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AMBIENT PARTICULATE MATTER (PM_{2.5}): PHYSICOCHEMICAL CHARACTERIZATION AND METABOLIC ACTIVATION OF THE ORGANIC FRACTION IN HUMAN LUNG EPITHELIAL CELLS (A549)

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ABSTRACT

To contribute to complete the knowledge of the underlying mechanisms of action involved in air pollution particulate matter (PM)-induced cytotoxicity, an aerosol was collected in Dunkerque, a French seaside City heavily industrialized. In this work, we focused our attention on its physical and chemical characteristics, its cytotoxicity, and its role in the induction of the volatile organic compound (VOC) and/or polycyclic aromatic hydrocarbon (PAH)-metabolizing enzymes in human lung epithelial cells (A549). Size distribution showed that 92.15% of the collected PM were PM_{2.5} and the specific surface area was 1 m²/g. Inorganic (i.e. Fe, Al, Ca, Na, K, Mg, Pb, etc.) and organic (i.e. VOC, PAH, etc.) chemicals were found in collected PM, revealing that much of them derived from wind-borne dust from the industrial complex and the heavy motor vehicle traffic. The thermal desorption study indicated that organic chemicals were not only adsorbed onto the surface but also highly incrustated in the structure of PM. The lethal concentrations at 10% and 50% of collected PM were 23.72 µg/mL (or 6.33 µg/cm²) and 118.60 µg/mL (or 31.63 µg/cm²), respectively. The VOC and/or PAH-coated onto PM induced significant increases in mRNA expressions of cytochrome P450 (*cyp*) *1a1*, *cyp2e1*, *cyp2f1*, *nadph quinone oxydo-reductase-1*, and *glutathione s-transferase-pi 1*, versus controls. Hence, we concluded that the metabolic activation of the very low doses of VOC and/or PAH-coated onto the inorganic condensation nuclei from Dunkerque City's PM is one of the underlying mechanisms of action closely involved in its cytotoxicity in human lung epithelial cells.

KEY-WORDS: A549 cells; particulate matter; physical and chemical characteristics; cytotoxicity; metabolic activation; volatile organic compounds; polycyclic aromatic hydrocarbons

INTRODUCTION

Air pollution particulate matter (PM) is a complex mixture of chemical and/or biological elements, such as metals, salts, carbonaceous material, volatile organic compounds (VOC), polycyclic aromatic hydrocarbons (PAH), endotoxins, which can in fact interact and, potentially, adsorb onto condensation nuclei (Alfaro-Moreno *et al.*, 2002; Schlesinger *et al.*, 2006; Soukup and Becker, 2001). However, the majority of toxicological studies published on PM toxicity have been conducted using complex mixtures, yet their physical and chemical characteristics are usually not well established and the influence of the elements-coated onto PM are often neglected (Hetland *et al.*, 2004).

The adverse effects of ambient PM on human health have been a concern for decades (Brunekreef and Holgate, 2002). Attempts to provide linkages between adverse health effects and PM properties have significantly increased (Englert, 2004). However, despite intensive investigation, the underlying mechanisms of PM-related adverse health effects are still incompletely understood, but a hypothesis currently under investigation is that organic compound-coated onto PM could played a key role in their toxicity (Liden *et al.*, 2003; Nel *et al.*, 2001; Osornio-Vargas *et al.*, 2003). Hence, a better knowledge of the physical and chemical characteristics of PM, which depend from the sources, the emission intensity, and the molecular interactions between inorganic and organic components, constitutes required information for the evaluation of PM toxicity (Buschini *et al.*, 2001; Schlesinger *et al.*, 2006). Nevertheless, the physical and chemical properties of air pollution PM revealed to be unsatisfactory to predict its biological reactivity (Baulig *et al.*, 2004).

Various lung target cells take significantly part in the metabolic activation of inhaled organic chemicals (Dahl and Lewis, 1993; Hukkanen *et al.*, 2000; Spivack *et al.*, 2003; Willey *et al.*, 1996). The superfamily of cytochrome P450 (CYP) is commonly known as being the main system, which catalyses the oxidation and the subsequent activation of such organic chemicals. In human lungs, VOC and PAH, which require metabolic activation to biologically reactive intermediates to elicit their cytotoxic effects, are mainly metabolized by CYP2E1/CYP2F1, and CYP1A1, respectively (Garçon *et al.*, 2004a; 2004b; Rossi *et al.*, 1999; Vakharia *et al.*, 2001a; 2001b). Produced epoxy compounds are thereafter rearranged either directly, out of phenol for benzene, or indirectly, through their conversion into dihydrodiols, mainly catalyzed by the microsomal epoxyde hydrolase (mEH) (Bauer *et al.*, 2003). Such metabolites will be able to undergo again an oxidation by the same CYP (Koop *et al.*, 1989). In lungs, the myelotoxic quinone-derivatives compounds are detoxified by the NADPH quinone oxydo-reductase-1 (NQO1) in hydroxyquinones (Bauer, 2003; Jaiswal, 2000; Wiemels *et al.*, 1999). Once metabolized by the phase I enzymes, ultimate reactive intermediates can rearrange themselves or exert direct adverse effects at molecular and/or cellular levels. The next metabolic step may involve the conjugation of such highly reactive metabolites in more hydrophilic metabolites, mainly catalyzed by the glutathione S-transferases (GST) (Hayes *et al.*, 1995a; 1995b; Thier *et al.*, 2003).

Hence, to improve the knowledge of the underlying mechanisms involved in PM-induced cytotoxicity, with a particular interest of the role of the organic chemicals, the PM sample was collected in Dunkerque, a French seaside City characterized by the proximity of a

lot of industrial activities and a heavy motor vehicle traffic. Its physical and chemical characteristics were carried out. We were interested in the PM-induced cytotoxicity in adherent A549 cells, and in the metabolic activation of the coated organic chemicals by these target cells, through the determination of the gene expressions of *cyp1a1*, *cyp2e1*, *cyp2f1*, *meh*, *nqo1*, *gst-μ1*, *gst-μ3*, and *gst-π1*. To better define the accurate role of the organic fraction, titanium dioxide (TiO₂) and PM having undergone a thermal desorption (dPM) were also included in the experimental design of this multi-step study as negative controls.

MATERIALS AND METHODS

Chemicals

Minimum essential medium (MEM) with Earle's salts, fetal bovine serum (FBS), L-glutamin, penicillin/streptomycin solution, and sterile Phosphate-Buffered Solution (PBS) were from InVitrogen-Life Technologies (Cergy Pontoise, France). Titanium (IV) oxide powder (99%) was from Acros Organics (Noisy Le Grand, France). Benzene and toluene were provided by Sigma-Aldrich (St-Quentin Fallavier, France). Cell Proliferation ELISA (BrdU), Cell Proliferation Reagent (WST-1), and Cytotoxicity Detection Kit (LDH), on the one hand, and all the reagents (except primers) for reverse transcription (RT) and polymerase chain reaction (PCR), on the other hand, were from Roche Diagnostics (Meylan, France). Proligo France SAS (Paris, France) synthesized primer pairs for PCR.

Methods

Figure 1 showed the experimental design of this multi-step approach.

PM Sampling, Physical and Chemical Characteristics, and Outgassing

PM Sampling: PM was collected in Dunkerque (51°04'N; 2°38'E), a French sea-side City located on the southern coast of the North-Sea, using high volume Sierra model 235 cascade impactor (Sierra Anderson, Smyrna, GA, USA). The sampling method has already been published elsewhere (Dagher *et al.*, 2005). Briefly, plates were mounted without any filters and no back up filter was used to maintain a constant aspiration flow rate (68 m³/h) for a period of 2 weeks. The lowest stage was doubled to increase the efficiency of smallest particle (PM_{0.33}) sampling. Collection was done continuously during 9 months, from January to September, to include seasonal changes, and impacting system was changed every 15 days; two identical systems were used alternatively. Meteorological data (i.e. wind speed, wind direction, temperature) were obtained from Météo France. The collection urban zone is characterized by the proximity of industrial activities (i.e. iron and steel industry, aluminum industry, oil refinery, basic chemistry, pharmaceutical industry, plant health production, food industry, etc.), and a heavy motor vehicle traffic. After sampling, impacting plates were dried under laminar flow bench during 48 h, and then PM was removed and stocked at 4°C. About 2 g of PM have been collected by this way and carefully homogenized.

PM physical characterization: Scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX; LeoTM 438 VP microscope and IXRF analysis

system; Zeiss SAS, Le Pecq, France) was used to perform single particle analysis and also to determine the PM size distribution. Prior to the analysis, PM was suspended in a n-hexane solution using ultrasonic treatment, and then filtered through a nucleopore filter to obtain well-distributed and dispersed PM, without agglomerates, as suitable for the SEM-EDX analysis. After the filter carbon-coating, the determination of PM size has been done in the automatic mode with SEM operating conditions as follows: accelerating voltage: 25 keV; intensity: 300 nA, and magnification: x 5000. The minimal observable size was 0.1 μm . The total number of studied particles in SEM-EDX was about 2500. Specific surface area has been measured according to the Brunauer, Emmett, and Teller method using a porosimeter Sorptomatic 1990 Series (Thermo Electron Corporation, Courtaboeuf, France).

PM chemical characterization: The analytical procedure for inorganic element determination consisted of the acidic total digestion of PM, and the subsequent analysis by inductively coupled plasma-atomic emission spectrometry (ICP-AES) using a Varian Liberty II (Varian S.A., Courtaboeuf, France). The ion composition was determined with ionic chromatography (Cl^- , NO_3^- and SO_4^{2-}), and visible spectrophotometry (NH_4^+). The analytical procedure for organic chemical determination consisted of a thermal desorption at 500°C coupled with cold trapping, prior gas chromatography-mass spectrometry (GC/MS) analyses on the Varian Saturn 4D apparatus (Varian France) equipped with a 60m J&W DB1 MS column. Thermogravimetric and differential thermal analysis (TG-DTA) and specific surface area determinations were carried out to evaluate the efficiency of the desorption method.

PM Outgassing: In order to better determine the role of the organic fraction-coated onto PM in the induced-adverse effects in A549 cells, PM having undergone a thermal desorption at 400°C under secondary vacuum were used as negative controls (i.e. desorbed particulate matter, dPM). Briefly, a progressive rise in temperature by 0,5°C/min was applied to PM up to 400°C, during 6 h, under a pressure of 10^{-7} Pa.

Cells and Culture Conditions

Adherent A549 cells derived from a type II-like human alveolar epithelial carcinoma (ATCC number: CCL-185) were cultured in sterile plastic flasks (Corning; Fisher Scientific Labosi SAS, Elancourt, France), in MEM with Earle's salts, containing: 5% FBS, 1% L-glutamin (200 mM), 1% penicillin (10000 IU/mL) and 1% streptomycin (10000 $\mu\text{g/mL}$) (In Vitrogen-Life Technologies), as described by Garçon *et al.* (2001). Exponentially growing cells were maintained at 37°C, in a humidified atmosphere containing 5% CO_2 . To avoid phenotypic changes affecting responsiveness to chemicals, all the A549 cells we used in the study of the cytotoxicity, on the one hand, and in the study of mRNA expression of VOC and/or PAH-metabolizing enzymes, on the other hand, derived from the same initial cell culture, not having undergone more than five passages.

Study of PM Cytotoxicity

PM cytotoxicity in A549 cells has been studied using four complementary methods: the incorporation of 5-bromo-2-deoxyuridine (BrdU) in cells (Cell Proliferation ELISA BrdU, Roche Diagnostics), the activity of the mitochondrial dehydrogenase (MDH) in cells (Cell Proliferation Reagent WST-1, Roche Diagnostics), the activity of lactate dehydrogenase (LDH) in cell-free culture supernatants (Cytotoxicity Detection Kit LDH, Roche

Diagnostics), and the colony forming method, as already described by Garçon *et al.* (2006). Briefly, to carry out the first three methods, A549 cells were seeded in 96-well culture microplates (Costar; Fisher Scientific Labosi SAS) at a density of 2×10^4 cells/200 μ L culture media containing 18.84, 37.68, 56.52, 75.36 or 150.72 μ g PM/mL (or 11.42, 22.64, 34.25, 45.67 or 91.35 μ g PM /cm²) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Non-exposed cells were used as negative controls and Triton X-100 (2%, v/v)-exposed cells as positive controls. Accordingly, 16 replicates were chosen at random as control cells, 8 replicates per PM concentration as exposed cells, and 8 replicates as positive controls. After 24, 48 or 72 h of incubation, the incorporation of BrdU and the activities of MDH and LDH were evaluated, following to the manufacturer's instructions. Survival rates of PM-exposed A549 cells were determined using the colony forming method (Shirali *et al.*, 1995). Briefly, proliferating cells were seeded in culture dishes (60 x 15 mm; Corning; Fisher Scientific Labosi SAS): 10 culture dishes were chosen at random as control cells, and 5 culture dishes per PM concentration (i.e. 18.84, 37.68, 56.52, 75.36 or 150.72 μ g PM/mL; or 4.61, 9.22, 13.83, 18.45 or 36.89 μ g PM/cm²) were designated at random as exposed cells. The values of the lethal concentrations at 10% and 50% (LC₁₀ and LC₅₀, respectively) were calculated comparing the number of crystal violet-stained cell colonies in exposed culture dishes with the number in non-exposed culture dishes.

Study of the mRNA Expression of VOC and/or PAH-Metabolizing Enzymes

Cell Exposure: Depending on the incubation time, A549 cells were seeded at different density (i.e. 6×10^6 , 3×10^6 or 1.5×10^6 cells/20 mL of culture media, respectively) in culture plastic flasks (Corning; Fisher Scientific Labosi SAS) and incubated at 37°C, in a humidified atmosphere containing 5% CO₂. After 3 h, culture supernatants were removed to eliminate non-adherent cells. Only living A549 cells were also incubated in the continuous presence of PM at their calculated LC₁₀ (i.e. 23.72 μ g PM/mL; or 6.33 μ g PM/cm²) or LC₅₀ (i.e. 118.60 μ g PM/mL; or 31.63 μ g PM/cm²) for 24, 48 or 72 h of incubation, without renewing the culture media. Non-exposed cells, TiO₂ (geometric diameter < 0.2 μ m)-exposed cells, and dPM-exposed cells were used as negative controls. A549 cells were exposed to TiO₂ at equivalent concentrations to inorganic LC₁₀ (i.e. Eq LC₁₀ = 19.42 μ g/mL; or 5.18 μ g/cm²) or LC₅₀ (i.e. Eq LC₅₀ = 97.13 μ g/mL; or 25.90 μ g/cm²), integrating thereby mass losses arising from the thermal desorption of the organic fraction. Benzene (7 μ M), toluene (7 μ M), and benzo(a)pyrene (B(a)P; 1 μ M)-exposed cells served as positive controls for the induction of VOC and/or PAH-metabolizing enzymes (Garçon *et al.*, 2001; 2004a; 2004b; Powley *et al.*, 1999). For each incubation time (i.e. 24, 48 or 72h), 8 culture flasks were chosen at random as control cells (i.e. incubated only with cultured media), 4 culture flasks per TiO₂ or dPM concentration as negative control cells, 4 culture flasks per PM concentration as exposed cells, and 4 culture flasks per VOC or PAH concentration as positive controls.

Observation of the Organic Fraction-Coated onto PM-Exposed Cells: After 24 h of incubation, PM-exposed cells were observed using a CKX41 microscope equipped with a DP12 digital camera and a U-RFLT50 UV source (Olympus France S.A.S, Rungis, France) with Rhodamin ($\lambda_{\text{excitation}}$: 570 nm; $\lambda_{\text{emission}}$: 590 nm).

Cell Sampling: After 24, 48 or 72 h of incubation, adherent cells were removed and centrifuged (500 g; 10 min; 4°C). Cell pellets were washed twice with 5-ml aliquots of cold phosphate-buffered saline (0.01 M; pH = 7.2), and frozen at -80°C until RNA extraction.

mRNA expression of VOC and/or PAH-metabolizing enzymes: Total RNA were extracted from A549 cell-aliquots using RNeasy Mini Kits (Qiagen). Conditions for the RT were published elsewhere (Garçon *et al.*, 2004a). Highly specific primer pairs sequences are reported in the Table 1. PCR thermocycling conditions were as reported by Iwanari *et al.* (2002; i.e. *cyp1a1*), Macé *et al.* (1998; i.e. *cyp2e1*), Nichols *et al.* (2003; i.e. *cyp2f1*), Spivack *et al.* (2003; i.e. *nqo1*, *gst-μ1*, *gst-μ3*, and *gst-π1*), and Dubois *et al.* (2000; i.e. *β-actin*). After gel electrophoresis and ethidium bromide intercalation, PCR-amplified products were visualized under UV light and analyzed by a computerized video/densitometry system (Software: GelAnalyst™ v3.1FR; Vasse Industries, Lille, France).

Statistical Analysis

Treatment attribution at random has been made following an 11 element-permutation table. Results are expressed as mean values and standard deviations. For each incubation time (i.e. 24, 48 or 72 h), data from cell cultures exposed to negative controls, PM, and positive controls were compared with those from non-exposed cell cultures. Statistical analyses were performed by the Mann-Whitney U test (Software: SPSS for Windows, v10.05, 2000; Paris, France). Statistically significant differences were reported with p values <0.05.

RESULTS AND DISCUSSION

PM Physical and Chemical Characteristics

Aerosol sampling was realized using a high volume cascade impactor designed to collect PM with a size $\leq 5 \mu\text{m}$. PM size distribution (Figure 2) revealed that it was in the size range 0.33-5.0 μm with a peak at 0.45 μm . Sampling restrictions were also respected insofar as 98.5% of PM have a size $< 5 \mu\text{m}$. The highest number of collected PM was also detected in size classes including PM with a size $\leq 2.5 \mu\text{m}$: 0-0.5 μm (33.63%), 0.5-1.0 μm (30.61%), 1.0-1.5 μm (14.33%), 1.5-2.0 μm (8.69%), and 2.0-2.5 μm (4.89%). Cumulative frequencies of PM₁ and PM_{2.5} (i.e. 64.24% and 92.15%, respectively) indicated that they were able to penetrate to the alveolar region of the lung to exert their toxicity (Harrison and Yin, 2000). According to epidemiological evidence, the greatest health risks correlate with smaller PM (Levy *et al.*, 2000). Electronic microscopy observations showed structure differences in Fe oxide particles according to their origins: the anthropogenic ones were also characterized by a spherical form due to high temperature manufacturing process like blast furnace (Figure 3). Reported low sizes conferred to collected PM a relatively high specific surface area (i.e. 1 m²/g for PM, and 50 m²/g for dPM), which could exacerbate their reactivity and allow the adsorption of other chemicals (Warren, 2002).

A total of 20 inorganic elements were found in Dunkerque City's PM (Figure 4). Among them, Fe, Al, Ca, Na, Pb, Mg, and Mn were the most abundantly present and

constitute not only elements usually associated with natural environment (i.e. Ca, Mg, Na), but also so-called anthropogenic inorganic elements (e.g. Al, Mn, Pb, Zn). Ionic species were detected: SO_4^{2-} , NO_3^- , Cl^- , NH_4^+ . An exhaustive determination of the organic chemical-coated onto PM was realized using a thermal desorption at 500°C coupled with cold trapping, prior GC/MS analyses (Table 2). TG-DTA curves performed under air evidenced the presence of several thermal phenomena (Figure 5). At first, at low temperature (146°C), an endothermic peak was ascribable to the gypsum dehydration in the collected PM (Figure 5a). In the temperature range $200\text{--}650^\circ\text{C}$, exothermic reactions were pointed at 315°C , 412°C et 498°C . These observations can be considered as a good evidence of the presence of organic compounds in the PM. As the experiment has been performed under oxidative conditions, the combustion of organic and carbonaceous compounds lead to exothermic phenomena recorded at 315°C and 412°C . The exothermic peak detected at 498°C could be related to the oxidation of amorphous carbon black. The comparison between the TG-DTA curves from collected PM (Figure 5a) and 500°C -GC/MS-analyzed PM (Figure 5b) revealed a decrease in the intensity of the first exothermic peak, showing partial desorption of the organic chemicals. Indeed, the PM thermal desorption at 500°C allowed a relative increase of the specific surface area (i.e. $28\text{ m}^2/\text{g}$ vs PM: $1\text{ m}^2/\text{g}$), suggesting that some organic chemicals remained coated. Various VOC (i.e. benzene, toluene, xylene, etc.), PAH (i.e. anthracene, fluoranthene, pyrene, etc.), and paraffins, were mainly detected. Accordingly, transition metals, VOC and PAH are often associated with PM, either through adherence to the core particle or as an integral component of the particle (Dreher *et al.*, 1997; Monn and Becker, 1999; Nel *et al.*, 2001). Moreover, PAH concentrations in Dunkerque City's PM were close to those in other PM collected in two locations in Paris (Baulig *et al.*, 2004). The chemical composition of Dunkerque City's PM was also closely related to the anthropogenic emission sources located near the sampling site; apart the heavy motor vehicle traffic, iron and steel industry, aluminum industry, oil refinery, basic chemistry, pharmaceutical industry, and food industry must be cited. The collected PM consisted in a very complex mixture of chemicals, generally present at relatively low doses, thereby rendering very difficult the share of toxicity ascribable to the different fractions (inorganic vs organic, notably).

PM Outgassing

To better define the role of the organic fraction, PM having undergone a thermal desorption at 400°C under secondary vacuum (i.e. dPM) was included as negative controls. The differences from the TG-DTA curves between dPM and PM showed that this outgassing method conserved inorganic structures but strongly desorbed organic chemicals (Figure 5c). The TG-DTA data showed the difference of weight losses suffered by PM and dPM between 100 to 550°C : 24.5 and 8.7% , respectively (Figures 5a and 5c). The remove of the organic fraction-coated onto PM using this outgassing method induced highly increase of the specific surface area; dPM: $50\text{ m}^2/\text{g}$ vs PM: $1\text{ m}^2/\text{g}$. Hence, the organic fraction was also not just coated-onto PM but firmly incrustated in the inorganic condensation nuclei present in the PM.

Observation of the Organic Fraction-Coated onto PM-Exposed Cells.

In the Figure 6a, PM seemed to be internalized by A549 cells. Using fluorescence illumination, the organic fraction-coated onto internalized particles could be observed (Figure

6b). Moreover, in dPM-exposed A549 cells, few remaining organic chemical-coated onto dPM were seen, suggesting the incomplete desorption of the organic fraction despite the employed outgassing method (data not shown).

PM Cytotoxicity

Thereafter, we focused our attention on the cytotoxicity of Dunkerque City's PM in A549 cells. Table 3 shows that the incorporation of BrdU significantly decreased in PM-exposed cells at concentrations $\geq 37.68 \mu\text{g PM/mL}$ (or $22.84 \mu\text{g PM/cm}^2$) in a concentration-dependent manner ($p < 0.05$). Similarly, MDH activity decreased in A549 cells 24 or 48 h after their exposure to PM at concentrations $\geq 37.68 \mu\text{g PM/mL}$ (or $22.84 \mu\text{g PM/cm}^2$; $p < 0.05$), or 72 h after their exposure to PM at concentrations $\geq 75.36 \mu\text{g PM/mL}$ (or $45.67 \mu\text{g PM/cm}^2$; $p < 0.05$). Concentration-dependent decreases in the incorporation of BrdU and activity of MDH in PM-exposed cells suggest the alteration of cellular division and mitochondrial metabolism, respectively. Significant increases of extracellular LDH activity were observed in A549 cells 72 h after their exposure to $150.72 \mu\text{g PM/mL}$ (or $91.35 \mu\text{g PM/cm}^2$; $p < 0.001$), and indicated a late disturbance of membrane integrity and/or permeability. Results of the colony-forming method showed a concentration-dependent effect in proliferating A549 cells. That method allowed the calculation of LC_{10} ($23.72 \mu\text{g PM/mL}$; or $5.81 \mu\text{g PM/cm}^2$) and LC_{50} ($118.60 \mu\text{g PM/mL}$; or $29.03 \mu\text{g PM/cm}^2$) (Figure 7). Dagher *et al.* (2005), studying the cytotoxicity of these PM in embryonic cells derived from human lung epithelium (L132 cell line), reported LC_{10} ($18.84 \mu\text{g/mL}$; or $4.61 \mu\text{g PM/cm}^2$) and LC_{50} ($75.36 \mu\text{g/mL}$; or $18.45 \mu\text{g PM/cm}^2$) quite different, mainly arising from their different origin (normal vs carcinoma).

mRNA Expression of VOC and/or PAH-Metabolizing Enzymes

One of the main findings of this work was, therefore, the ability of the PM to induce the gene expression of *cyp1a1*, *cyp2e1*, *cyp2f1*, and *nqo1*. Indeed, a statistically significant induction of *cyp1a1* transcript was observed in A549 cells, 24, 48 and 72h after their exposure to dPM at its EqLC_{10} ($p < 0.05$) and EqLC_{50} ($p < 0.01$), to PM at its LC_{10} ($p < 0.01$) and LC_{50} ($p < 0.01$), and to B(a)P ($p < 0.01$), compared to controls (Table 4). With regards to *cyp2e1* gene expression, a significant increase was observed in A549 cells 24 h after their exposure to PM at its LC_{10} ($p < 0.05$) and LC_{50} ($p < 0.05$), 48 h after their exposure to PM at its LC_{10} ($p < 0.05$) and LC_{50} ($p < 0.01$), and 72 h after their exposure to PM at its LC_{50} ($p < 0.01$), versus controls (Table 4). Cell exposure to benzene or toluene caused an induction of *cyp2e1* gene expression only after 24 h of exposure, versus controls ($p < 0.05$). An increase of *cyp2f1* transcript was observed in A549 cells, 24 h after their exposure to PM at its LC_{50} ($p < 0.05$), 48 h after their exposure to PM at its LC_{10} ($p < 0.05$) and LC_{50} ($p < 0.01$), and 72 h after their exposure to PM at its LC_{50} ($p < 0.01$), compared to controls (Table 4). Cell exposure to benzene induced enhanced mRNA expression of *cyp2f1* gene after 24 h of incubation, versus controls ($p < 0.05$). No significant variations of *meh* transcripts were reported (data not shown). A significant increase in *nqo1* mRNA expression were observed in A549 cells, 24, 48 and 72 h after their exposure to PM at their LC_{10} and LC_{50} ($p < 0.05$ or $p < 0.01$), and 24 and 48 h after their exposure to benzene ($p < 0.05$), and B(a)P ($p < 0.05$ and $p < 0.01$, respectively), versus non-exposed cells (Table 4). In agreement with these data, the transcription of *cyp1a1* gene seen in cells exposed to dPM confirmed that the employed outgassing method was not

enough efficient to remove total PAH. In other respects, it is important to highlight that the gene induction of VOC functionalization enzymes (i.e. CYP2E1 and CYP2F1) by PM at their LC₅₀ continued until 72 h. In contrast, the induction of the gene transcripts of *cyp2e1* and *cyp2f1* by PM at their LC₁₀ was limited to 48 h. This result suggested the role of the physical vector of PM, which can enhance both the penetration and the retention of VOC into the cells, thereby enabling them to exert a more durable induction (Garçon et al., 2004a; 2004b; Vakharia *et al.*, 2001a; 2001b). This hypothesis was supported by the observations of PM-exposed cells by fluorescence microscopy, which showed the organic compounds-coated onto PM, even after their internalization. Within the aerosol, VOC-coated onto PM would be less sensitive to an early volatilization, as suggested by the fast but short induction of *cyp2e1*, *cyp2f1* and *nqo1* genes in benzene and/or toluene-exposed A549 cells. With agreement with Hukkanen *et al.* (1997; 2002), Runge *et al.* (2001), and Sheets *et al.* (2004), we observed a great difference between the levels of *cyp2e1* and *cyp2f1* transcripts in exposed A549 cells, suggesting the key role played by CYP2E1 in the metabolic activation of VOC. According to Jaiswal (2000) and Joseph *et al.* (1994), the gene expression of *nqo1* increased in A549 cells exposed to the VOC-coated onto PM. However, cell exposure to toluene did not induce any gene expression of *nqo1*. This can be explained by the limited role of this enzyme in the metabolic activation of toluene, which is mainly converted into benzyl alcohol after the oxidation of its methyl function; this latest is further oxidized in benzaldehyde and benzoic acid. The CYP2E1 and CYP1A1 respectively catalyze the oxidation of benzene and B(a)P in epoxides, which can be converted into diols, then in catechols and finally in quinone derivatives. The significant induction of *nqo1* gene expression by PM, benzene or B(a)P is also in agreement with the literature. In contrast, *meh* gene has already been described as not inducible by organic chemicals (Bauer *et al.*, 2003; Guengerich *et al.*, 1996).

The epoxides producing through the first phase of the metabolic activation of organic chemicals are thereafter conjugated by GST- μ 1, GST- μ 3, GST- π 1 and GST- τ 1, while phenols, hydroquinones, catechols and trihydroxybenzenes are conjugated with sulphates or glucuronides, before their excretion (Henderson *et al.*, 1989; Rossi *et al.*, 1999). The gene expression of *gst- μ 1* could not be detected in A549 cells. Accordingly, *gst- μ 1* is deleted in about half of Caucasians, and its gene expression cannot be observed in A549 cells (Bell *et al.*, 1993). Similarly, whatever the considered exposure and incubation time, there was no significant variation of *gst- μ 3* gene expression (data not shown). However, Macé *et al.* (1998) reported that this isoenzyme plays a major role in the detoxification. Statistically significant induction of *gst- π 1* gene transcription was reported in A549 cells 48 or 72 h after their exposure to PM at its LC₁₀ (p<0.01 and p<0.05, respectively) and LC₅₀ (p<0.01), benzene (p<0.01 and p<0.05, respectively), toluene (p<0.01 and p<0.05, respectively), and B(a)P (p<0.05) (Table 5). In human lung, GST- π 1 is also the most active followed by GST- μ 1 and GST- μ 3, whereas GST- τ 1 lacks of activity (Piipari *et al.*, 2003).

In conclusion, the physical and chemical characteristics of Dunkerque City's PM indicated that much of this PM derived from wind-borne dust from the industrial complex, and the heavy motor vehicle traffic. Taken together, these results supported that the metabolic activation of very low doses of VOC and/or PAH-coated onto the inorganic condensation

nuclei of the Dunkerque City's PM is one of the underlying mechanisms of action closely involved in its cytotoxicity in human lung epithelial A549 cells.

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FIGURES

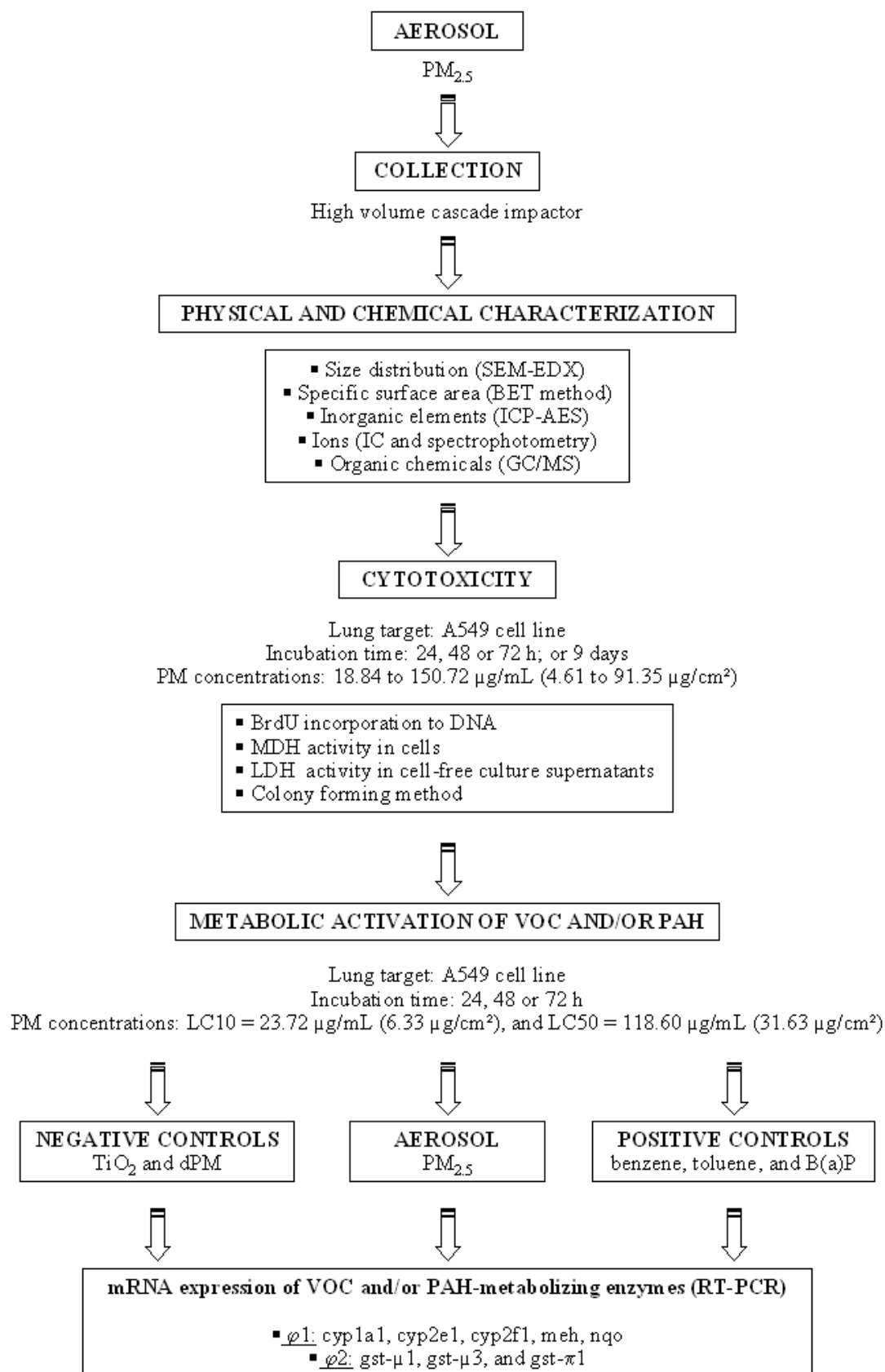


Figure 1: This work consisted in a multi-step approach including the collection of particle matter (PM), the determination of its physical and chemical characteristics, the evaluation of its cytotoxicity in A549 cells, and the study of the metabolic activation of volatile organic compound (VOC) and/or polycyclic aromatic hydrocarbon (PAH)-coated onto collected PM by A549 cells. (Scanning electron microscopy coupled with energy dispersive X-ray, SEM-EDX; Brunauer, Emmett, and Teller, BET; inductively coupled plasma-atomic emission spectrometry, ICP-AES; ionic chromatography, IC; gas chromatography-mass spectrometry, GC/MS; 5-bromo-2-deoxyuridine, BrdU; mitochondrial dehydrogenase, MDH; lactate dehydrogenase, LDH; desorbed particulate matter, dPM; benzo(a)pyrene, B(a)P).

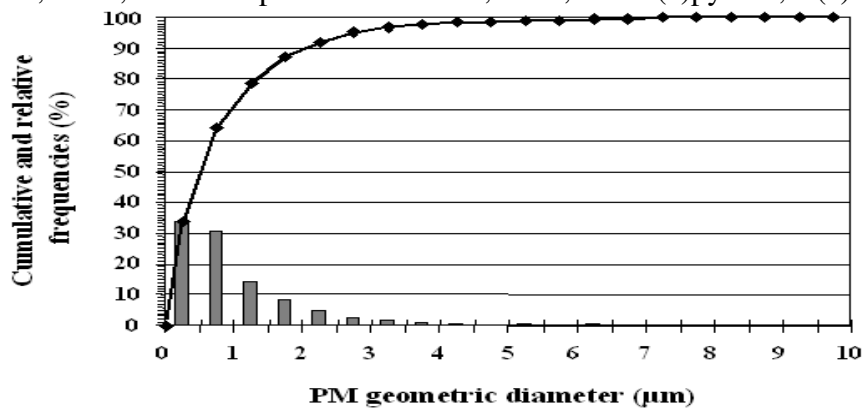


Figure 2: Size distribution of the particle matter (PM) collected in Dunkerque, a French seaside city located on the southern coast of the North-Sea, using high volume cascade impactor, is depicted as cumulative frequency (%; curve) and relative frequency (%; histogram). PM size distribution has been determined using scanning electron microscopy.

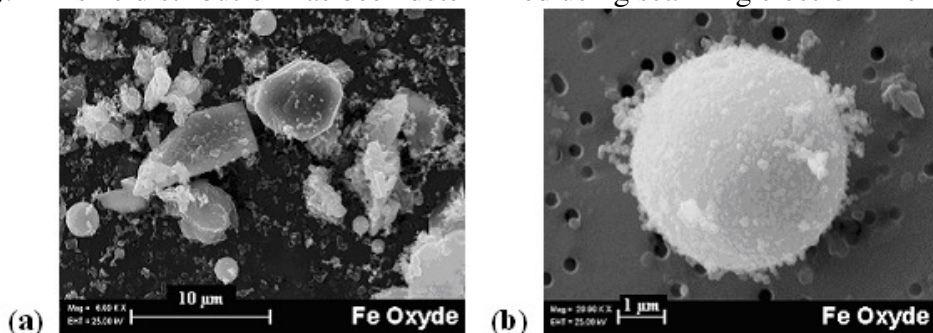


Figure 3: Scanning electron microscopy coupled with energy dispersive X-ray analysis of Fe oxides in collected particle matter: (a) natural origin; (b) anthropogenic origin.

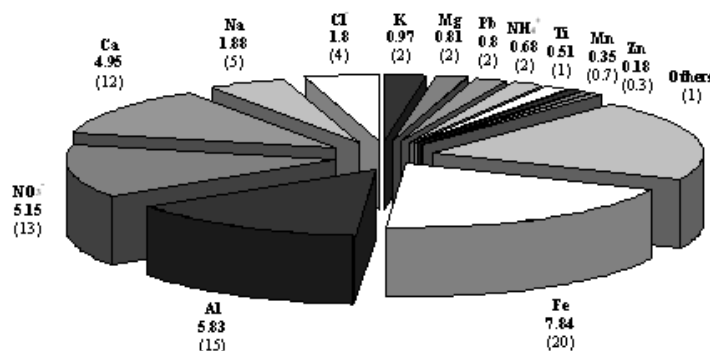


Figure 4: Inorganic compounds detected in collected particle matter (PM) by inductively coupled plasma-atomic emission spectrometry (ICP-AES), ionic chromatography (Cl⁻, NO₃⁻ and SO₄²⁻), or visible spectrophotometry (NH₄⁺) (percentage m/m of total PM in bold, and of the inorganic fraction in brackets).

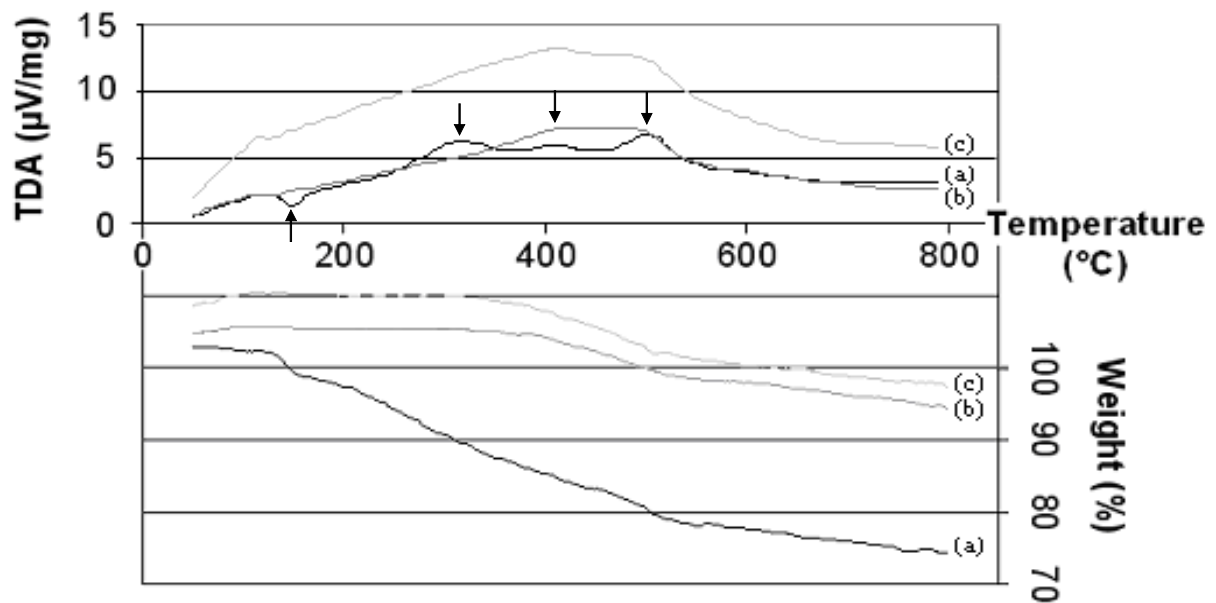


Figure 5: Thermogravimetric and differential thermal analysis (TG-DTA) of the particle matter (PM): (a) native collected PM, (b) 500°C-GC/MS-analyzed PM, and (c) 400°C-outgassed under secondary vacuum-PM (desorbed PM; dPM). (↑: endothermic peak; ↓: exothermic peak).

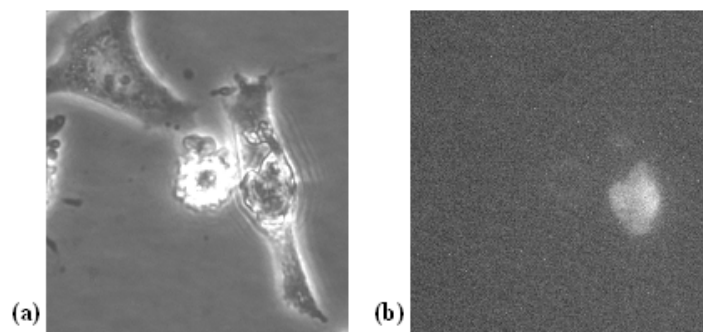


Figure 6: Contrast phase microscopy observations of particle matter-exposed A549 cells (Magnification: 400 x): (a) White light (b) Rhodamin (excitation: 570 nm; emission 590 nm).

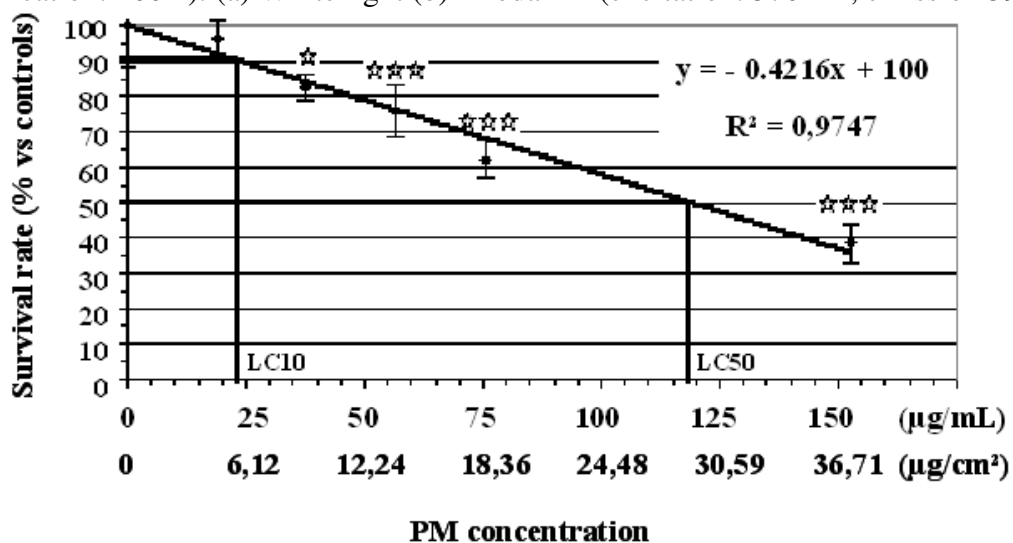


Figure 7: Survival rates (%) of A549 cells 9 days after their incubation in the continuous presence of increasing concentrations of collected particle matter (PM; 18.84, 37.68, 56.52, 75.36 or 150.72 $\mu\text{g PM/ml}$; or 4.61, 9.22, 13.83, 18.45 or 36.89 $\mu\text{g PM/cm}^2$) without renewing the culture media. Non-exposed cells were used as negative controls. These values are depicted as mean values and standard deviation of 10 cell cultures for negative controls and 5 cell cultures for every PM concentrations. Lethal concentrations at 10% and 50% (LC_{10} and LC_{50} , respectively) were calculated comparing the number of crystal violet-stained cell colonies in exposed culture dishes with the number in non exposed culture dishes: $\text{LC}_{10} = 23.72 \mu\text{g PM/ml}$ (or $5.81 \mu\text{g PM/cm}^2$) and $\text{LC}_{50} = 118.60 \mu\text{g PM/ml}$ (or $29.03 \mu\text{g PM/cm}^2$). (Mann-Whitney U test; vs controls; *: $p < 0.05$; ***: $p < 0.001$).

Table 1: Sequence of the Primers for the Study of the mRNA Expression of VOC and/or PAH-Metabolizing Enzymes

<i>Gene</i>	<i>Primers sequences</i>	Size (bp)	<i>Reference</i>
<i>cyp 1a1</i>	S 5'-TCT TTC TCT TCC TGG CTA TC-3' AS 5'-CTG TCT CTT CCC TTC ACT CT-3'	596	Iwanari <i>et al.</i> , 2002
<i>cyp 2e1</i>	S 5'-TGC CAT CAA GGA TAG GCA AG-3' AS 5'-AAT GCT GCA AAT GGC ACA C-3'	356	Macé <i>et al.</i> , 1998
<i>cyp 2f1</i>	S 5'-GCT GCG GAA AAC TGA AGG-3' AS 5'-GCC AAA GAG CAG GTT ATG TGT-3'	427	Nichols <i>et al.</i> , 2003
<i>meh</i>	S 5'-TGA GCG GAT GAA GGT CTA TG-3' AS 5'-TCA AAG CCA TGT TGC TTA CC-3'	401	NCBI GenBank
<i>nqo1</i>	S 5'-TGA AGA AGA AAG GAT GGG AGG-3' AS 5'-AGG GGG AAC TGG AAT ATC AC-3'	223	Spivack <i>et al.</i> , 2003
<i>gst-μ1</i>	S 5'-ACT TTC CCA ATC TGC CCT AC-3' AS 5'- TTC TGG ATT GTA GCA GAT CA-3'	191	Spivack <i>et al.</i> , 2003
<i>gst-μ3</i>	S 5'-ACT TTC CTA ATC TGC CCT ACC -3' AS 5'-TAA CAC ACC TGC TCT CTC C-3'	805	Spivack <i>et al.</i> , 2003
<i>gst-π1</i>	S 5'-CAC CAA CTA TGA GGC GGG CAA-3' AS 5'- ATC AGC AGC AAG TCC AGC A-3'	159	Spivack <i>et al.</i> , 2003
<i>β-actin</i>	S 5'-ATC ATG TTT GAG ACC TTC AA-3' AS 5'-CAT CTC TTG CTC GAA GTC CA-3'	314	Dubois <i>et al.</i> , 2000

Table 1 presents the sequences of the Sense (S) and Antisense (AS) primers for the study of the mRNA expression of volatile organic compounds (VOC) and/or polycyclic aromatic hydrocarbons (PAH)-metabolizing enzymes: *cytochrome P450 (cyp) 1a1*, *cyp 2e1*, *cyp 2f1*, *microsomal epoxyde hydrolase (meh)*, *nadph quinone oxydo-reductase 1 (nqo1)*, and *glutathione S-transferase (gst)- μ 1*, *gst- μ 3*, and *gst- π 1*. The primers for *meh* (Accession no. NM_000120) were designed by sequence information of the National Center for Biotechnology Information (NCBI) Genbank (Accession n°NM_000120) and Primer 3 Input v02 software (bp = base pairs).

Table 2: Organic Compounds Detected in the Collected PM after Thermal Desorption and Gas Chromatography-Mass Spectrometry Analyses

Compound	C (ppm)	Compound	C (ppm)
Benzene	106.5	Phenanthrene	28.3
Toluene	42.2	Dibutylphthalate	110.1
Nonene	9.3	Eicosane	1.4
Xylene	65.4	Fluoranthene	4.7
Styrene	13.5	Henicosane	5.2
1-Ethenyltrimethylbenzene	15.4	Docosane	17.4
Decane	64.9	Tricosane	8.7
Diethylbenzene	170.3	Tetracosane	39.7
Limonene epoxyde	20.5	Pentacosane	12.2
Tetramethylbenzene	89.5	Bis(Ethyl.Hexyl)phtalate	41.4
Naphtalene	38.1	Acenaphtene	11.1
Methylnaphtalene	141.9	Fluorene	6.3
Dimethylnaphtalene	90.2	Pyrene	4.7
Heptadecane	15.1	Benzo(a)anthracene	4.9
Anthracene	47.1	or Chrysene	

Table 2 shows the list of the organic compounds detected in the collected particulate matter (PM). The concentrations are expressed in ppm ($\mu\text{g/g}$).

Table 4: Metabolic Activation of the VOC and/or PAH-Coated onto PM: Cytochromes P-450

		EXPOSURE									
		C	TiO ₂ EqLC ₁₀	TiO ₂ EqLC ₅₀	dPM EqLC ₁₀	dPM EqLC ₅₀	PM LC ₁₀	PM LC ₅₀	Benzene 7μM	Toluene 7μM	B(a)P 1μM
<i>cyp 1a1</i>	24h	1 ± 0.340	0.922 ± 0.350	1.205 ± 0.502	1.620* ± 0.342	2.159** ± 0.671	2.889** ± 0.309	3.382** ± 1.054	1.150 ± 0.390	0.835 ± 0.269	3.235** ± 0.849
	48h	1 ± 0.231	0.894 ± 0.264	0.824 ± 0.053	1.342** ± 0.254	1.683** ± 0.406	2.341** ± 0.203	2.669** ± 0.679	0.980 ± 0.166	0.766 ± 0.168	2.165** ± 0.334
	72h	1 ± 0.269	0.869 ± 0.232	1.018 ± 0.278	1.578* ± 0.541	1.916** ± 0.545	2.419** ± 0.719	2.893** ± 1.020	0.978 ± 0.176	0.843 ± 0.374	2.65** ± 0.251
<i>cyp 2e1</i>	24h	1 ± 0.396	1.333 ± 0.244	1.345 ± 0.287	1.330 ± 0.121	1.048 ± 0.158	1.682* ± 0.521	2.009** ± 0.376	1.896* ± 0.665	1.616* ± 0.463	1.500 ± 0.215
	48h	1 ± 0.233	0.958 ± 0.166	0.930 ± 0.200	0.792 ± 0.103	0.813 ± 0.153	1.614* ± 0.527	1.962** ± 0.319	1.170 ± 0.300	1.490 ± 0.516	1.531 ± 0.504
	72h	1 ± 0.331	0.867 ± 0.182	0.826 ± 0.261	0.796 ± 0.081	1.343 ± 0.183	1.150 ± 0.038	2.290** ± 0.212	1.417 ± 0.476	0.933 ± 0.169	0.782 ± 0.086
<i>cyp 2f1</i>	24h	1 ± 0.294	1.113 ± 0.334	1.076 ± 0.297	0.905 ± 0.042	1.267 ± 0.219	1.564 ± 0.543	2.329* ± 0.625	1.465* ± 0.510	1.287 ± 0.104	1.660 ± 0.569
	48h	1 ± 0.180	0.989 ± 0.194	1.046 ± 0.236	1.054 ± 0.354	1.103 ± 0.262	1.358* ± 0.271	1.638** ± 0.319	1.105 ± 0.253	1.088 ± 0.200	1.024 ± 0.130
	72h	1 ± 0.279	1.069 ± 0.157	1.037 ± 0.164	0.967 ± 0.156	0.959 ± 0.283	1.088 ± 0.240	1.700* ± 0.322	0.931 ± 0.208	1.075 ± 0.143	0.882 ± 0.140

Gene expressions of *cytochrome P450 (cyp) 1a1*, *cyp2e1*, and *cyp2f1* (fold-induction vs controls) in A549 cells 24, 48 or 72 h after their exposure. Cells exposed to TiO₂ (<0.2μm) or to desorbed particulate matter (dPM) at equivalent concentrations to inorganic LC₁₀ (Eq LC₁₀ = 19.42 μg PM/mL or 5.18 μg PM/cm²) or LC₅₀ (Eq LC₅₀ = 97.13 μg PM/mL or 25.90 μg PM/cm²), integrating weight losses due to the organic desorption, were used as negative controls. Other cells were exposed to particulate matter (PM) at their LC₁₀ (i.e. 23.72 μg PM/mL or 6.33 μg PM/cm²) or LC₅₀ (i.e. 118.60 μg PM/ml or 31.63 μg PM/cm²). Moreover, cells exposed to benzene, toluene and benzo(a)pyrene (B(a)P) served as positive controls for the induction of the mRNA expression of volatile organic compounds (VOC) and/or polycyclic aromatic hydrocarbons (PAH)-metabolizing enzymes. These values are depicted as mean values and standard deviation of 8 replicates for controls (C), and 4 replicates for negative controls, every PM concentrations, and positive controls (Mann-Whitney U-test; vs controls; *: p<0.05; **: p<0.01).

Table 5: Metabolic Activation of the VOC and/or PAH-Coated onto PM: Other Enzymes

		EXPOSURE									
		C	TiO ₂ EqLC ₁₀	TiO ₂ EqLC ₅₀	dPM EqLC ₁₀	dPM EqLC ₅₀	PM LC ₁₀	PM LC ₅₀	Benzene 7μM	Toluene 7μM	B(a)P 1μM
<i>nqo1</i>	24h	1 ± 0.119	0.977 ± 0.223	0.964 ± 0.153	1.081 ± 0.157	0.932 ± 0.102	1.252** ± 0.139	1.350* ± 0.253	1.264* ± 0.162	1.032 ± 0.148	1.204** ± 0.249
	48h	1 ± 0.289	1.226 ± 0.383	1.289 ± 0.272	0.909 ± 0.263	1.429 ± 0.342	1.584* ± 0.274	1.759* ± 0.427	1.368* ± 0.133	1.254 ± 0.300	1.427* ± 0.145
	72h	1 ± 0.328	0.894 ± 0.434	1.129 ± 0.412	1.220 ± 0.244	1.159 ± 0.310	1.475* ± 0.343	1.742** ± 0.236	1.447 ± 0.537	1.206 ± 0.388	0.832 ± 0.341
<i>gst-πI</i>	24h	1 ± 0.093	0.986 ± 0.101	0.991 ± 0.083	1.042 ± 0.100	1.039 ± 0.100	1.003 ± 0.057	1.092 ± 0.094	1.060 ± 0.134	0.996 ± 0.078	1.192 ± 0.238
	48h	1 ± 0.062	0.985 ± 0.082	0.941 ± 0.150	0.996 ± 0.078	1.017 ± 0.066	1.302** ± 0.097	1.265** ± 0.073	1.193** ± 0.151	1.102** ± 0.097	1.135** ± 0.044
	72h	1 ± 0.094	1.063 ± 0.136	1.042 ± 0.144	0.958 ± 0.077	1.073 ± 0.142	1.312* ± 0.122	1.335** ± 0.046	1.153* ± 0.079	1.279* ± 0.189	1.289* ± 0.121

Gene expressions of the *NADPH quinone oxido-reductase-1 (nqo1)* and *glutathione S-transferase-pi I (gst-πI)* (fold-induction vs controls) in A549 cells 24, 48 or 72 h after their exposure. Cells exposed to TiO₂ (<0.2μm) or to desorbed particulate matter (dPM) at equivalent concentrations to inorganic LC₁₀ (Eq LC₁₀ = 19.42 μg PM/mL or 5.18 μg PM/cm²) or LC₅₀ (Eq LC₅₀ = 97.13 μg PM/mL or 25.90 μg PM/cm²), integrating weight losses due to the organic desorption, were used as negative controls. Other cells were exposed to particulate matter (PM) at their LC₁₀ (i.e. 23.72 μg PM/mL or 6.33 μg PM/cm²) or LC₅₀ (i.e. 118.60 μg PM/ml or 31.63 μg PM/cm²). Moreover, cells exposed to benzene, toluene and benzo(a)pyrene (B(a)P) served as positive controls for the induction of the mRNA expression of volatile organic compounds (VOC) and/or polycyclic aromatic hydrocarbons (PAH)-metabolizing enzymes. These values are depicted as mean values and standard deviation of 8 replicates for controls (C), and 4 replicates for negative controls, every PM concentrations, and positive controls (Mann-Whitney U-test; vs controls; *: p<0.05; **: p<0.01).