**Fine atmospheric particles emitted by industrial, traffic and urban sources in France: characterization and genotoxicity**

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Health risks associated with inhalation of fine particulate matter of 2.5 micrometers in diameter or smaller depend on their atmospheric levels and physicochemical properties. The relationships between chemical compositions and genotoxic activities of particles emitted by mineral industries, traffic and urban sources during summer and winter in the region of Provence-Alpes-Côte d'Azur (France) were investigated.

The fine particles were separated in respect to water-soluble (13 minerals and metals) and organic-extractable (16 polycyclic aromatic hydrocarbons) components that were quantified. The chromosome damaging properties of the hydrophilic and lipophilic extracts were assessed using the centromeric micronucleus assay on a human lung fibroblast cell line.

The composition of the fine particulate matter was variable and depended upon the sources and seasons. Both the hydrophilic and lipophilic extracts induced chromosome damage: (1) in hydrophilic extracts, Ca and Zn affected chromosome losses induction, (2) acenapthylene affected chromosome damage (breakages and losses) induction and naphthalene affected chromosome damage and losses induction in lipophilic extracts without metabolic activation, and (3) benzo[a]pyrene affected chromosome losses induction in lipophilic extracts with metabolic activation. Fine particulate matter arising from coal-fired power station, road traffic, and other urban sources were the most efficient to induce chromosome breakage.

Keywords: fine atmospheric particulate matter ($PM_{2.5}$); atmospheric particles; mineral industries; genotoxicity; cytokinesis-block micronucleus assay

## 1. Introduction

Health risks posed by environmental contaminants depend on their concentration, on the duration of exposure, and on the physiological / behavioral characteristics of the target. Routes of human exposure to pollutants are mainly by inhalation, non-dietary ingestion, and dermal adsorption (Kang, Cheung, and Wong 2011). Health risks associated with fine atmospheric particulate matter ($PM_{2.5}$) exposure by inhalation depend on the aerodynamic size fraction of the particles, the atmospheric levels, and the physical properties and chemical composition (Brüggemann et al. 2009).

$PM_{2.5}$ composition is heterogeneous and complex, depending on the emission sources, the distance from the sources, and the seasons during which it is generated (Brüggemann et al. 2009). $PM_{2.5}$ may consist of an inorganic core of ions, metals, or inorganic carbonaceous species, coated with organic compounds such as polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOC), as well as crustal and biological elements (Lepers et al. 2014). André et al. (2011) reported that some VOC and PAHs (i.e. benzene, benzo[a]pyrene, naphthalene, benz[a]anthracene, fluoranthene) adsorbed onto $PM_{2.5}$ collected in the urbano-industrialized area of Dunkerque (France) were classified as carcinogenic agents by the International Agency for Research on Cancer (IARC), and many of them act by inducing genotoxicity (Pope III et al. 2002; Valavanidis, Fiotakis, and Vlachogianni 2008). During phase I of metabolism involving cytochrome $P_{450}$ isoforms, some of the lipophilic compounds are bioactivated to electrophilic metabolites that interact with intracellular macromolecules such as DNA, proteins, or lipids (Zhang et al. 2009). In 2013, exposure to outdoor air pollution has been considered by IARC to cause lung cancer in humans, and particulate matter, a major component of outdoor air pollution, was evaluated separately and also classified as carcinogenic to humans (WHO 2013).

The region of Provence-Alpes-Côte d'Azur (PACA) in the southeastern part of France is particularly affected by the procedure of litigation initiated by the European Commission due to frequent exceedances of $PM_{2.5}$ in six areas, including the Aix-Marseille area (DREAL 2010). The World Health Organization (WHO) 2005 guideline limits aim to achieve the lowest $PM_{2.5}$ concentrations possible, i.e. 10 $\mu g/m^3$ as annual mean, and 25 $\mu g/m^3$ as daily mean (WHO 2014). Between Aix-en-Provence and Marseille, the "Bassin Minier de Provence" (BMP) is located, a former coal mining area. Although the last mine pit closed in 2003, the region is still occupied by a coal-fired power station, cement works, and an alumina

plant, industries that generate particulate matter (Reis et al. 2014). As BMP constitutes a characteristic urban-industrialized territory where different pollution sources of mineral industries, traffic road and domestic emissions coexist, this area was chosen to investigate the relationships between origins, physicochemical characteristics, and genotoxicity of $PM_{2.5}$.

Numerous studies investigating $PM_{2.5}$ genotoxicity focused on the implication of organic compounds (i.e. PAHs, phthalate esters) in the mutagenic potentials of atmospheric fine particles (Borgie et al. 2015; Lepers et al. 2014; Líbalová et al. 2014; Oh et al. 2011). However, as far as we know, no study on $PM_{2.5}$ emitted by mineral industries genotoxicity was published; moreover most if not all the publications relative to $PM_{2.5}$ reported mutagenic and/or chromosome damaging effects without discriminating chromosome breakage and losses. The importance of such study is given by the fact that a very large proportion of carcinogens act through DNA or chromosome damaging properties. Additionally, a single gene or chromosome mutation is still considered to be able to give rise to a clonal expansion and to a tumor. No threshold value can be established for direct DNA damaging agents. Consequently, it appears necessary to evaluate the contribution of the different emission sources of $PM_{2.5}$ and the genotoxic potential of the $PM_{2.5}$ lipophilic and hydrophilic extracts to identify hazards that may lead to increased risk of cancer through $PM_{2.5}$ inhalation.

The present work aimed at determining the relationship between the chemical compositions of five $PM_{2.5}$ samples collected under three main sources (industrial, traffic and urban) during two seasons (summer 2010 and winter 2011), and their genotoxic activities. To this end, $PM_{2.5}$ were extracted as hydrophilic and lipophilic phases and the objectives of this research were to: (1) quantify the concentrations of 13 inorganic elements (Na, Al, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Mo, and Pb) in the water-soluble fractions; (2) quantify the concentrations of the16 US-EPA recommended PAHs (acenaphthylene, acenaphthene, anthracene, chrysene, fluoranthene, fluorine, phenanthrene, pyrene, naphthalene, benz[a]anthracene, benz[a]pyrene, benzo[b+k]fluoranthene, benzo[g,h,i]perylene, dibenz[a,h]anthracene, indeno[1,2,3,c,d]pyrene) in the organic-extractable fractions; (3) assess the DNA-damaging ability of both hydrophilic and lipophilic extracts obtained from the $PM_{2.5}$ collected on filters using the cytokinesis-block micronucleus (CBMN) assay, in case of the lipophilic fraction with and without metabolic activation, on the normal human lung fibroblast (NHLF) cell line, highly relevant to assess genotoxic effects induced in human through inhalation; and (4) evaluate potential associations between the chemical composition of the $PM_{2.5}$ and their genotoxicity.

## 2. Materials and methods

### *2.1 Study area and PM2.5 sampling*

Samplings were carried out within five sites related to a particular type of exposure close to, respectively, (a) three mineral industries (cement works, alumina plant and coal-fired power station), (b) a heavy traffic road, and (c) an urban site considered free of industrial components. These sites were located in the BMP (Fig. 1 and Table 1).

[Figure 1 near here]

[Table 1 near here]

Samples were collected during seven consecutive days in summer (July) and in winter (January). A high-flow sampler (30 $m^3$/h, DA 80, Digital, San Diego, CA, USA) was used to collect $PM_{2.5}$ on 150 mm quartz fiber filters (QFH 150, untreated, ALBET, Dassel, Germany) and was programmed to collect samples for 24 h (00:00 – 23:59). Filters were weighed before and after collection and were stored protected from light at -20°C for further chemical and biological investigations.

### *2.2 Extraction of the hydrophilic and lipophilic fractions*

To extract the hydrophilic and lipophilic fractions of the collected $PM_{2.5}$, the procedure applied to each filter was already described in previous studies (Gutiérrez-Castillo et al., 2005; Zheng et al., 1997). Briefly, each filter was divided into two equal parts that were agitated for 30 min in an ultrasonic bath either with 20 mL Milli-Q deionized water to extract hydrophilic compounds, or with dichloromethane to extract lipophilic compounds. Soluble components were separated from the insoluble ones by centrifugation at 2500 rpm for 10 min. The supernatants were filtered using nylon 0.45 µm syringe filters. For the hydrophilic fractions, the resultant filtrate was freeze-dried in vacuum, and then weighed; for the lipophilic ones, the resultant filtrate was totally evaporated using a stream of $N_2$ gas, then weighed. The hydrophilic and lipophilic pellets were then dissolved in either Milli-Q deionized water or DMSO, respectively, at a concentration of 5 mg/mL, then filtered using 0.22 µm syringe with hydrophilized poly(tetrafluoroethylene) (PTFE) filters. These stock solutions were stored at -20°C until analysis. Blank filters were handled and treated under the same procedure. This procedure was carried out on the filters collected during the 7 days of sampling for each site and season. The 7 stock solutions obtained for each site and season were pooled to perform the cytotoxicity and genotoxicity assays.

### *2.3 Chemical characterization*

#### *2.3.1 Inorganic elements*

A quarter of filter was immersed in 5 mL concentrated $HNO_3$ (Optima, Fisher Scientific, Illkirch, France) for 5 minutes, then sonicated for 15 minutes before dilution to 50 mL with ultrapure water.

Inorganic elements, minerals and metals (Table 3) were analyzed in triplicate by inductively coupled plasma-mass spectrometry (ICP-MS) (Series II ICP/MS, Thermo-Electron, Les Ulis, France). Operational parameters are given in Table 2. Calibration curves for the 13 individual elements were determined with a multi-element solutions (SCP Sciences, Baie-d'Urfé, Canada) in 5% $HNO_3$ at concentrations from 1 to 500 µg/L of each element. The background level of each inorganic element was determined on blank filters and subtracted from the level found for each sample. Results were calculated using Plasmalab software (Thermo-Electron).

[Table 2 near here]

#### *2.3.2 Organic components*

All reagents were of analytical grade. Acetone and n-hexane were from VWR International (Fontenay sous bois, France), dichloromethane from Merck (Darmstadt, Germany).

The dry filters were extracted using an accelerated solvent extraction system (ASE 350, Dionex, Sunnyvale, CA, USA), with acetone / dichloromethane (50:50, v:v) at 100°C and at a pressure of 103 kPa. The extracts were concentrated under $N_2$ stream at 25°C to 0.5 mL using a Turbovap II (Caliper Lifesciences, Hopkinton, MA, USA). The residues were adjusted to 1 mL using n-hexane.

PAHs (Table 4) were analyzed in triplicate by gas chromatography-mass spectrometry (GC-MS) equipped with a capillary column (30 m × 0.25 mm, 0.25 µm film thickness, Elite 5 MS) (Autosystem XL-TurboMass, Perkin Elmer, Norwalk, CT, USA). Chromatographic conditions were as follows: splitless injection (30 s), the splitless injector being temperature programmed from 50°C (0.1 min) to 250°C at 200°C/min), holding isothermally for 10 min. Helium was used as carrier gas in constant flow mode of 1 mL/min). The GC oven was kept isothermally at 40°C for 2 min, then temperature programmed to 120°C at 45°C/min, and at 5°C/min up to 310°C, holding isothermally for 20 min. The mass spectrometer was operated in the electron ionization mode (70 eV) and simultaneously scanned in both full scan and the

selected ion monitoring mode. PAHs were identified by retention time and m/z ratios of standards, and then quantified using deuterated internal standards as surrogates (chrysene $d_{12}$ and phenanthrene $d_{10}$; CIL, Andover, MA, USA).

Calibration curves for the 16 individual PAHs were determined with a solution of all 16-PAHs (Supelco, Bellefonte, PA, USA). The blanks always (unused filters) returned results that were below the detection limit; and the coefficient of variation between the PAHs concentrations of triplicate samples was less than 10%.

### *2.4 Cytotoxicity and genotoxicity assays*

#### *2.4.1 Cell culture and cell viability*

NHLF cells were maintained in Fibroblast Basal Medium (FBM™) supplemented with 0.1% human fibroblast grow factor-B, 0.1% insulin, 2% Fetal Bovine Serum (FBS), and 0.1% gentamicin/amphotenicin-B at 37°C under 5% of $CO_2$. All reagents were from Clonetics (Lonza, Basel, Switzerland).

Cell viability was assessed using a XTT assay kit (2H-Tetrazolium, 2.3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-hydroxide, inner salt) according to the manufacturer's instructions (Xenometrix, Hégenheim, France). Cells, grown in a 96-well plate at a density of $10^5$ cells/well, were incubated with the tested materials (hydrophilic extracts at 3 - 6 - 12 - 25 µg/mL; and lipophilic extracts at 1 - 2.5 - 5 - 10 µg/mL) for 24 h before adding the XTT reagent. After 3 h incubation, cell viability was quantified with a scanning multi-well spectrophotometer microplate reader (Multiskan, Thermo Scientific, Waltham, MA, USA) using 450 nm and 690 nm emission filters. Three independent experiments were performed for each extract.

#### *2.4.2 CBMN assay in combination with centromere labeling*

4'-6-Diamidino-2-phenylindole (DAPI), goat anti-human Alexa Fluor® 488, trypsin 0.25% EDTA, FBS, and Phosphate Buffered Saline (PBS) Dulbecco's were obtained from Life Technologies (Saint Aubin, France). Paraformaldehyde (PFA) 4% in PBS was from EMS (Hatfield, PA, USA). Bovine Serum Albumin (BSA) fraction V was purchased from Eurobio (Courtaboeuf, France). Vectashield was provided from Vector Laboratories (Burlingame, CA, USA). S9 microsomal rat liver extract and cofactors were purchased from Xenometrix (Hégenheim, France). Human anti-kinetochore antibodies (CREST) were obtained from

Laboratory of Immunology at the Hôpital de la Conception, Marseille (France). The other reagents were from Sigma-Aldrich (Lyon, France).

The CBMN assay was performed according to the original method described by Fenech (2007), with minor modifications for centromere labeling that was performed as described by González et al (2011). Cells were seeded at a concentration of 2.5 x $10^4$ cells/chamber in LabTek™ 4-Chamber Slide™ (Nuc International, Villebon-sur-Yvette, France), and cultured under standard conditions (37°C, 5% $CO_2$). After 24 h culture, slides were treated with increasing concentrations of the extracts (0.375 - 0.75 - 1.5 - 3 µg/mL for hydrophilic extracts; 0.125 - 0.25 - 0.5 - 1 µg/mL for lipophilic extracts), vehicle control (0.25% $H_2O$ and DMSO, respectively), and appropriate clastogenic (10 ng/mL mitomycin C, MMC) and aneugenic (25 nmol/L colchicine, col.) positive controls. At the end of the treatment (24 h), the medium was removed and replaced by fresh medium containing 3 µg/mL cytochalasin B to inhibit cell division after mitosis. After additional 24 h incubation, the culture medium was further changed and, after 2 h incubation, cells were washed in PBS and fixed (4% PFA). To discriminate chromosome losses and breakages, cells were incubated with CREST serum (1: 1000 in 1% BSA/PBS) for 30 min. Cells were washed in 0.5% Triton X-100/PBS and incubated with secondary antibody goat anti-human Alexa Fluor® 488 (1: 200 in 1% BSA/PBS) for 1 h. Cells were then washed in 0.5% Triton X-100/PBS and incubated with a 0.06 µg/mL solution of phalloidin-TRITC (tetramethylrhodamine B isothiocyanate) for 30 min to stain the cytoplasm. After two washes in 0.5% Triton X-100/PBS, cells were incubated with a solution of DAPI 0.5 µg/mL) for 10 min to stain the nuclei. Finally, the slides were mounted in Vectashield and stored in dark at 4°C until analysis.

The experiments were performed also upon metabolic activation by using the following adaptations: NHLF cells were treated with S9 mix (10% S9 supplemented with 5 mmol/L G6P, 4 mmol/L NADP, 33 mmol/L KCl, and 8 mmol/L $MgCl_2$-6 $H_2O$ diluted in phosphate buffer) and increasing concentrations of lipophilic extracts (0.125 - 0.25 - 0.5 - 1 µg/mL) for 3 h. As positive control, cells were incubated with benzo[a]pyrene (BaP, 5 µg/mL), which served as proclastogenic control agent. Cells were then washed twice in PBS and cultured for 21 h. Addition and removal of cytochalasin-B were performed as described previously in absence of metabolic activation.

Micronuclei (MN) contained in binucleated cells were scored using a fluorescence microscope BX 60 (Olympus, Rungis, France) equipped with a black-and-white camera (Andor Luca S), and appropriate filters for DAPI, phalloidin-TRITC, and Alexa Fluor® 488.

Before the analysis, the cytokinesis block proliferation index was determined on 500 viable cells to provide the average number of cell divisions completed by the cells, as previously described (Benameur et al. 2011; Kirsch-Volders et al. 2003). Micronucleus analysis results are expressed as a frequency of binucleated cells with micronuclei (BNMN) per 2000 binucleated cells; scoring criteria were in accordance with those previously described (Fenech 2007). Taking advantage of the CREST staining, micronuclei containing whole chromosome(s), which are positively labeled (centromeric micronuclei, C+MN), and acentric chromosome fragments, which are not marked due to the absence of centromere (acentromeric micronuclei, C−MN) were discriminated (González et al. 2011; Iarmarcovai, Botta, and Orsière. 2006; Mateuca et al. 2006; Natarajan et al. 1996). Two chambers were prepared per concentration, and MN were counted for 1000 binucleated cells on each chamber. Results were expressed as mean of n = 2 slides × 3 independent experiments.

*2.4.3 Statistical analysis*

Cytotoxic and genotoxic results are expressed as the mean value of three independent experiments ± standard deviation (SD). Data were submitted to statistical evaluation using one-way or two-way ANOVA. Normality and homoscedasticity were assumed. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, USA) software was used to perform statistical analysis and to draw the graphics. The significance was compared to negative controls. Values were considered statistically significant at $p < 0.05$.

Relationships between water-soluble or organic-extractable components and the genotoxicity observed on NHLF cells after the exposure to, respectively, the hydrophilic fractions or the lipophilic ones were both investigated using a method of factorial analysis. Principal component analysis (PCA) is a mathematical technique adapted to quantitative variables that transforms n possibly correlated variables into a (smaller) number of uncorrelated variables referred to as principal component (Jolliffe 2014; Reis et al. 2015). Analyzing the correlation of each original variable with the first (and more important) PCA components, it is possible to visualize the correlations between the n geochemical variables in simple bi-plots.

**3. Results**

### *3.1 PM2.5 concentrations, inorganic elements and organic components*

The $PM_{2.5}$ concentrations were quite similar in summer and winter for the three industrial sites (14 and 13 μg/m$^3$ in S1, 13 and 14 μg/m$^3$ in S2 and 15 and 12 μg/m$^3$ in S3, in summer and winter, respectively), while they doubled in winter for the traffic and urban sites (13 up to 28 μg/m$^3$ for both S4 and S5). From these data it was evident that in our study area, $PM_{2.5}$ concentrations exceeded WHO air quality $PM_{2.5}$ annual average, set at 10 μg/m$^3$, for summer and winter seasons. Moreover the traffic and urban sites in winter also exceeded the short-time exposure mean, set at 25 μg/m$^3$ (WHO Regional Office for Europe 2006).

In the water-soluble extractions, concentrations of Na, Al, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Mo, and Pb were determined in each site for both seasons (Table 3).

[Table 3 near here]

Among the 13 analyzed water-soluble compounds extracted from the five sites on both season, Na and Ca were the most abundant elements in all sites (447.14 ng/m$^3$ and 993.19 ng/m$^3$, respectively), followed by trace elements Fe, Al, Cu and Zn. Ti, V, Cr, Mn, Ni, Mo and Pb were the less abundant elements (mean concentrations $\leqslant$ 5 ng/m$^3$). The highest total concentration of inorganic elements was found in the road traffic site (3007.08 ng/m$^3$), and the lowest in the alumina plant site (608.96 ng/m$^3$), both in winter. Higher amounts of water-soluble inorganic species were found in $PM_{2.5}$ collected during summer for all sites (i.e. S2: 1622.76 ng/m$^3$ in summer and 608.96 ng/m$^3$ in winter), except for the road traffic site. The highest Al concentration (110.13 ng/m$^3$) was found for the alumina plant in summer. Notably, Na (238.77 – 622.62 ng/m$^3$), Al (1.62 – 110.13 ng/m$^3$), Ca (358.15 – 2125.90 ng/m$^3$), Fe (13.17 – 138.55 ng/m$^3$), and Cu (0.72 – 46.36 ng/m$^3$) showed a wide range of values, reflecting the heterogeneity of the water-soluble extracts among the five sites under investigation.

The OHM-BMP mean levels for Na (469.18 ng/m$^3$) and Ca (1075.56 ng/m$^3$) were higher than the ones reported for other industrial or urban sites in Dunkerque (Dergham et al. 2012), but similar with another study performed in Marseille (Salameh et al. 2015). Mean levels of Ti, V, Cr, Ni, Cu, Zn an Pb were similar to the majority of those reported in the literature (Dergham et al. 2012; Salameh et al. 2015), but lower than the ones from China (Liu et al. 2015).

The 16 PAHs recommended by US-EPA were quantified in the organic-extractable fractions (Table 4), and among these naphthalene, benz[a]anthracene, chrysene, benzo[b+k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, dibenz[a,h]anthracene and benzo[g,h,i]perylene are carcinogenics, probably or possibly carcinogenic to humans (Gutiérrez-Castillo et al. 2005), and counted for 31% - 69% of the total PAHs concentrations.

[Table 4 near here]

Among the 16 PAHs analyzed, benzo[b+k]fluoranthene was the most abundant PAH in all sites in winter and in three sites (S1, S2 and S5) in summer. The total PAHs ($\sum$PAHs) concentrations ranged from 0.099 ng/m$^3$ to 0.492 ng/m$^3$, these concentrations were comparable to the average value 0.217 ng/m$^3$ reported for Beirut (Borgie et al. 2015). Higher levels of total PAHs were observed in winter compared to summer, and this observation was noted for all sites, except for the coal-fired power station, which presented similar concentrations in both seasons. The two largest seasonal effects were observed in the alumina plant and road traffic sites, with a 2.8 and 2.7-fold increase during winter compared to summer, respectively; for the other sites the differences were less pronounced, with 1.1 to 1.5 fold-increase.

### *3.2 Cytotoxicity assessed by XTT*

The *in vitro* cytotoxicity of hydrophilic and lipophilic $PM_{2.5}$ extracts was assessed by performing a XTT assay on NHLF cells in order to define the range of concentration to be tested in the CBMN assay in combination with centromere labeling (Fig. 2). Hydrophilic and lipophilic extracts from the five studied sites were tested and the data were pooled to generate a single graph per extract.

[Figure 2 near here]

As shown in Fig. 2, both hydrophilic and lipophilic extracts exerted a concentration related decrease in NHLF cells viability. Further, SD values showed that the cytotoxicity was similar among the 5 sites. Less than 50% of survival cells were observed after 24 h exposure to hydrophilic extracts at concentrations greater than 6 µg/mL in summer and 3 µg/mL in winter. After 24 h exposure to lipophilic extracts, less than 50% of survival cells were noted at concentrations greater than 1 µg/mL in both seasons.

XTT was performed to define the working extracts concentrations to use for the genotoxicity study (CBMN assay). To avoid any DNA or chromosome damage indirectly induced by a

cytotoxic effect, the highest working concentrations was fixed at 3 µg/mL for hydrophilic extracts and at 1 µg/mL for the lipophilic ones, in accordance with the highest recommended concentration to be tested in case of cytotoxicity in the OECD guideline 487 (55 ± 5 %) (OECD 2014).

### *3.3 Genotoxicity assessed by the CBMN assay in combination with centromere labeling*

DNA integrity was evaluated at chromosomal level by CBMN assay in combination with centromere labeling (Fig. 3), an *in vitro* test that determines the frequency of MN formation in exposed NHLF cells and allows discrimination between direct and indirect chromosome damage. Further, as many organic-extractable components, including PAHs, need to be bioactivated to exert genotoxic properties, cells were exposed to lipophilic extracts in absence and in presence of metabolic activation.

[Figure 3 near here]

The hydrophilic extracts obtained from $PM_{2.5}$ at sites 1, 2, 3 and 5 induced a significant formation of BNMN at all tested concentrations (0.375, 0.75, 1.5 and 3 µg/mL) in summer. This was the same for site 3 in winter. Differently, in winter no BNMN induction was observed for sites 1, 2 and 4. At 0.75, 1.5 and 3 µg/mL, the hydrophilic extracts induced BNMN formation in summer for site 4 and in winter for site 5.

The lipophilic extracts without metabolic activation obtained from sites 1 and 3 induced a significant BNMN formation at 0.25, 0.5 and 1 µg/mL in both seasons. A statistically significant BNMN induction was noted at 1 µg/mL for sites 2 and 4, compared to the negative control in summer, while in winter no BNMN induction was observed for site 4 only. Site 5 showed BNMN induction at 0.5 and 1 µg/mL in summer, while only at the highest concentration (1 µg/mL) in winter.

Using a metabolic activation, the lipophilic extracts obtained from sites 1 and 2 induced BNMN only at the highest tested concentration (1 µg/mL) in both seasons. On site 3, significant BNMN increases were detected at 0.25, 0.5 and 1 µg/mL for both seasons, while this induction was noted at 0.5 and 1 µg/mL for site 5. Site 4 showed BNMN induction at all tested concentrations in summer, while only at the two highest concentrations (0.5 and 1 µg/mL) in winter.

The effects of hydrophilic and lipophilic extracts on the formation of centromere positive (C+MN) or centromere negative (C-MN) micronuclei were further analyzed.

Results showed that all the hydrophilic extracts obtained in summer induced C+MN formation in binucleated NHLF cells after 24 h exposure, indicating an induction of aneugenic events such as chromosome migration abnormalities leading to chromosome losses for all tested concentrations, while no induction was noted in winter. Without metabolic activation, the lipophilic extracts obtained in summer from site 1 caused aneugenic events at 0.25, 0.5 and 1 µg/mL. At 0.5 and 1 µg/mL, extracts from sites 1 and 3 induced C+MN formation in winter. In presence of metabolic activation, the lipophilic extracts obtained from all sites did not induce aneugenic events.

Hydrophilic extracts obtained from $PM_{2.5}$ at sites 3 and 4 induced C-MN formation in binucleated NHLF cells after 24 h exposure, indicating that clastogenic events (chromosome breakage resulting from a partial chromosome loss) occurred at 1.5 and 3 µg/mL in summer, while this induction was noted for site 5 only at 3 µg/mL. In absence of metabolic activation, the lipophilic extracts obtained from site 3 induced C-MN formation at 0.25, 0.5 and 1 µg/mL in summer and at 1 µg/mL in winter. Clastogenic events were also noted at the highest concentration (1 µg/mL) in winter for site 5.

In presence of metabolic activation, C-MN formations were noted at 0.5 and 1 µg/mL for the lipophilic extracts obtained from sites 4 and 5 in both season, and in winter for site 3. Clastogenic events were observed at 0.25, 0.5 and 1 µg/mL on site 3 in summer and at only at the highest tested concentration (1 µg/mL) on site 2 in winter.

The induction of BNMN by hydrophilic extracts were higher than those induced by lipophilic extracts. These enhanced increases in BNMN were mainly consecutive to the aneugenic effects (C+MN) of hydrophilic extracts in summer. For the lipophilic extracts (with or without metabolic activation), most of the significant induction of BNMN was due to chromosome breakage (C-MN), whatever the season was. Moreover, independently from the hydrophilic / lipophilic extractions, sites 3, 4 and 5 were more efficient to induce chromosome breakages compared to sites 1 and 2. In these two latest sites, chromosome losses effects were predominant mostly in summer.

### *3.4 Correlation between chemical composition of $PM_{2.5}$ and in vitro genotoxicity*

PCA was performed to unravel possible relationships between soluble components extracted from $PM_{2.5}$ and their genotoxicity, in terms of chromosome damage (Fig. 4). The data matrix used to carry out the PCA was composed by either the 13 inorganic elements (Fig. 4(a)) or the 16 PAHs (Fig. 4(b) and 4(c)). C+MN, C-MN and BNMN induced by hydrophilic

extracts, lipophilic extracts without metabolic activation, and lipophilic extracts with metabolic activation, were projected as supplementary variables on Fig. 4(a), 4(b) and 4(c), respectively.

[Figures 4(a), 4(b), 4(c) near here]

First of all, the results showed that the chromosome breakage (C-MN) induced by hydrophilic extracts were associated to an inorganic elements cluster composed by Ca, Cu, Cr and Zn (Fig.4 (a)). Then, the results showed that the global chromosome damage (BNMN, C+MN and C-MN) induced by lipophilic extracts without metabolic activation were associated to an organic components cluster composed by acenaphtene / fluorene, and acenapthylene / napthalene to a certain extent (Fig.4 (b)). Finally, the results showed that chromosome damage (BNMN) induced by lipophilic extracts with metabolic activation were associated to an organic components cluster composed by fluoranthene and benzo(ah)anthracene, while the chromosome losses and breakages (C+MN and C-MN) seemed to be affected by dibenzo(ah)anthracene, chrysene and pyrene (Fig.4 (c)).

Spearman's correlation coefficients ($r$) were calculated between chromosome damage (C+MN, C-MN and BNMN) induced by the three extracts (hydrophilic, lipophilic with and without metabolic activation) and the soluble components determined in these extracts to better support the interpretation of PCA results (Supplementary data - Tables 1).

Ca and Zn were significantly ($p < 0.05$) correlated with C-MN ($r = 0.39$ for both), which indicated an ability to induce chromosome breakages in hydrophilic extractions (C-MN). Mn and Mo were significantly ($p < 0.01$ and $p < 0.001$, respectively) negatively correlated ($r = -0.48$ and $r = -0.59$, respectively) with chromosome losses. Further, a negative significant ($p < 0.01$) correlation ($r = -0.42$) was evidence between Mo and chromosome damage (BNMN).

Acenaphtylene was significantly ($p < 0.05$) correlated with a global induction of chromosome damage ($r = 0.39$, $r = 0.33$ and $r = 0.32$ for BNMN, C+MN and C-MN, respectively) in lipophilic extracts without metabolic activation; and naphthalene was significantly ($p < 0.05$) correlated with BNMN and C-MN ($r = 0.31$ and $r = 0.31$, respectively). Benzo(a)pyrene showed significant ($p < 0.05$) correlations with C-MN ($r = 0.40$) in lipophilic extracts with metabolic activation, suggesting that this PAH affected chromosome breakages (C-MN), while anthracene showed significant ($p < 0.05$) negative correlation ($r = -0.36$) suggesting a protection of NHLF cells against C-MN formation.

The joint interpretation of the results produced by the statistical tools and the geometrical ones allowed further detailing the effects detected in NHLF cells exposed to the hydrophilic and lipophilic (with and without metabolic activation) extracts. Strong correlations were observed between these two methods for the hydrophilic and lipophilic extracts without metabolic activation. Thus, relationships between soluble components and chromosome damage were evidenced: (1) Ca and Zn were positively correlated with C-MN induction in hydrophilic extracts, while Mo and Mn were negatively correlated with chromosome damage (BNMN / C+MN, and C+MN induction, respectively); (2) naphthalene and acenaphtylene were positively correlated with chromosome damage in lipophilic extracts without metabolic activation (BNMN / C-MN, and BNMN / C-MN/ C+MN, respectively); (3) when considering the lipophilic extracts with metabolic activation, benzo(a)pyrene was associated with an increase in chromosome breakage. Benzo(a)pyrene clastogenicity is well documented in the literature (group 1 in IARC classification) and used as positive control to determine gene and chromosome mutations in various *in vivo* or *in vitro* genotoxic assays. Spearman's correlations showed a positive correlation between acenaphtylene and C-MN in the lipophilic extracts with metabolic activation, while they are opposed in the PCA graph. Thus, this relationship was not considered significant. A significant ($p < 0.05$) negative correlation ($r = -0.36$) was found between anthracene and C-MN.

## 4. Discussion

Air pollution and $PM_{2.5}$ were recently classified as carcinogenic to humans (group 1) by the IARC (IARC 2013), bringing them on central concern. Particulate matter, a major component of outdoor air pollution, was evaluated separately and was also classified as carcinogenic to humans (Group 1) (IARC 2013). Besides the $PM_{2.5}$ atmospheric levels and the duration of exposure, the chemical compositions of the $PM_{2.5}$ play a role in the occurrence of adverse effects including cancer. The process of carcinogenesis is initiated by mutagenic and epigenetic events. DNA and/or chromosome damaging agents could indeed induced inheritable mutations that ultimately cause cancer and other genetic disorders. Therefore, our work aimed to characterize the chemical composition of water- and organic-extractable fractions of the $PM_{2.5}$ emitted by mineral industries, road traffic and urban sources and to determine the relationships between the chemical composition and the genotoxic activity. Aneugenic and clastogenic effects of hydrophilic and lipophilic (with and without metabolic activation) extracts from five $PM_{2.5}$ samples collected in the PACA region known for its annual $PM_{2.5}$ levels exceedances were discriminated. The mechanisms involved in

chromosome losses and breakage are truly different. In the first case, the molecular targets of aneugens are mostly proteins involved during the mitosis. In the second case, the molecular target of the clastogens is the DNA, with the induction of double strand breaks before or during the DNA repair processes (Fukasawa 2005; Iarmarcovai, Botta, and Orsière 2006; Saunders et al. 2000).

In our study, $PM_{2.5}$ sampling was performed in BMP that has been affected by the dynamics of Aix-Marseille metropolitan area, due to the economic development of the region to new residential practices. As a result, urbanization has been spreading over forest and agricultural areas, and mineral industry, road traffic and urban pollutions are becoming the three main environmental issues among the new residents (Reis et al. 2014). The geographical locations of the different sites were optimally defined according to the following criteria: site topology, prevailing wind direction, and logistical constraints such as electric power and accessibility.

$PM_{2.5}$ concentrations ranged from 12 to 15 µg/m$^3$ for all sites in summer and for cement works, alumina plant and coal-fired power station sites in winter, while $PM_{2.5}$ levels increased up to 28 µg/m$^3$ for the road traffic and urban sites in winter. The combination of wood heating systems and green waste burning by the population surrounding in winter probably accounted for these two highest $PM_{2.5}$ concentrations (AIRPACA Qualité de l'Air 2012). In the PACA region, winter is particularly favorable to the increase of $PM_{2.5}$ levels due to anthropogenic emissions as well as meteorological conditions favorable to the accumulation of pollutants (AIRPACA Qualité de l'Air 2012). WHO air quality $PM_{2.5}$ annual average set at 10 µg/m$^3$ was greatly exceeded in BMP area, even if the $PM_{2.5}$ levels measured in our study were lower than those reported for capitals affected by traffic (23 - 26 µg/m$^3$) or for a mining area in South Africa (20 µg/m$^3$) (Borgie et al. 2015; Kaonga and Kgabi 2010; Szigeti et al. 2013).

On the water-soluble phase, 13 inorganic elements (minerals and metals) were analyzed and variable quantities of elements were detected at the five sites. Among the minerals and metals present within the five samples, Na and Ca were the most abundant compounds; the highest concentrations in inorganic elements were detected in the road traffic site regardless of the seasons. Higher water-soluble compounds concentrations were noted in summer for all sites, except for the road traffic where the concentrations were similar. The average minerals and metals concentrations were relatively low when compared to those reported in the literature for urban environment (Gutiérrez-Castillo et al. 2006; Pakkanen et al. 2001).

The organic-extractable fraction from the $PM_{2.5}$ allowed us to measure the total PAHs levels that ranged from 0.099 to 0.332 ng/m$^3$ in summer and from 0.180 to 0.492 ng/m$^3$ in winter. These concentrations were far lower than those reported for urban (i.e. 8.8 ng/m$^3$ in summer and 12.3 ng/m$^3$ in winter in Mexico (Gutiérrez-Castillo et al. 2006); 337 ng/m$^3$ in Guangzhou (Li et al. 2006) and industrial sites (i.e. 0.407 ng/m$^3$ in summer and 2.167 ng/m$^3$ in winter (Teixeira et al. 2013)). Among the 16 PAHs analyzed, 8 belonged to groups 1 and 2 of the IARC, and they could be partially responsible of the genotoxicity observed in lipophilic extracts. In accordance to the literature, highest PAHs levels were observed in winter (Lepers et al. 2014; Li et al. 2006; Teixeira et al. 2013). These variations could be partly due to the atmospheric conditions such as low temperatures, lower intensity of solar radiation, and decreased PAHs photo-degradation that favor the increase of PAHs concentrations in $PM_{2.5}$ (Teixeira et al. 2013). The two sites (S4 and S5) with the smoked filters showed the highest PAHs concentrations, certainly due to wood combustion from residential heating and green waste burning, that represents 57% of PAHs emissions winter in PACA region (AIRPACA Qualité de l'Air 2012).

In our study, the highest total inorganic elements concentrations in $PM_{2.5}$ extracts were observed in summer, except for the road traffic site. For each site, $PM_{2.5}$ were generated by various emission sources and this should account for the high heterogeneity of each inorganic element. For example, the highest Cu concentrations noted in the road traffic site in both seasons can be related to dusts generated by the wear of tires and breaks (Borgie et al. 2015; Sharma et al. 2007). Regarding Al, the highest level was noted in summer in the alumina plant site, and Al was not detected in winter in this site. The mass fraction of Al, also quantified in $PM_{10}$ and soil particles (< 100 µm) during the study, was higher in both seasons in the alumina plant site compared to the other sites in soil particles only (data not shown), suggesting that Al mass fraction increased with the particles size. Moreover, speciation of each element could also contribute to the observed heterogeneity. For example, the presence of a Zn phase more soluble in the road traffic and urban sites than in the three other sites (i.e. $ZnCl_2$ compared to Zn oxides) could explain, at least partly, the highest concentrations of Zn in these two sites.

The chromosome damaging properties of the hydrophilic and lipophilic (with and without metabolic activation) extracts from $PM_{2.5}$ samples collected in three industrial, one traffic and one urban sites in the BMP region were evaluated in NHLF cells by CBMN assay with micronucleus labeling. Data obtained in this study showed that the extracts were able to

induce significant increase in chromosome damage by the formation of BNMN cells either by chromosome breakage (clastogenesis) or chromosome loss (aneugenesis). Although hydrophilic extracts induced higher BNMN compared to lipophilic ones (with or without metabolic activation), inorganic elements should not be considered as stronger genotoxicants than organic ones. Indeed, chromosome damage induced by hydrophilic extracts were mostly chromosome losses whereas lipophilic extracts mostly induced chromosome breakage. In addition, concentrations analyzed were higher for the hydrophilic extracts (up to 3 µg/mL) compared to lipophilic ones (up to 1 µg/mL).

Aneugenic effects were more frequently observed with the hydrophilic extracts in summer. They could occurred through a variety of cellular targets, including alteration of protein structures involved in cell division and migration of chromosomes centrosome, spindle, kinetochore, and cell membrane (Mateuca et al. 2006). None of the quantified inorganic elements were positively correlated with chromosome losses (C+MN) by using PCA and Spearman's correlations. It should be noted that, first, the seasonal effect was not assessed by this combination of statistical analyses, second, although 13 inorganic elements were quantified in water-soluble fractions, induction of C+MN could be due to others chemicals. In addition, a chromosome damaging effect of a chemical was considered significant when both a positively significant Spearman's coefficient and a PCA association were noted. Ca and Zn appeared positively correlated with chromosome breakages (C- MN). It has been reported that, Zn act as a protector of genome stability at low concentrations, and induced weak clastogenic effects at high concentrations (Roney et al. 2006). Moreover, Zn has shown few associations with health endpoints, in relation to cardiovascular endpoints as well as respiratory outcomes (Rohr and Wyzga 2012).

The lipophilic extracts with or without metabolic activation induced mainly clastogenic events. Acenapthylene and naphthalene were significantly correlated with the induction of chromosome breakage for lipophilic extracts in absence of metabolic activation, while benzo[a]pyrene was correlated to clastogenic events in presence of metabolic activation. Benzo[a]pyrene, known to act after metabolic activation, was classified by IARC as carcinogenic to human (Gutiérrez-Castillo et al. 2006; IARC 2013). Surprisingly, some inorganic elements and PAHs showed negative associations with chromosome damage. However, components of the complex chemical composition of the $PM_{2.5}$ may interact with each other to produce antagonist, synergistic or additive effects (Gutiérrez-Castillo et al. 2006).

PCA and Spearman's correlations showed the influence of each component independently but not in combination, thus some inorganic elements (i.e. V, Pb, and Cr) or PAHs known for their genotoxic properties maybe acting in association were not revealed by our statistical tests. Moreover, the genotoxicity exerted by the lipophilic extracts could also be due to nitro- and oxi-PAHs that were not quantified in this study, but still present in the extracts (Lepers et al. 2014).

Regardless the chemical composition of the extracts, sites 3, 4 and 5 were more efficient to induce chromosome breakages compared to sites 1 and 2, where in contrast the chromosome losses were predominant. Clastogenic compounds interact with chromosome and lead to DNA double-strand breaks that are clearly associated with increased risk of carcinogenesis. Additionally, as no threshold can be established for direct DNA and/or chromosome damaging agents, preventive strategies and further investigations (i.e. inflammation, oxidative stress, epigenetic effects…) should be considered in such situations. Beyond mineral industries, human activities, mainly related to combustion (i.e. road traffic, waste burning, heating) appeared to be responsible of genotoxic properties highlighted in the BMP area.

For future research it would be interesting to investigate the whole particles, without extraction to highlight synergistic, additive or antagonistic effects of the particles components. Also, concerning the chemical characterization, nitro and oxidized PAHs concentrations should be determined as well as metals speciation's to obtain a finer characterization and maybe strong correlation between chemical composition and genotoxic potencies useful to define the sources of particulate emissions to be reduce in priority to decrease health risks.

Acknowledgements

This work was supported by the Call for Research Project 2009 "Health-Environment-Work" common program from the Agence française de sécurité sanitaire de l'environnement et du travail (AFSSET) and the Agence De l'Environnement et de la Maîtrise de l'Energie (ADEME) under Grant [0974C0260]. Funding for this research were also provided by the Labex DRIIHM, Réseau des Observatoires Hommes – Milieux – Centre National de la Recherche Scientifique (ROHM–CNRS), ECCOREV, and companies Ciments Lafarge, Rio Tinto Alcan and SNET, and the agglomeration of Aix Community.

The authors would like to thank Grégory Gille, Patricia and Daniel Lozano, Jérémie Soubise and Claire Blouvac for them help in carrying out samples. The authors also want to express their gratitude to Kankoé Sallah for his statistical analysis performed, as well as Chiara Uboldi for her precious help to write this article.

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