depending on PM composition can influence its toxicity expression. There are major mechanisms generally beginning with an oxidative stress induced by both metabolic activation of organic compounds coated onto PM (André et al., 2011; Billet et al., 2007), or by environmentally persistent free radicals characterized on or inside particles (Gehling and Dellinger, 2013). PM samples studied in this work were collected at an urban site under the influenced of various particulate emission sources. It indeed contained numerous chemical components as PAHs, furans, and dioxins as well as inorganic compounds including ions and metals, black carbon, crustal and biological elements. Important Fe, Ca and Al concentration levels can be partly explained by both natural and anthropogenic sources, such as resuspension of crustal dust or raw material handled in the Dunkerque industrial area (Hleis et al., 2013). The proximity of the sampling site to the North Sea could explain the relatively high proportion of Na from sea salts in $PM_{0.3–2.5}$ (Ledoux et al., 2009). According to previous studies (Kfoury et al., 2016; Ledoux et al., 2017), industrial emissions related to two steel manufacturing plants located in Dunkerque mainly account for a major proportion of Fe, Zn, Mn, Pb and Cr concentrations. Elements as Ni and V are known to be markers of heavy fuel oil combustion from shipping or oil refinery activities (Mazzei et al., 2008; Vallius et al., 2005; Wu et al., 2007) whereas Pb, Cu, Zn are also related to the abrasion of mobile part of vehicles, such as brakes and tires (Gietl et al., 2010; Thorpe and Harrison, 2008).

Long-term adverse health effects have been linked to particulate-bound PAHs. Since PAH compounds are recognized as having carcinogenic and mutagenic properties, the 16 PAHs measured in this study were selected based upon their mutagenic and carcinogenic potential as defined by the United States Environmental Protection Agency. PAHs were

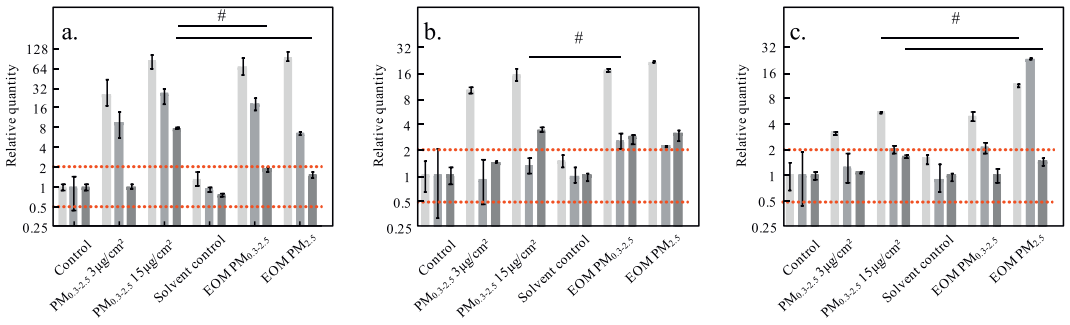

Fig. 3 – Cytochrome P450 (CYP) 1A1 (a), CYP1B1 (b) and NAD(P)H: quinone oxidoreductase 1 (NQO1) (c) mRNA levels (Relative Quantity *vs* untreated control cells) after BEAS-2B cells exposure for 6 hr (light grey), 24 hr (dark grey) or 48 hr (dim grey) to $PM_{0.3–2.5}$ at 3 (C3) or 15 µg/cm² (C15), to solvent control (DMSO, 0.1% *v/v*), or to Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and of $PM_{2.5}$ at an equivalent concentration to 15 µg/cm². Results are expressed as median [Q1;Q3] from 4 experiments, with 18S as housekeeping gene. For RQ, significant threshold is under/over the dot line. Significant differences between $PM_{0.3–2.5}$ and EOMs are highlighted by # ($p < 0.05$, Mann Whitney U test). PM: particulate matter.

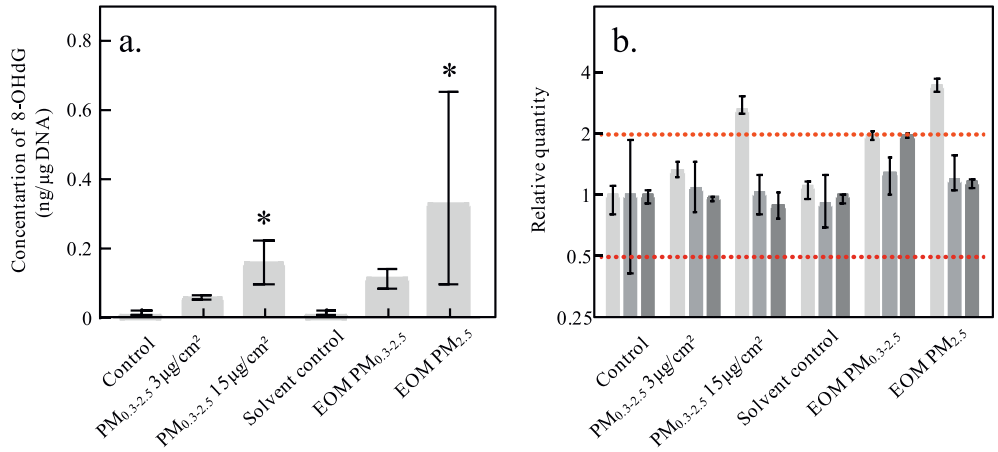

Fig. 4 – (a) 8-Hydroxy-2′-deoxyGuanosine (8-OHdG) concentrations (ng 8-OHdG/µg DNA) in BEAS-2B cells after exposure for 48 hr to $PM_{0.3–2.5}$ at 3 (C3) or 15 µg/cm² (C15), to DMSO (0.1% *v/v*), or to Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and of $PM_{2.5}$ at an equivalent concentration to 15 µg/cm². Results are expressed as median [Q1;Q3] from 4 experiments; Mann–Whitney U-test; versus unexposed control controls; * = $p < 0.05$, between $PM_{0.3–2.5}$ and EOMs # = $p < 0.05$. (b) 8-oxoguanine DNA glycosylase 1 (OGG1) mRNA level (Relative Quantity *vs* untreated control cells) after BEAS-2B cells exposure for 6 hr (light grey), 24 hr (dark grey) or 48 hr (dim grey) (c) to $PM_{0.3–2.5}$ at 3 (C3) or 15 µg/cm² (C15), to solvent control (DMSO, 0.1% *v/v*), or to Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and of $PM_{2.5}$ at an equivalent concentration to 15 µg/cm². Results are expressed as median [Q1;Q3] from 4 experiments, with *18S* as housekeeping gene. For RQ, significant threshold is under/over the dot line. Significant differences between $PM_{0.3–2.5}$ and EOMs are highlighted by # ($p < 0.05$, Mann Whitney U test). PM: particulate matter.

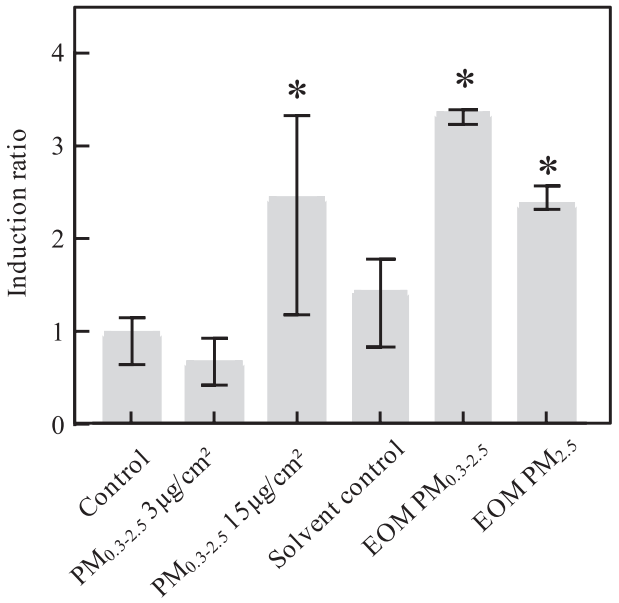

Fig. 5 – Histone H2A.X phosphorylation (Ser139) (Relative concentrations *vs* untreated control cells) in BEAS-2B cells after exposure for 72 hr to $PM_{0.3–2.5}$ at 3 (C3) or 15 µg/cm² (C15), to solvent control (DMSO, 0.1% *v/v*), or to Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and of $PM_{2.5}$ at an equivalent concentration to 15 µg/cm². Results are expressed as median [Q1;Q3] from 4 experiments; Mann–Whitney U-test; versus unexposed control controls; * = $p < 0.05$, between $PM_{0.3–2.5}$ and EOMs # = $p < 0.05$. PM: particulate matter.

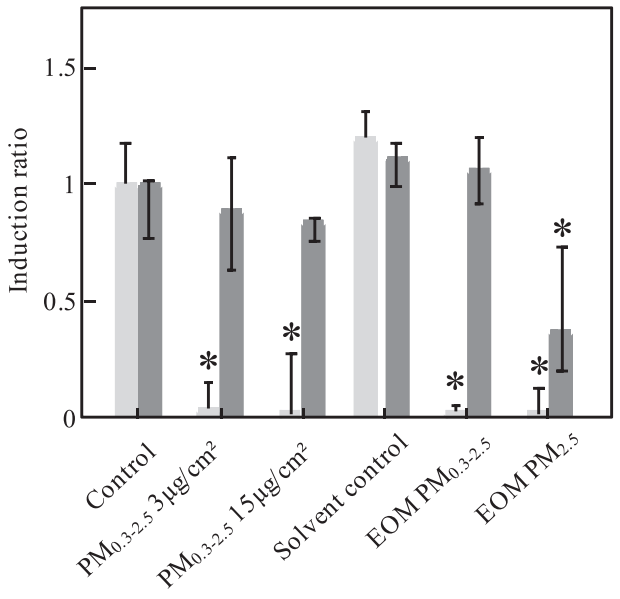

Fig. 6 – Telomerase activity (light grey) and Telomeres length (dim grey) in BEAS-2B cells after exposure for 72 hr to $PM_{0.3–2.5}$ at 3 (C3) or 15 µg/cm² (C15), to solvent control (DMSO 0.1% *v/v*), or to Extractable Organic Matter (EOM) of $PM_{0.3–2.5}$ and of $PM_{2.5}$ at an equivalent concentration to 15 µg/cm². Results are expressed as median [Q1;Q3] from 4 experiments; Mann–Whitney U-test; versus unexposed control controls; * = $p < 0.05$, between $PM_{0.3–2.5}$ and EOMs # = $p < 0.05$. PM: particulate matter.

much more distributed in the ultrafine fraction of PM probably due to the contribution of traffic sources in the smallest particles (Kinney et al., 2000). The high proportion of BbF let suggest that diesel exhaust was a major source of PAHs (Duval and Friedlander, 1981) and evidenced the impact of road traffic on the composition of the organic fraction of PM, as confirmed using diagnostic ratios. Indeed, InPy/BghiP, BbF/BkF and BaP/BghiP values were respectively close to 1, superior to 0.5 and between 0.5 and 0.6 in agreement with a significant contribution of traffic due to diesel combustion (Ravindra et al., 2008). A coal combustion contribution could not be excluded from the Fla./Pyr (~1) and BaP/BkF (~0.6) ratio values (Cecinato et al., 2014; Ravindra et al., 2008) and could be explained by the use of coal in the integrated steelworks. Finally, Fla/Pyr (~1) and InPy/BghiP (~1) ratios could also suggest the influence of biomass combustion related to the use of wood for domestic heating (Yunker et al., 2002). Our results clearly indicated that $PM_{2.5}$ composition in this urban area was influenced by road traffic and industrial emissions. Some of the PAHs quantified in our collected PM are also classified as certain or probable carcinogens. A large weight of evidence links exposure to $PM_{2.5}$ and mutagenicity and genotoxicity (Landkocz et al., 2017; Lepers et al., 2014; Melki et al., 2017). According to Brown et al., these results indicate that high molecular weight PAHs (>228 g/mol) isolated from PM represent the greatest biological activity of air particulate samples (Brown et al., 2005). Even if the mechanism of action of each PAH is not fully known, some of them are known ligands of the aryl hydrocarbon receptor (AhR). Benzo(*a*)pyrene carcinogenic equivalence concentrations (BaP-CEC) reflecting the most accurate relative cancer potency attributed to PAHs were proposed (Nisbet and LaGoy, 1992). Using these BaP-CEC factors, we could evaluate the toxicity of both each individual PAH and chemical mixtures that mediate cell responses through the AhR (Brown et al., 2001) (Fig. 2.b). Due to its strong interaction with the AhR, DahA is more toxic than BaP and appeared as the major toxic PAH detected in the two PM samples. Bbf with the highest atmospheric concentration was only in the third position in terms of toxicity. Finally, PAHs with 2 or 3 rings are considered as little part of the PM toxicity.

The *in vitro* genotoxicity of PM samples was assessed in a human lung cell model. At first, the induction of XMEs' gene expression was measured since some of the organic constituents of the collected particles are known to be procarcinogens, requiring activation to exert their toxicity. For example, BaP can be oxidized to BPDE capable of forming bulky adducts to DNA. These adducts could be at the origin of certain mutations. CYP1A1 gene expression was induced after exposure to $PM_{0.3–2.5}$ in a dose-dependent manner. This XME, specialized in the phase I oxidation of PAHs (Ding and Kaminsky, 2003), is induced by activation of the AhR. The EOM of the two PM samples strongly induced the gene expression, which proves the major role of the PAHs in their own biotransformation, particularly DahA, BaP and DbF, strong inducers of AhR. CYP1A1 gene induction decreased after 6 hr of exposure, but it remained significant when cells were exposed to the total collected $PM_{0.3–2.5}$, compared to the EOMs. This could be explained by synergistic mechanisms between the metal compounds of the particles and the PAHs coated thereon or by a progressive release of PAHs in the culture medium or in the exposed cells (Billet et al., 2007). Organic extraction indeed caused a higher bioavailability, however released PAHs could be quickly converted, as seen in our results. Finally, *CYP1A1* induction could also be explained by the dioxins present in the collected PM. The CYP 1A1 and 1B1 have both shown the capacity to carry out bioactivation of PAHs (Uppstad et al., 2010). NQO1 also participated in the biotransformation of the organic compounds present on PM samples, being induced mainly at shortest times of exposure. This could be explained by the rapid activation of PAHs by the first phase XME. Comparing the similar results obtained after exposure to $PM_{0.3–2.5}$ and to its EOM, we can conclude that if the extraction increases PAH bioavailability, this effect is compensated by a potential synergy between compounds. The explanation of these results could be completed by those obtained by Xu et al. who showed the major role of the aromatic organic compounds compared to the aliphatic and polar fractions in the genotoxicity of $PM_{2.5}$ (Xu et al., 2008). NQO1 is indeed active in the bronchiolar region after exposure to BaP or to diesel exhaust particles (Iba and Caccavale, 2013). Finally, it is also noteworthy that exposure to EOM of $PM_{2.5}$ produced the highest gene expression induction for each XME, in agreement with its higher PAH concentration.

The metabolization of organic compounds produces ROS leading to an oxidative stress. This mechanism is a well-known cause of various diseases, especially cancer. PM is known to induce oxidative stress in the lung cells (Dergham et al., 2012; Kouassi et al., 2010). This damages DNA and leads to apoptosis and other symptoms. In addition, oxidative stress may induce inflammatory injuries and epigenetic changes. Results showed that exposure to both PM sample and their EOMs produced an oxidation of DNA, with the formation of 8-OHdG. This increase was dose-dependent and higher when cells were exposed to $PM_{2.5}$ (that includes ultrafine particles) compared to $PM_{0.3–2.5}$. Oxidative DNA adducts, such as 8-OHdG, can be removed by base excision repair performed by the DNA glycosylase OGG1 (Nakabeppu et al., 2006). The gene expression of this repair enzyme was shortly induced after exposure to $PM_{0.3–2.5}$ and to $PM_{2.5}$ EOM, in agreement with previous studies (Sorensen et al., 2003). This increase was significant after exposure to the highest $PM_{0.3–2.5}$ concentration, but not after exposure to its EOM. This could be explained by a synergy between the organic and the inorganic components of PM (Valavanidis et al., 2008), and by some reactions due to the metallic compounds, such as Fenton reaction catalyzed by Fe (Ovrevik et al., 2006). Finally, the EOM of $PM_{2.5}$ also induced 8-OHdG formation and OGG1 gene expression. The major role of PAHs in these inductions was clearly shown in population study by Yang et al. (Yang et al., 2015). Organic compounds linked to PM, and notably PAHs, were often associated with genotoxic responses (Høgsberg et al., 2013; Ramos de Rainho et al., 2013).

Another genotoxic insult caused by PAH exposure is the formation of DNA bulky adducts. In this study, we do not observe the formation of DNA bulky adducts (no observable spot) after exposure of BEAS-2B to $PM_{0.3–2.5}$, or to EOMs during 72 hr, despite the high sensitivity of the post-labeling used method, validated by BPDE positive control. This result is surprising, regarding the concentrations of PAHs, and the induction of XMEs able to bioactivate PAHs detected on PM in electrophilic compounds able to link to DNA. Several hypotheses could explain why DNA adducts were not observed. First, despite the induction of the gene expression of XMEs, their catalytic activity was not measured and could be inhibited.

Several studies have highlighted that some PAHs inhibited CYP1A1 and CYP1B1 activity (Shimada and Guengerich, 2006). According to the involved PAH, this inhibition can be either direct, caused by the parent molecules, or mechanism based, caused by the generated metabolites. PM contained a lot of different PAHs such as BkF, a gene inducer of CYP1A1, but also an antagonist to the metabolization of BaP, and therefore to the formation of BPDE DNA adducts (Tarantini et al., 2011). Another explanation could be the activation of repair enzymes between 6 and 72 hr of exposure. For example, PAH-DNA adducts can be removed by nucleotide excision repair by Xeroderma Pigmentosum-A (Pavanello et al., 2005). This result could also be explained by the co-exposure of BEAS-2B to a mixture of PAHs and dioxins (Wu et al., 2008).

Even if we did not observe DNA adducts of PAHs, this could be due to the used protocol optimized to detect BaP adducts. There could be other adducts which can induce DDR in PM-exposed BEAS-2B cells. To better understand the DNA damage mechanisms potentially involved, a specific measurement of DDR was carried out: H2A.X phosphorylation. Exposure of the cells to total particles, or EOMs at the highest exposure concentration resulted in an increase of phosphorylation. These results showed that exposure to PM or extracts is genotoxic. We were then interested in the indirect genotoxicity, measuring telomerase activity. This is a specific reverse transcriptase responsible for maintaining the telomere length (Wyatt et al., 2010). The role played by telomerase in carcinogenesis is ambivalent, as both its overexpression and its down-expression can have deleterious effects. Telomere shortening contributes to a persistent DDR and an irreversible loss of division potential of somatic cells, and thereby to an increased risk of chronic diseases. However, there are only a few studies of the modulation of telomerase activity by exposure to PM. In BEAS-2B cells, there was a significant decrease in telomerase activity after 72 hr of exposure to both PM and EOMs. In another study, rat lung epithelial cells were incubated with ultrafine Carbon Black before the measurement of nuclear and mitochondrial telomerase activities (Büchner et al., 2013). The exposure to particles decreased telomerase activity in both the nucleus and the mitochondria. Such a decrease in telomerase activity, due to PM exposure, is associated with genomic instability and cellular tetraploidization, typical of epithelial cancers (Frias et al., 2012; Pereira and Ferreira, 2013; Young, 2010). The length of the telomeres was then measured in our study. Although there was a tendency to decrease telomere length in these samples, significant length reduction was observed only in the case of exposure to the EOM of $PM_{2.5}$. This underlies the role of the organic fraction in this indirect genotoxicity. Amongst this fraction, the aromatic compounds are thought to be the most genotoxic (Xu et al., 2008). Leclercq et al. measured telomere length in normal human bronchial epithelial exposed to $PM_{2.5}$ collected during autumn-winter and spring–summer seasons (Leclercq et al., 2017). They showed telomere shortening in both PM-exposed cells, but with a stronger intensity after exposure to autumn-winter PM which contained 3 fold higher concentrations of PAHs. The role of PAHs on telomeres shortening was also shown after chronic exposure to PAHs of Polish male non-current smoking coke oven workers and matched control (Pavanello et al., 2010). The exposed workers exhibited lower telomeres length which decreased with longer duration of work as a cokeoven worker.

4. Conclusions

$PM_{2.5}$ constitutes a complex mixture of fine and ultrafine particles composed of various chemical compounds including metals, ions, and organics. The present multidisciplinary work aimed to study the influence of size and composition on the toxicity of PM sampled on a multi-influenced site. On the one hand, the comparison between the physico-chemical characteristics of the total collected PM and the EOM of the two aerosols ($PM_{0.3–2.5}$ and $PM_{2.5}$) evidenced that $PM_{2.5}$ comes from several sources. Natural, such as soil resuspension and marine sea-salt emissions, but also anthropogenic origins such as sea shipping, road traffic, and industrial sources were identified in the $PM_{2.5}$ samples, with differences related to the ultrafine fraction, containing ten times more PAHs than $PM_{0.3–2.5}$. On the other hand, from a toxicological point of view, fine particles ($PM_{0.3–2.5}$) induced several mechanisms of action implied in the genotoxicity of air pollution. These events, including oxidative stress and inflammation, are also related to lung cancer early events. The organic extracts showed a more pronounced effect in some assays than the collected particles. Differences between native PM and EOMs can be due to bioavailability of organic compounds in the extract. The toxicity of PM-EOMs is even bigger for the sample including the ultrafine fraction ($PM_{2.5}$). These results confirm the major effect of organic compounds on toxic effects, but also the potential contribution of the inorganic fraction of the PM as a vehicle which maintains longer the effects in exposed cells.

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