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Pamela Melki

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DOCTORAL THESIS

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Faculty of Sciences: Department of Biology- Department of Chemistry (UOB)

by

Pamela N. MELKI

HEALTH IMPACT OF AIRBORNE PARTICULATE MATTER IN NORTHERN LEBANON: FROM A PILOT EPIDEMIOLOGICAL STUDY TO PHYSICO-CHEMICAL CHARACTERIZATION AND TOXICOLOGICAL EFFECTS ASSESSMENT

JURY MEMBERS:

Charbel AFIF	Associate professor, University Saint Joseph	Examiner
Véronique ANDRE	Associate professor, University of Caen	Examiner
Zeina DAGHER	Professor, Lebanese university	Examiner
Hervé SABOURIN	Professor, Université de Poitiers	Examiner
Dominique COURCOT	Professor, University of the Littoral Côte d'Opale	Thesis co-director
Roula ABDEL MASSIH	Associate professor, University of Balamand	Thesis co-director
Sylvain BILLET	Associate professor, University of the Littoral Côte d'Opale	Thesis co-director
Samer AOUAD	Associate professor, University of Balamand	Thesis co-director
Frédéric LEDOUX	Associate professor, University of the Littoral Côte d'Opale	Thesis co-director

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LIST OF ABBREVIATIONS

2AA	2-Amino-Anthracene
Ac	Acenaphthene
AcI	Acenaphthylene
AhR	Aryl Hydrocarbon Receptor
Ant	Anthracene
AP	Apurinic/aprimidinic sites
AP-1	Activator protein 1
APE	AP-endonuclease
ARE	Antioxidant response elements
As	Arsenic
ASK1	Apoptosis signaling kinase 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
B[a]An	Benz[a]anthracene
B[b]Fl	Benzo[b]fluoranthene
B[k]Fl	Benzo[k]fluoranthene
B[a]P	Benzo[a]pyrene
BCA	Bicinchoninic Acid
β -gal	β -galactosidase
B[ghi]Pe	Benzo[g,h,i]perylene
BER	Base excision repair
B-NHEJ	Backup NHEJ
BPDE	B[a]P-7,8-diol-epoxide
BrdU	5-bromo-2-deoxyuridine
Ca ²⁺	Calcium
CaCO ₃	Calcium carbonate/ Calcite
CaSO ₄	Gypsum
CAT	Catalase
Cd	Cadmium
χ^2	<i>Chi</i> -squared test
Chk	Checkpoint protein
Chr	Chrysene
CI	Confidence interval
Cl ⁻	Chlorides
CCD	Charge-coupled device
C _{max}	Carbon maximum number
CO	Carbon monoxide
CO ₂	Carbon dioxide
COMT	Catechol-O-methyltransferase
COPD	Chronic obstructive pulmonary disease
CPI	Carbon preference index
Cr	Chromium
C _t	Threshold cycle

Cu	Copper
CVD	Cardiovascular diseases
CYP	Cytochrome P450
D _{ae}	Aerodynamic diameter
DDR	DNA damage response
DEP	Diesel exhaust particles
DiB [a,h]An	Dibenz[a,h]anthracene
DL	Detection limit
DL-PCBs	Dioxin-Like polychlorinated biphenyls
ΔMFI	Median Fluorescence Intensity
DMS	Dimethyl sulphide
DNA-PK	DNA dependent protein kinase
DNMTs	DNA methyltransferases
5'-dRP	5'-Deoxyribose phosphate terminal
DSBs	DNA double-strand breaks
EC	Elemental carbon
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EEA	European Environment Agency
EOM	Extractable organic matter
ETS	Environmental tobacco smoke
FP	Fine particles
F	Fluorine
Fla	Fluoranthene
Flu	Fluorene
FP-I	Industrial influenced fine particles
FP-R	Rural influenced fine particles
GC-MS	Gas Chromatography-Mass Spectrometry
GHG	Greenhouse gases
GPx	Glutathione peroxidase
HCl	Hydrochloric acid
Hg	Mercury
HNO ₃	Nitric acid
HRR	Homologous recombination repair
H ₂ SO ₄	Sulfuric acid
ICP-AES	Inductively Coupled Plasma- Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
ICW	In-Cell Western
IF	Induction Factor
IgE	Immunoglobulin E
I.I.	Industrial Impact
<i>iNOS</i>	Inducible nitric oxide synthase
InPy	Indeno[1,2,3-cd]pyrene
ISCLs	Inter-strand cross-links
I-TEQ	International Toxic Equivalent Quantity
K ⁺	Potassium
K ₂ SO ₄	Potassium sulfates

LDH	Extracellular lactate dehydrogenase
LINE 1	Long interspersed nucleotide element 1
MAPKKK	MAP kinase kinase kinase
MDA	Malondialdehyde
MDH	Mitochondrial dehydrogenase
ME	Major elements
Mg ²⁺	Magnesium
MgSO ₄	Magnesium sulfates
MR	Mutagenicity Ratio
MRN	MRE-Rad50-Nbs1
MTH1	MutT Homolog 1
Na ⁺	Sodium
NaCl	Sodium chloride
NaNO ₃	Sodium nitrate
Nap	Naphthalene
Na ₂ SO ₄	Sodium sulfates
NER	Nucleotide excision repair
2-NF	2-Nitrofluorene
NH ₃	Ammonia
NH ₄ ⁺	Ammonium
NHEJ	Non homologous end-joining
NH ₄ NO ₃	Ammonium nitrate
Ni	Nickel
NMVOC	Non-Methane Volatile Organic Compounds
NO	Nitrogen oxide
NO ₂	Nitrogen dioxide
NO ₃ ⁻	Nitrates
N ₂ O	Nitrous oxide
NO _x	Nitrogen oxides
NQO1	NADPH quinone oxidoreductase
4-NQO	4-Nitro-Quinoline-Oxide
NSCLC	Non-Small Cell Lung Carcinomas
SCLC	Small Cell Lung Carcinoma
O ₃	Ozone
OC	Organic carbon
OGG1	Oxoguanine glycosylase 1
8-OHdG	8-hydroxy-2'-deoxyguanosine
OR	Odds ratio
8-oxoG	8-oxo-7,8-dihydroguanine
PAHs	Polycyclic aromatic hydrocarbons compounds
PAL	Alkaline phosphatase
PARP	Poly-ADP-ribose polymerase
Pb	Lead
PC1	Principal Component 1
PCA	Principal Component Analysis
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo- <i>p</i> -dioxins

PCDFs	Polychlorinated dibenzo-furans
PCNA	Proliferating cell nuclear antigen
Ph	Phenanthrene
γ H2AX	Phosphorylated H2AX
PIKKs	Phosphatidylinositol 3-kinase-like protein kinase
PM	Particulate matter
PNK	Polynucleotide kinase
PO_4^{3-}	Phosphates
POPs	Persistent organic pollutant
Pyr	Pyrene
RAL	Relative Adduct Levels
ROS	Reactive oxygen species
RRs	Relative risks
RTLTF	Respiratory tract lining fluid
SAM	S-adenosylmethionine
Sb	Antimony
SD	Standard deviation
Se	Selenium
SE	Southeast
SEM-EDX	Scanning electron microscope with energy dispersive X-ray analysis
SI	Water-soluble Ions
SiO_2	Quartz
SO_2	Sulfur dioxide
SO_4^{2-}	Sulfates
SOD	Superoxide dismutase
SW	Southwest
TC	Total carbon
TE	Trace Elements
TEF	Toxic Equivalent Factor
TSG	Tumor suppressor genes
UFP	Quasi-ultrafine particles
U.S. EPA	United States-Environmental Protection Agency
V	Vanadium
VOCs	Volatile organic compounds
WHO	World Health Organization
XPC	Xeroderma pigmentosum, complementation group C
XRCC1	X-ray repair cross-complementing group 1
XRD	X-ray diffraction
XRE	Xenobiotic response element
Zn	Zinc

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FOREWORD

The scale and seriousness of the impact of air pollution on health that have been detected by scientific investigations over the past decade are the subject of media reports and policy debate throughout the world. According to the World Health Organization, more than 7 million premature deaths each year are related to the effects of urban outdoor and indoor air pollution.

Air pollutants can be encountered in two forms: gases and particles. This thesis focuses on airborne particulate matter (PM), a complex mixture of particles originating from natural sources (marine aerosols, soil erosion, volcanic eruptions, biogenic particles...etc.) as well as from anthropogenic ones (road traffic, industrial emissions...etc.). Most of the inhaled PM is removed by mucociliary clearance, yet the PM_{2.5} fraction, the so-called alveolar fraction with aerodynamic diameter less than 2.5 µm is largely retained in lungs. PM_{2.5} accounts thus for 96% of the number of particles observed in human pulmonary parenchyma (Feng *et al.*, 2016). In addition, the great specific surface area of PM_{2.5} facilitates the binding of toxic compounds, such as polycyclic aromatic hydrocarbons (PAHs), leading to consider these particles as a conveyor of contaminants inside humans.

On the basis of the close relationship between human exposure to high concentrations of PM_{2.5} and increase lung cancer mortality, PM was recently classified as a Group I carcinogen (Loomis *et al.*, 2014). Observational epidemiology, controlled human exposures to pollutants, animal toxicology and *in vitro* mechanistic studies were performed to evaluate the impact of air particles on health. Most of the evidence focuses on respiratory and cardiovascular effects attributed to short- and long-term exposures, as well as on the development of pregnancy-related outcomes (Analitis *et al.*, 2006; Cui *et al.*, 2016), premature mortality and reduced life expectancy (Brunekreef and Holgate, 2002). Considering that PM is highly heterogeneous, the potential health risks of PM_{2.5} promoting respiratory diseases and lung cancer, as well as its chemical characteristics, remain elusive; and the genotoxic mechanisms induced by PM_{2.5} are not fully identified.

Lebanon is one of the developing countries, where exposure to environmental pollution represents a major source of health risk. Northern Lebanon is affected by several sources of pollution, such as cement factories and quarries, phosphate fertilizer industry, transport section, and other human activities. However, toxicity, and knowledge about air pollutants and their impact on health remains very patchy in the region.

Within the scope of this thesis, we will test the health impact of airborne particulate matter in Northern Lebanon, from a pilot epidemiology study to chemical and toxicological analyses. The principal objectives of this work aim to:

- Examine the levels of perceived air pollution annoyance and health risk and evaluate the relationship between the development of respiratory diseases and living close to industrial activities
- Assess the mutagenic and genotoxic effects of fine particulate matter (PM_{2.5-0.3}) samples collected in a background area (rural) or under industrial influence
- Relate the PM composition to the observed genotoxic effects

In order to achieve these objectives, a perception and epidemiological survey was conducted in two areas in Northern Lebanon, the first one under industrial influence and the second one under rural influence. Simultaneous sampling of fine particles (PM_{2.5-0.3}) and quasi-ultrafine particles (PM_{0.3}) was done by cascade impaction at two sites in Northern Lebanon: Zakroun, a site affected by industrial influences, and Kaftoun, a background rural site. After sampling, physicochemical and toxicological analyses were performed.

Therefore, this thesis report is divided into four chapters.

Based on the literature, the first chapter presents characteristics of atmospheric particles, their sources, their physicochemical characteristics, and their impact on health while highlighting the cellular mechanisms induced by PM_{2.5}. Finally, the chapter focuses on the situation in Lebanon and Northern Lebanon, to expose the hypothesis, objectives and methodology applied in this work.

The second chapter describes in details the performed perception and epidemiological survey. This chapter includes a detailed explanation of the study procedure, results and discussion of air pollution perception and epidemiology and the impact of respondents' gender, age and occupations, showing prevalence of the respiratory symptoms, the past illnesses, and the family history and children health.

The third chapter deals with the physicochemical characteristics of collected PM. After presenting materials and methods used for sampling and analysis, metals, ions and organics composition is discussed.

The mutagenic and genotoxic effects of fine particulate matter are described in the fourth chapter. It presents and discusses results regarding mutagenicity and genotoxicity using two classical bacterial assays, and *in vitro* genotoxicity on human lung cells searching DNA alterations and their repair.

The different results of the thesis are then discussed extensively to provide potential mechanisms that might contribute to the pathogenesis of PM-associated respiratory diseases and uncover the relationship between PM composition and observed genotoxic effects. This thesis ends with a general conclusion and the perspectives of this study.

CHAPTER 1

INTRODUCTION

This first chapter is divided into six parts. It presents definitions and characteristics of air pollution and atmospheric particles. It particularly aims at exposing different aerosol sources, their physicochemical characteristics and their global assessment. The next parts are devoted to the description of the impact of the atmospheric particles on health, while highlighting the cellular mechanisms induced by PM_{2.5}. The chapter also focuses on the situation in Lebanon and Northern Lebanon, where this study is conducted. Afterwards, the exposition of the hypothesis, objectives and methodology will conclude this chapter.

1.1 Atmospheric pollution

1.1.1 Background and evolution of air pollution

According to the World Health Organization, air pollution is the contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere (WHO, 2011). The U.S. Environmental protection agency defined the air pollution as a mixture of solid particles and gases in the air (US EPA, 2013a). According to Article 2 of the Law on Air and the Rational Use of Energy (LAURE) in France, air pollution is defined as the “introduction by man, directly or indirectly, in the atmosphere and confined spaces, of substances having adverse consequences likely to endanger human health, to harm living resources and ecosystems, to influence climate change, to damage the properties and, to cause excessive odors” (*LOI n° 96-1236 du 30 décembre 1996 sur l’air et l’utilisation rationnelle de l’énergie*, 1996). This law highlights explicitly the role of man in the air pollution however it was later amended by the Grenelle II Act. The words “or presence” were added to the existing article so that pollutants of natural origin are recognized and analyzed as well as the anthropogenic pollutants. Article 1 of this law, states: “The goal is the implementation of the right recognized for each one to breathe air that is not harmful to his health. This action is in the public interest to prevent, monitor, reduce or eliminate air pollution, preserve air quality and for these purposes, save and rational use of energy”.

The first mentioned traces of air pollution are the dust and fumes generated from the mines of ancient Rome (Italy) in the seventh century before Christ (Stromberg, 2013). However, it is from the nineteenth century that the industrial revolution became significant. Then, chronic pollution started to affect the cities of industrialized countries, particularly in Europe and the United States, and more recently the cities of the developing countries. Our modern society has known various massive air pollution episodes, such as those of the valley of the Meuse (Belgium) in 1930, Donora (US) in 1948, Poza Rica (Mexico) in 1950, London (UK) in 1952, Cincinnati (US) in 1968, New York (US) in

1953, 1962-1963, 1966 (Greenburg *et al.*, 1962; McCABE, 1952; Murray, 2009; Nemery *et al.*, 2001; US Department of Health, 1969). Air pollution episodes gave rise to a large number of deaths and hospitalizations; this is an evidence of the most deleterious effects of air pollution on health. The London smog is also one of the most significant in the history of air pollution episodes; it occurred in December 1952 for five days and have caused nearly 4,000 deaths (Lean, 2014; Logan, 1953). Recent analysis suggests that this episode actually caused 12,000 deaths (Bell and Davis, 2001). Currently, air pollution is directly or indirectly responsible for 40,000 deaths/year in some European countries such as Austria and Switzerland (Cohen *et al.*, 2005; Samet *et al.*, 2000; WHO, 2014a). In France, air pollution is responsible for 48,000 deaths/year, which corresponds to 9% of the annual mortality (Archy world news, 2016). Most countries in the Arab region, and in particular the capitals and other major cities, such as Beirut, Sana'a, Damascus, Cairo, Baghdad and Manama, also suffer from different degrees of air pollution where air pollutants often exceed the World Health Organization (WHO) guidelines (Chaaban, 2011).

Air pollution is a problem for all of us, considering that the average adult breathes over 11.5 m³ of air per day. WHO reports that in 2012, 7 million premature deaths annually are linked to air pollution, that is one in eight of the total global deaths. This finding more than doubles previous estimates and confirms that air pollution is now the world's largest single environmental health risk (WHO, 2014a).

1.1.2 Levels of air pollution

Air pollution has no barrier. Emission inventories identify the transmitters contributing most significantly to the emissions of a given substance (EEA, 1997a). Among all potential emitters of pollutants, there are two sources: natural and anthropogenic. Thus, certain atmospheric pollutants can migrate away from their emission sources and interfere at different spatial scales. Three types of air pollution are distinguished based on the scale of study: the local, the regional and the global (Cote *et al.*, 2008; Elichegaray *et al.*, 2009; Ramanathan and Feng, 2009).

Local air pollution is encountered in the immediate vicinity of emission sources. This form of pollution is mainly due to human activities and occurs close to the emission sources such domestic heating, industrial waste, gases associated with transport, waste incineration, etc. The emissions of these sources reach a distance less than few kilometers. In this type of pollution, the most characteristic pollutants are: the suspended particulate matter (PM), sulfur dioxide (SO₂), carbon monoxide (CO), nitrogen oxides (NO_x), volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons compounds (PAHs), elemental carbon or carbon soot, and metals.

The regional pollution covers larger territories; it affects places distant few kilometers to few hundred kilometers from the emission source of pollutants. This regional pollution results of the physico-chemical transformation of primary pollutants to secondary pollutants. The ozone (O₃) is one of these secondary pollutants that are formed by the presence of NO_x and VOCs. In addition for

example, nitric acid and sulfuric acid are the result of the presence of NO_x and SO₂. Sulfur dioxide is oxidized in the atmosphere to form sulfuric acid (H₂SO₄) and nitrogen dioxide (NO₂) is oxidized to nitric acid (HNO₃), which in turn reacts with ammonia (NH₃) to form ammonium nitrate (NH₄NO₃).

The global scale is related to phenomena that may affect the balance of the Earth's ecosystem as a whole. It contributes to climate change, and the global warming caused by the accumulation of greenhouse gas emissions (particularly carbon dioxide). It affects the stratospheric ozone layer due to the carbon dioxide (CO₂), the methane, the nitrous oxide (N₂O) and halogenated gases from domestic activities. This reduction of the ozone layer can cause damage in the human body such as skin cancer.

1.1.3 Main air components

Air pollution is dominated by a few major components whose levels fairly reflect the overall status of air pollution and the various emission sources. These factors are considered as air pollutants because they do not naturally exist in the air or they do at very low concentration, and they are harmful to health and the environment. Air pollutants can be encountered in two forms: gases and particles. Their composition includes CO, NO_x, SO₂, O₃, heavy metals, PAHs, VOCs, polychlorinated dibenzo-*para*-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), polychlorinated biphenyls (PCBs) and PM. Once pollutants are released into the air, they can interact with each other and the environment in complex ways depending on temperature, humidity, photochemistry and other environmental conditions. Pollutants can be categorized into: primary pollutants and secondary pollutants which are produced from the interaction of primary pollutants. The presence of all these pollutants modifies the physicochemical characteristics of the atmosphere.

In the atmosphere, all gaseous or particulate components will be diluted, transported and processed, depending on weather conditions (Kondratyev *et al.*, 2006). Many atmospheric factors influence the way air pollution is dispersed, including wind direction and wind speed, type of terrain and heating effects. Lack of rainfall and wind are adverse weather conditions preventing the dispersion of primary pollutants and can lead to episodes of intense pollution often summarized under the term "high pollution". Dispersion and transport of air pollutants are carried out in a slice of altitude ranging between 1 and 2 km. The latter is indeed the most disturbed layer, constantly agitated by turbulent motions both horizontal and vertical. In this layer, pollutants may also undergo chemical transformations more or less complex. Some pollutants have a lifetime which is high enough to promote their displacement in the layers with a higher altitude even in the stratosphere (air layer between 8 and 40 km in altitude).

Atmospheric suspended particles have an impact on human health and influence the chemistry of the troposphere. Therefore, the upcoming section focuses on the definition of atmospheric particles, their physicochemical characteristics and their life cycle.

1.2 Atmospheric particles

1.2.1 Definition

Airborne “Particulate matter” or PM is a complex mixture of particles and liquid droplets. The term “aerosols” is also used but the scientific definition refers to liquid or solid particles suspended in a carrier gas or a gas mixture (Kodas, 1990). When considering the particle independently of the carrying gas, the term “particles” is more adequate.

It is convenient to classify atmospheric particles by their aerodynamic properties and according to their formation process. According to their forming process, airborne particles can be classified as primary or secondary aerosols. Primary aerosols are emitted directly from the emission sources into the atmosphere in particulate form. Whereas secondary aerosols refer to particles generated in the atmosphere, formed by the conversion mode gas-particle due to the condensation of natural and anthropogenic vapors, or generated by the evolution of a primary particle.

1.2.2 Physical properties

1.2.2.1 Granulometry and particle size classification

The field of particle size spans several orders of magnitude. A frequently used classification is based on the aerodynamic properties of the particles thus the particle size distribution. This classification is important because it is related to the transport and removal of particles from the air; it governs their deposition within the respiratory system; and it is associated with the chemical composition and sources of particles (Brown *et al.*, 2002; Harrison and Yin, 2000). These properties are conveniently summarized by the equivalent aerodynamic diameter (D_{ae}) that is the size of a unit-density sphere (1 g/cm^3) with the same aerodynamic characteristics (same settling velocity in air) as the considered particle (Hinds, 1999). The particle aerodynamic diameter is given by the Equation 1.1 where D_g is the particle geometric diameter, ρ_p is the density of the particle, ρ_0 is the reference density (1 g/cm^3), and k is the shape factor, which is 1.0 in the case of a sphere. It is important to note that various types of aerosol instrumentation report different measures of particle diameter depending on the employed methodology and application. Therefore, a universal measurement of aerosol particle size does not exist and as a result, the sizing definition will be specified for particle diameters (Möller *et al.*, 2008).

$$D_{ae} = D_g k \sqrt{\frac{\rho_p}{\rho_0}} \quad (1.1)$$

The size of suspended particles in the atmosphere varies over four orders of magnitude, from a few nanometers to tens of micrometers (Figure 1.1). The largest particles, called the coarse fraction (or mode) ($D_{ae} > 2.5 \text{ }\mu\text{m}$), are mechanically produced by the break-up of larger solid particles. These particles can include wind-blown dust from agricultural processes, uncovered soil, unpaved roads or mining operations, near coasts and evaporation of sea spray; it can also include pollen grains, mould

spores, and plant and insect parts. Because of their relatively large size, coarse particles settle out of the atmosphere in a reasonably short time by sedimentation, except on windy days, where fallout is balanced by reentrainment. Smaller particles or fine particles ($D_{ae} < 2.5 \mu\text{m}$) consist of particles generated by industrial and urban activity but also biogenic particles. This class of particles can be divided into an accumulation mode ($0.1 < D_{ae} < 2.5 \mu\text{m}$) and a nucleation mode ($D_{ae} < 0.1 \mu\text{m}$) (*Aitken* mode). Particles with diameters between 0.1 and 2.5 μm are formed due to the coagulation of particles smaller than 0.1 μm and from the condensation of vapors onto existing particles. They can also be introduced directly into the atmosphere, mainly through the incomplete combustion of wood, oil, coal, gasoline and other fuels. The accumulation mode is so named because particle removal mechanisms are least efficient in this regime, causing particles to accumulate there until they are ultimately lost through rain or other forms of wet deposition. Particles extending from 0.01 to 0.1 μm are formed from ambient-temperature gas-to-particle conversion as well as condensation of hot vapors during combustion processes. These particles act as nuclei for the condensation of low vapor pressure gaseous species, causing them to grow into the accumulation range. The lifetime of these particles is short, as they are lost principally by coagulation with larger particles.

The nucleation and Aitken mode particles account for the majority of atmospheric particles by number. Due to their small sizes, they rarely account for more than a few percent of the total mass. Hence if the toxicological effects are determined primarily by the number of particles, rather than their mass, these small particles could ultimately prove to be of high importance. The accumulation mode particles account, generally, for a significant fraction of the total aerosol mass and have the greatest surface area. This makes these particles of significant importance to gas phase deposition and atmospheric heterogeneous chemistry. Most of the aerosol mass is found in the coarse mode, where large particles contribute significantly to the optical properties of atmospheric aerosols.

Different terminology is used to describe particles with respect to their size (Figure 1.1). In ambient air, total suspended particulates (TSP) correspond to all suspended particles regardless of size. Particles with an aerodynamic diameter of less than 1 μm are called PM_{10} (e.g. diesel particles). Ultrafine particles, nanoparticles or $\text{PM}_{0.1}$ are particles with an aerodynamic diameter less than 0.1 μm . They are generated by gas-to-particle conversion processes that are not yet well defined. Their lifetime could be in the order of minutes due to their rapid coagulation or random impaction onto surfaces (Kulmala *et al.*, 2004). Those having an aerodynamic diameter less than 2.5 μm are called fine particles and $\text{PM}_{2.5}$. Those having an aerodynamic diameter less than 10 μm will be referred to as coarse particles or PM_{10} . $\text{PM}_{(2.5-10)}$ are the particles having a D_{ae} between 2.5 and 10 μm .

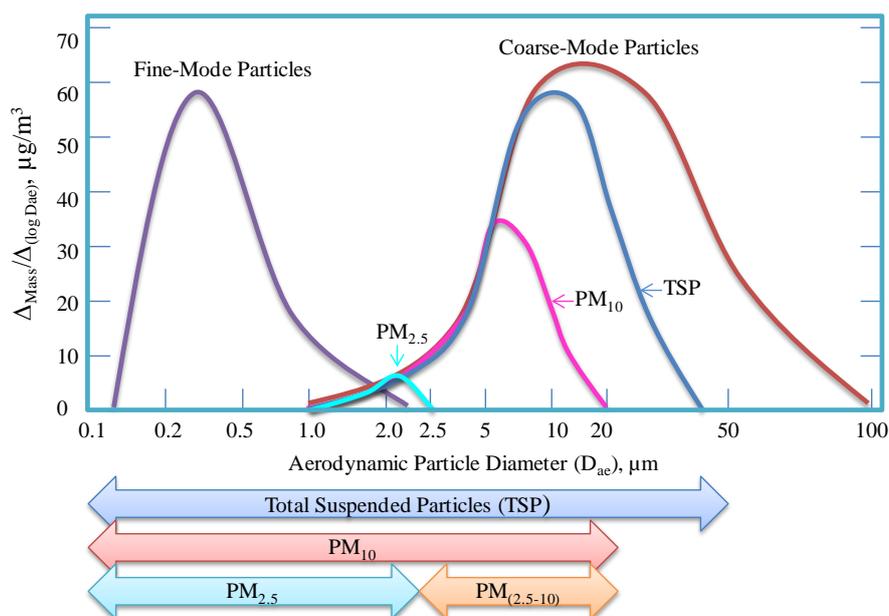


Figure 1.1: Schematic representation of the size distribution of particulate matter in ambient air (US EPA, 1996)

1.2.2.2 Textural properties

Whatever their chemical composition, the solid particles can be characterized according to their textural properties. The concept of texture includes: the specific surface area (m^2/g), the specific pore volume (cm^3/g) and porosity, the shape of the pores, and the pore distribution or the pore volume distribution as a function of pore size.

The specific surface area (m^2/g) is the total surface area per unit mass of a divided solid, it corresponds on one hand to the geometric surface of the grains, depending on the particle size distribution and shape (such as asbestos), and on the other hand to the surface developed by the pore walls (Corn *et al.*, 1971). The specific surface area promotes the adsorption or the attachment of molecules from the gas phase. In the solid-gas interface, the molecules from the gas phase can either bounce off or set on the surface of the solid for a longer or shorter time, causing an over-concentration on the surface of the solid. Depending on the nature and intensity of the bonding forces in play, there are physical adsorption and chemical adsorption. The larger the specific surface area, the greater the capability to adsorb the molecules is important. The particles having a diameter less than $1 \mu\text{m}$ are generally characterized by a high specific surface value, which will promote the adsorption of gaseous molecules. The soot have a microporous and mesoporous structure that contributes to the total surface area, it can exceed $100 \text{ m}^2/\text{g}$ (Rockne *et al.*, 2000). These particles are likely to have a high number of sorption sites for different gaseous organic compounds in the troposphere, such as VOCs, light PAHs, PCBs and PCDD/F. These textural properties determine the penetration, retention and excretion of the particles in the respiratory tract, and are therefore responsible for their health effects (Novák *et al.*, 2014; Wang *et al.*, 2013). Table 1.1 shows that the relative number of particles and the relative

surface area of the aerosol increase the smaller the particle diameter is. The shape of the particles is important for the penetration of the particles in the lung, for example inhaled asbestos fibers penetrate varying levels of the body's organs, depending on the size and shape of the fibers. As the lungs and other organs attempt to eliminate the presence of the fibers through a gradual exertion, the asbestos fibers either clear the respiratory areas and exit through mucus or further scar the organ as it remains lodged.

Table 1.1: The relative number of particles and the relative surface area of the aerosol according to the particle diameter

Diameter (μm)	Relative number	Relative surface area
10	1	1
1	10^3	10^2
0.1	10^6	10^4
0.01	10^9	10^6

1.2.3 Mechanisms of particle formation

Atmospheric particles properties are not stable in the environment and they do not have a constant composition throughout their life, both their size and chemical nature can be modified by various physico-chemical processes. Although some particles get directly into the atmosphere from natural and anthropogenic aerosols, a large number of particles are formed in the atmosphere (Metzger *et al.*, 2010). The process of formation of PM generally consists of three mechanisms: nucleation, condensation and coagulation as shown in Figure 1.2.

The nucleation leads to a significant fraction of particles (in number) in the atmosphere formed by the homogeneous nucleation from the gas phase; it is the genesis of particles. The condensation is a process where gas molecules condense on the particles. This mechanism increases the diameter of the particles while retaining their number. The coagulation is a process that increases the diameter of primary particles by reducing their number. This is the formation of a particle from the combination of two or more elemental particles. Due to the Brownian motion, the particles migrate permanently in the atmosphere and can collide with other particles. The rate of the coagulation depends on the concentration of particles, their composition, their speed of movement, and their specific surface area. These mechanisms of formation of PM lead to different classes of particles according their aerodynamic diameter. Chemical transformation corresponds to homogeneous or heterogeneous reaction of nucleation or condensation of gases naturally or anthropogenically emitted. This phenomenon is called gas-particle conversion that leads to the formation of small particles that will grow in size by physical transformation. Mainly oxidation reactions take place with three major classes: compounds based on nitrogen (NO_2 and NH_3), those based on sulfur (SO_2) and organic compounds.

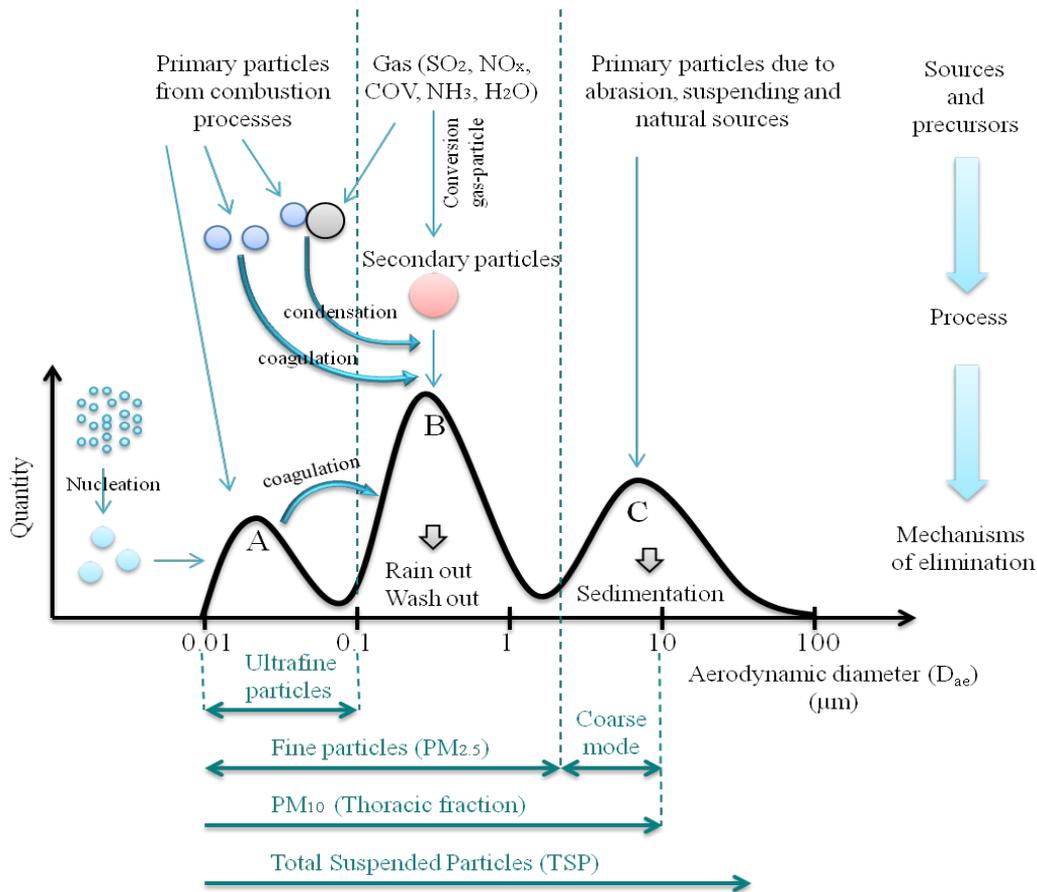


Figure 1.2: Schematic representation of the particle size distribution of aerosols and their mechanisms of formation (Modified from Whitby and Cantrell, 1976)

1.2.4 Composition of atmospheric particles related to their emission sources and their evolution in the atmosphere

The composition of atmospheric particles is very heterogeneous and varies according to their emission sources, their physico-chemical changes in the atmosphere and their size fractions (Gummeneni *et al.*, 2011; Thurston *et al.*, 2011) and the location. Predominantly found in the particle species are sulfate, nitrate, ammonium, crustal elements, the biogenic fraction, water and carbonaceous material. Trace elements such as metals and dioxins are also present (Hueglin *et al.*, 2005; Mazzei *et al.*, 2008).

1.2.4.1 Emission sources

Primary PM sources are derived from both human (anthropogenic) and natural (non-anthropogenic or biogenic) activities. Secondary PM sources directly emit air contaminants into the atmosphere that form or help form PM. Global natural emissions of PM account for about 90% of the total flux, whereas anthropogenic PM account for the remaining 10% of the PM mass emitted yearly in the atmosphere of the planet (Table 1.2). At the local level, given the presence of many emission

sources, anthropogenic sources are often actually more important than natural ones; this is particularly the case in urban areas and heavily industrialized sites. The origin determines the chemical composition of aerosols, while the production mechanisms are responsible for their size and shape characteristics.

Table 1.2: Particles flux estimation in the atmosphere (Delmas *et al.*, 2007)

Origin	Sources	Flux (Mt/yr)		
		Averaged estimation	Min and Max estimation	
Natural	Primary	Mineral dust	1500	1000-3000
		Sea salt	1300	1000-10000
		Volcanic ash	33	4-10000
	Secondary	Biogenic debris	50	26-80
		Biogenic sulfates	90	80-150
		Volcanic sulfates	12	5-60
		Secondary organic aerosols	55	40-200
		Nitrates	22	15-50
		Total	3060	2170-23540
	Anthropogenic	Primary	Soot	20
Industrial dust			100	40-130
Biomass combustion			80	60-160
Secondary		Anthropogenic sulfates	140	170-250
		Anthropogenic nitrates	36	25-65
		Organic aerosols	10	0.5-25
	Total	390	300-710	

1.2.4.1.a Natural sources

Globally, natural sources of **primary aerosol** dominate the mass emissions of PM. Natural sources are mainly sea salt, soil erosion, volcanic eruptions, wildfires and biogenic sources. Because approximately 75% of the Earth's surface is ocean, sea salt is the biggest contributor (Lewis and Schwartz, 2004).

- Sea salts, commonly known as sea spray, are particles arising during the bursting of air bubbles on the surface of water due to the movement of waves. There are two types of sea salt particles. The jet drops consist of a brine core (dominated by sodium and chloride) surrounded by an organic film and these are the particles formed from wave crashing and other mechanical processes. In contrast, film drops are primarily organic and formed from less harsh mechanical processes such as bubble bursting through the sea surface microlayer (Lewis and Schwartz, 2004). When freshly generated from the sea, sea salt particles possess the composition of the sea water. This composition is modified during their stay in the atmosphere due to the reaction with gaseous and acidic species such as NO_x, leading to chloride depletion. In general, they consist essentially of sodium chloride (NaCl), sodium, potassium or magnesium sulfates (Na₂SO₄, K₂SO₄ and MgSO₄) (Bliefert and Perraud, 2008). Hygroscopic properties of these compounds determine the particle size.

- Soil erosion, is another natural source of primary aerosol, generated primarily through wind action in dry conditions or in arid regions which cover about a third of the surface of the continents

(Mahowald *et al.*, 2005). The composition of these particles depends on the geological characteristics of the area (Pietrodangelo *et al.*, 2013). The chemical composition of atmospheric aerosols from soil erosion indicates that they are on the whole, consisting of characteristics of the crustal elements, mainly Al, Si, Fe, Ti, Ca, Na, Mg and K (Usher *et al.*, 2003). The particles can be suspended in the air if the wind speed exceeds 3 m/s. Sand particles with a size between 0.1 and 5 μm can travel distances more than 5000 km (Dall'Osto *et al.*, 2010).

- Volcanic eruptions are also sources of primary particles but in a limited time contribution. The volcanic particles are mainly composed of glass and also contain pyroxene, feldspar, plagioclase and aluminosilicates. Two types of solid particles are formed, dust and fly ash, as well as gaseous emissions mainly SO_2 , CO_2 , H_2O , H_2S , etc. These compounds can act as a precursor gas at the gas-particle conversion.

- Wildfires, as volcanic eruptions, have a limited spatial and temporal impact. The difference between natural and anthropogenic aerosols produced under these conditions is difficult to establish. The composition of smoke from forest fires varies and this smoke is composed of primary particles, in the form of ash and soot, and gases that may lead to the formation of secondary particles by gas-particle conversion. The amount of the particles produced and their composition depends on the characteristics of the burnt plant timber (Alves *et al.*, 2010).

- The origin of the biogenic particles are variable such as pollens, spores, fragments of animals and plants, as well as bacteria, algae, protozoa, fungi and viruses. The largest quantities of these particles are observed outdoors between spring and fall (Després *et al.*, 2012).

The natural sources of **secondary particles** are the sources of precursors involved in the gas-particle conversion. These gases are mainly composed of sulfur, nitrogen and hydrocarbons. Thus, the natural emissions of sulfur come from the marine flora (dimethyl sulphide), terrestrial fauna and flora, volcanic eruptions and biomass burning. These sulfur compounds in the atmosphere react to form SO_2 , which after oxidation lead to the formation of sulphate particulate. During the decomposition of vegetation and fertilizers, natural emissions of N_2O take place. N_2O is formed during electrical phenomena in the atmosphere during storms and by the oxidation of natural NH_3 and nitrogen oxide (NO) emitted from earth's crust. Secondary organic aerosols may be formed by oxidation of organic compounds emitted by forests and grasslands, volcanic eruptions and burning biomass.

1.2.4.1.b Anthropogenic sources

Anthropogenic emissions of **primary particles** result mainly from the transportation sector (cars, trains, ships, and aircraft), industrial waste (metallurgy, foundries, refineries, and mining), petroleum products (housing, industrial, commercial), incineration sites and agricultural activities. Road transport contributes to particulate emissions not only through the exhaust gas, but also by the wear of tires and brakes, as well as the resuspension of dust covering the roads. It is responsible for

many types of particles: fine carbon particles (resulting from the operation of the engines), coarse particles (from road abrasion), particles from abrasion of tires and brake, characterized by their high copper (Cu) in the case of brakes, or zinc for the tires (Davis *et al.*, 2001). For example in France, the residential sector (heating wood, oil or coal) is the largest emitter (39%) of PM_{2.5}, while the manufacturing industry emits 29%, road transport 19% and the agriculture and forestry activities are responsible for 10% of emissions. However, the respective contributions of different sectors are different for PM₁₀, 33% is emitted from industries, 27% from residential and tertiary sector, 19% in agriculture and 17% by road transport (CITEPA, 2010). The manufacture of non-metallic minerals and construction materials (cement, bricks) emit 15% of PM₁₀ and PM_{2.5} (CITEPA, 2010).

The industries are responsible for the issuance of a vast majority of heavy metals. However, the nature of the metals emitted depends on the type of industry, for example arsenic (As) is derived from the raw materials used in the production of certain glasses, as well as ferrous and non-ferrous metals; the cadmium (Cd) is issued by the sectors of steel, non-ferrous metallurgy and production of ceramics; the chromium (Cr) is mainly (51.5%) due to the production of ferrous metals, in particular steel mill and foundry; the mercury (Hg) is mainly produced by the chemical industry (30%), mostly the production of chlorine, by ferrous metallurgy (23%), and non-metallic minerals and materials construction (20%), that 43% are related to cement; the nickel (Ni) of industrial origin is associated with steel plants; the lead (Pb) comes from 71% of the ferrous metallurgy; the selenium (Se) is mainly emitted by glass production and construction materials; the zinc (Zn) is essentially derived from the ferrous metallurgy (CITEPA, 2010). Particles from agriculture are mainly related to soil tillage, they are mostly coarse particles of the same composition as terrigenous particles. The nature of industrial particles depends on the process, but combustion particles generally are dominated by black or elemental carbon and heavy organic material such as polycyclic aromatic hydrocarbons (Bond *et al.*, 2007). In cement plants and phosphate fertilizer complexes, raw material, fossil fuel and waste fuel cause significant emission of heavy metal such as Pb and Cd. Heavy metals can have different sources other than the industries. Pb, Cu, and Zn are related to the abrasion of mobile part of motor vehicles (brakes, tires ...). Tire rubber is rich in Zn as well as in Cu, Pb, Mn, Co, Ni and Cd as well as antimony (Sb) which is identified as coming almost exclusively from the excessive use of the vehicle brakes (Gietl *et al.*, 2010). Tires used as combustible waste in industries kilns to supplement traditional carbonaceous fuels could be the source of these cited heavy metals. The main sources of Ni and vanadium (V) are the burning of fossil fuels and oils (Al-Momani *et al.*, 2005; Pacyna, 1984). Lead, used to be added to petrol, was the source of high levels of lead in the air of major cities. As a result of EPA's regulatory efforts to remove lead from on-road motor vehicle gasoline, emissions of lead from the transportation sector dramatically declined by 95% between 1980 and 1999, and levels of lead in the air decreased by 94% between 1980 and 1999. The major sources of Pb emissions to the air today are ore and metals processing and piston-engine aircraft operating on leaded aviation gasoline.

Secondary particles can be formed by anthropogenic emissions and gaseous compounds by gas-particle conversion (Pankow, 1994). Anthropogenic emissions of sulfur based compounds are totally due to SO₂ emissions. Sulfur is emitted from burning of coal and petroleum products, refineries and processing of non-ferrous ores. Nitrogen-based gas such as NO and NO₂ emitted into the atmosphere by combustion processes (fossil fuels, biomass) and NH₃ emissions from the combustion of biomass, animal husbandry and agriculture are precursors of anthropogenic particles (Benson *et al.*, 2011). Burning fossil fuels (transport, industry), waste incineration and combustion of wood and coal (cooking and heating) are sources of anthropogenic particles rich in organic compounds.

1.2.4.2 Composition

1.2.4.2.a Inorganic and organic fraction

Atmospheric aerosols are generally composed of variable amounts of metals, ions and carbon: sulphate, ammonium, nitrate, sodium, chloride, trace metals, crustal elements, water and carbonaceous material.

- The major elements:

The major elements adsorbed on the particles are Al, Ca, Fe, K, Mg and Na. Soluble ions could be adsorbed on the particles such as F⁻, Cl⁻, NO₃⁻, SO₄²⁻, PO₄³⁻, and NH₄⁺. The sources of these elements are mentioned in *paragraph 1.2.4.1*.

The sulphate present in the particle composition is derived predominantly from the atmospheric oxidation of anthropogenic sulphur-containing compounds such as SO₂ and natural sulphur-containing compounds such as dimethyl sulphide (DMS) (Mamane *et al.*, 2008). It comes mainly from the combustion of fossil fuels containing sulfur (diesel, coal). The main sources of SO₂ are industrial plants and domestic heating, electricity generation and motor vehicles (WHO, 2014b). Industrial processes produce sulfur effluents such as oil refineries and certain manufacturing processes. SO₂ may have natural source, the most important are forest fires and volcanic eruptions. As a result of chemical reactions in the atmosphere, SO₂ is converted to sulfate in a liquid or a solid form.

Nitrate is formed mainly from the oxidation of atmospheric NO₂. Nitrogen oxides include the nitrogen monoxide or nitric oxide and nitrogen dioxide; they are the products of oxidation of the nitrogen (air or fuel) with oxygen under conditions of elevated temperature. These compounds are mainly emitted during combustion phenomena including the transport sector (vehicles) and combustion installations (heating, power stations...) (WHO, 2011). The automobile emission is the major contributor of nitrogen oxide, accounting for about 50% of the total. Electric power plants contribute about 33% and the percentage share of the industrial establishments, commercial institutions and residential units is 12%, 14% and 1% respectively.

Sulphates and nitrates are initially formed as H₂SO₄ and HNO₃, but are progressively neutralized by atmospheric ammonia forming the corresponding ammonium salts, NH₄NO₃,

NH_4HSO_4 and $(\text{NH}_4)_2\text{SO}_4$. NH_3 can also react with hydrochloric acid (HCl) to form NH_4Cl . The reaction of formation of NH_4Cl and NH_4NO_3 is reversible: these salts are volatile and may lead to the reformation of their gaseous precursors at low pressures of NH_3 or at elevated temperature. Chlorides is found adsorbed on the particles as a result of ammonia neutralization of HCl vapor, which is emitted from sources such as incinerators and power stations and the main source of chlorides is sea spray even at locations hundreds of miles from the coast.

In a marine and coastal atmosphere, the HNO_3 can also react with NaCl to form sodium nitrate (NaNO_3) (Dasgupta *et al.*, 2007) and release HCl.

In a calcium-rich atmosphere, HNO_3 can react with CaCO_3 to lead to the formation of $\text{Ca}(\text{NO}_3)_2$ (Hodzic *et al.*, 2006). During a dust transport from deserts, CaCO_3 can react with H_2SO_4 to form CaSO_4 and with NaCl and release HCl (Hwang and Ro, 2006).

- The trace elements:

The trace elements such as Ag, As, Ba, Cd, Ce, Co, Cr, Cu, La, Mn, Nb, Ni, P, Pb, Rb, Sb, Sc, Se, Sn, Sr, Te, Ti, V and Zn are also adsorbed or condensed on the surface of the particles and may play an important role in the toxicity of the aerosol. Heavy metals are released during the burning of fossil fuels and wood, cement, agriculture, some industrial processes and waste incineration. The sources of these elements are mentioned in *paragraph 1.2.4.1.b*.

- The carbonaceous fraction:

The carbonaceous fraction of the aerosols consists of both elemental and organic carbon. Elemental carbon (EC) is emitted directly into the atmosphere, predominantly from combustion processes. While particles containing organic carbon (OC) can be directly emitted into the atmosphere from primary sources such as biomass burning and combustion processes, they can also be introduced by secondary organic aerosol formation. The latter process occurs when VOCs undergo atmospheric oxidation reactions, forming products that have low enough volatility to form aerosol via either nucleation or gas-to-particle partitioning to pre-existing particles (Jaoui and Kamens, 2002). It is usually produced by the incomplete combustion of carbon-based fuels including petrol, diesel, and wood and from the combustion of natural (such as the terpene found in the essential oils of plants, especially conifers and citrus trees) and synthetic products such as cigarettes. The main anthropogenic sources of OC are transport (diesel vehicles), industries and wood heating.

- The organic fraction:

VOCs are emitted as gases from certain solids or liquids. They can be adsorbed on PM, they are emitted into the atmosphere from anthropogenic and biogenic sources (Seinfeld and Pandis, 2012). VOCs include a variety of substances belonging to different chemical families (aromatic hydrocarbons, ketones, alcohols, alkanes, aldehydes, etc). They are organic compounds containing one or more carbon atoms that have high vapor pressures and therefore evaporate readily to the

atmosphere. VOCs are emitted from the combustion of fuels, or by evaporation of solvents in certain products during their manufacture, storage or use.

PAHs are a group of over 100 compounds with two or more fused aromatic benzene rings that are present in the atmosphere in very small quantities (ng/m^3 of air) but in a higher concentrations than VOCs. They can appear in two forms: adsorbed on suspended particles and in gas phase: low-molecular-weight PAHs (two and three rings) occur in the atmosphere predominantly in the vapor phase, whereas multi-ringed PAHs (five rings or more) are largely bound to particles. Intermediate-molecular-weight PAHs (four rings) are partitioned between the vapor and particulate phases, depending on the atmospheric temperature (Srogi, 2007). In the atmosphere, PAHs can react with pollutants such as ozone, nitrogen oxides and sulfur dioxide, yielding diones, oxy-, nitro- and dinitro-PAHs, and sulfonic acids, respectively (Agency for Toxic Substances and Disease Registry, 1996). PAH formation can result from natural processes, but they are mainly formed by man-made sources: incomplete combustion process of carbonaceous materials at high temperature, pyrolysis of fossil fuels and other organic materials (tobacco or charbroiled meat) (Nikolaou *et al.*, 1984), waste incineration and combustion of wood and coal (Ravindra *et al.*, 2008).

PCDDs, PCDFs and PCBs are persistent organic pollutant (POPs) and share a similar base structure of two aromatic benzene rings with variable chlorine substituents. “Dioxins” generally refer to a group of seven PCDDs, ten PCDFs, and twelve “dioxin-like” PCBs, which are grouped together based on similar structural characteristics and mechanisms of action. The congener 2,3,7,8-TCDD is the most widely studied dioxin and is therefore often referred to simply as “dioxin” (Safe, 1990). The number and configuration of the chlorine compounds play a large role in determining properties of each PCDD, PCDF, and PCB congener. PCDDs and PCDFs are not commercially produced or manufactured chemicals; rather, they are released into the environment as a by-product of combustion (burning) of organic matter in the presence of chlorine at high temperatures or other industrial processes such as waste burning incinerators. Natural combustion (forest fires, volcanic activity) are also anecdotal source of emission of PCDDs and PCDFs. The main human way of exposure to PCDD and PCDF is related to alimentation (90 %). PCBs, on the other hand, were manufactured until 1979 for use in numerous commercial and industrial applications (insulating materials). Before they were banned, PCBs were released into the environment during manufacture and use and are still released into the environment through poorly maintained hazardous waste sites or leaks from old equipment (US EPA, 2013b). Worldwide contamination by dioxins and related compounds, such as PCDDs, PCDFs and coplanar PCBs has been of great concern due to their persistency in the environment, highly bioaccumulative nature and adverse effects on wildlife and humans (Lohmann *et al.*, 2001; Ueno *et al.*, 2005).

1.2.4.2.b Biologic fraction

Forests correspond to the majority of sources of organic particles (Cachier *et al.*, 1985). This fraction consists of small organisms such as bacteria, spores, pollen, seeds, cellulose fragments of plants, algae, protozoa, fungi, as well as fragments of insects (Huffman *et al.*, 2010). Jones and Harrison showed the correlation between the mechanisms responsible of the addition of the biologic fraction and the meteorological factors (Jones and Harrison, 2004). This probably includes wind abrasion, the biological activity of microorganisms on the surface of plants and the plant physiological processes such as transpiration and guttation. These processes can produce particles containing biogenic elements such as sodium, magnesium, phosphorus, sulfur, potassium, calcium, zinc, and rubidium (Beauford, 1977). Furthermore, a significant fraction of the aerosol is comprised of second biogenic particles formed by the gas-particle conversion of organic biogenic gas.

1.2.5 Dispersion and elimination of atmospheric particles

Several factors are involved in the dispersion of atmospheric particles: atmospheric pressure, wind, atmospheric stability and temperature, rainfall and precipitation, sunshine and photochemistry (European Environment Agency, 2016).

Low pressures often generate a fairly strong turbulence, which will promote good dispersion of airborne pollutants. In contrast, the dominance of anticyclones increasing the pressure of the atmosphere will decrease the dispersion of pollutants and subsequently will lead to the formation of pollution episodes (Seinfeld and Pandis, 2012).

Wind is one of the most important meteorological parameter for the transport and dispersion of pollutants. The slow movements of wind will stir the air masses and allow dilution of pollutants while movements on a larger scale will ensure the transport of pollutants over long distances sometimes hundreds to thousands of miles (Triantafyllou and Kassomenos, 2002). There is a clear relationship between wind speed and concentration levels of pollutants. Pollutant dispersion increases with speed and wind turbulence and the wind speed increases with altitude. At low wind speed, whose direction is often variable, pollutants stagnate which causes a stabilization or deterioration of air quality by cumulation.

The stability of the atmosphere plays a key role in the dispersion, the temperature affects the chemistry and the temperature inversions strongly limit the dispersion of air pollutants. Atmosphere is described as “stable” if a parcel of air, away from its position, tends to return to its starting position (Perrino *et al.*, 2001). These air movements are guided by thermodynamic laws. If the air mass lifted is colder than the surrounding environment, it will be denser and therefore sink down to its starting level (stable atmosphere). If the air mass lifted is warmer than the surrounding environment, it will be lighter and therefore suffer an elevation (unstable atmosphere). Thus, the stability of a mass of air depends on its temperature and elevation, relative to the temperature of the stationary air surrounding it. The vertical thermal structure of the troposphere holds a significant stake in the vertical mixing of

air masses, and therefore in the dilution of pollutants (Perrino *et al.*, 2008). Under normal circumstances of dissemination in the troposphere, temperature decreases with altitude. This does not hinder the vertical distribution of air masses, and therefore the pollutants until they reach a certain equilibrium in a way that the density of the ambient air is equal to the upstream air. But there may be some situations of temperature inversion starting from a certain height. In this case, a layer of warm air is located above a layer of cold air and acts as a thermal cover. Under normal circumstances, the polluted upward air is then blocked by the layer of warm air. This phenomenon contributes to local pollution and can lead to pollution peaks. It occurs under special weather situations such as early morning following a clear night with no wind, in this case, the air close to the ground layers cooled immediately while the upper layers are warmer. It also occurs during anticyclonic conditions (sunny day, area of high pressure) with low winds. These pollution episodes are called smog.

Rainfall is generally associated with an unstable atmosphere, which allows a good dispersion of air pollution. Moreover, they lead to some soil pollutants, and can sometimes speed up the release of other pollutants. The concentrations of pollutants in the atmosphere decreases significantly in rain including dust and soluble elements such as SO₂. Moisture affects the processing of primary pollutants, sometimes at the origin of acid rain (sulfuric acid formed from SO₂ and nitric acid from NO_x) (Aymoz, 2005). The lack of precipitation promotes fly ash and/or re-airborne dust.

The sun causes convections which are at the origin of vertical and horizontal movements of the air. It also affects the chemistry of pollutants; due to the solar energy, particularly through the action of ultraviolet rays, it can alter certain molecules in the air and promote the photochemical formation of tropospheric ozone. Reactions of this type are the cause of the secondary production of many pollutants in the troposphere like the formation of photochemical smog resulting in a temperature inversion which is characterized by hot and dry weather, clear clouds and little wind.

The removal of the particles depends on several parameters including weather conditions (wind, temperature, humidity) and their size. Various mechanisms help to reduce the number of particles in the atmosphere by dry or wet deposition. Dry fallout is the transport of particles to surfaces without precipitation (to soil, vegetation, and buildings). The main mechanisms leading to the deposition of particles are the sedimentation, the impaction and the Brownian diffusion. Particle deposition depends on atmospheric characteristics, the surface (chemical and biological reactivity, characteristic of the land) and physical properties of the particles (size, shape, density) (Ruijrok *et al.*, 1995). The sedimentation, by gravity, is the major removal mode of the coarse particles; they have a short residence time in the atmosphere, a few minutes to a few days. Large particles, between 2.5 and 20 µm, are deposited by impaction (under the effect of the wind). Particles, between 0.1 and 2.5 µm, have a shelf life in the environment for several days until their deposition by wet fallout. The ultrafine particles have a short life due to their rapid transformation into larger particles. Wet deposition corresponds to particles precipitation by rain, fog or snow by two phenomena called “washout” and “rainout”. The washout can be described as the interception of atmospheric particles by raindrops and

rainout is the condensation of water vapor on the particles resulting in the formation of water droplets, which are removed during precipitation (capture of particles by cloud droplets) (Aymoz, 2005).

Thus, the particles are in constant transformation, their life depends on their chemical composition, surface condition and their responsiveness to the surrounding environment and the meteorology. Particles chemically change (homogeneous and heterogeneous reactions) and physically transform (nucleation, condensation, coagulation) before removal from the atmosphere by dry or wet deposition. The following part will describe the global situation of air pollution and particles concentration.

1.3 Global assessment of air pollution

1.3.1 Consequences of air pollution

Air pollution has a negative impact on the ozone layer, ecosystems, climate, buildings, and our health. It can be harmful even when it is not visible or scented. Recent scientific studies have shown that some pollutants can harm public health and welfare even at very low levels. There is no general threshold of harm but there is a growing relationship between the level of pollution and its effect especially in case of cumulative phenomena as chronic exposure. In mid 80s, observations noted that the ozone layer had thinned on the poles of the planet forming a “hole”, due to release of man-made substances commonly known as Ozone Depleting Substances (ODS) into its atmosphere such as the chlorofluorocarbons (CFCs). Animal and plants species differ in their sensitivity to air pollution and its biogeochemical effects, such as soil and water acidification. For instance, lichens differ in sensitivity to sulfur dioxide (Thormann, 2006), vascular plants differ in sensitivity to ozone (King *et al.*, 2005), fish and other aquatic biota differ in sensitivity to lake acidity (Baker and Bernard, 1989), and grasses and forbs differ in sensitivity to added nitrogen (Wedin and Tilman, 1996). This shows that air pollution can affect the biodiversity of the ecosystem, either in shifting species composition or outright loss of sensitive species. Air pollution contributes in the acidification of lakes, eutrophication of estuaries and coastal waters, and mercury bioaccumulation in aquatic food webs. In terrestrial ecosystems, the effects of air pollution on biogeochemical cycling are also very well documented, but the effects on most organisms and the interaction of air pollution with other stressors are less well understood (Lovett *et al.*, 2009). Ozone can profoundly alter plants’ operations, particularly photosynthesis and stomatal regulation. Atmospheric deposition can have positive effects such as being a significant nutrient. It may profoundly alter ecosystems and their functioning by changes induced in the soil (acidification) and by changes in the conditions of competition between plant and microbial species. Atmospheric particles lead to quantitative and qualitative change in the radiation that may significantly alter photosynthesis, crop production, and evapotranspiration. PM can also lead to changes in the rainfall, at the regional scale, by multiplying condensation nuclei and in extreme cases, by changing circulation patterns. Ecosystems play a role in the emission of pollutants

and in the deposition processes. Pollution affects the climate by increasing the average temperature of the planet due to man-released gases or atmospheric greenhouse gases (GHGs) (carbon dioxide, methane, nitrogen oxides, etc.) that increase the greenhouse effect. The main pollutants affecting materials are sulphur dioxide and sulphates, nitrogen oxides and nitrates, chlorides, carbon dioxide, ozone and PM (Brimblecombe, 2003). The materials most sensitive to pollutants are calcareous building stones and ferrous metals. The most important effect of air pollution is on health and this effect is complex because people are subject to varied exposures (Chen *et al.*, 2008). People react differently to air pollution based on their age, health status and at the same time on the dose of pollution and the effects of pollutants which may explain the variability of individual responses. *More details in the paragraph 1.4.*

1.3.2 The situation in developed countries

Most of the world's population will be subject to degraded air quality in 2050 if human-made emissions continue as usual. The average world citizen 40 years from now will experience air pollution similar to that of today's average of East Asian citizen (European Geosciences Union, 2012). Currently, urban outdoor air pollution causes 1.3 million estimated deaths per year worldwide, according to the World Health Organization (Poizzer *et al.*, 2012; WHO, 2014c). Historically, air pollution in developed countries resulted mainly from industrial activity, and from burning fossil fuels for industrial and domestic heating (GOV UK, 2011). Indeed, the important economic development in the Northern countries during the second half of the twentieth century generated a high density of industrial and commercial activities. However, emission intensities, particularly in industry, have been reduced through technological change and environmental regulations, contributing to improved air quality over the last 20 to 50 years.

According to a press release from the World Health Organization held in September 2011 in Geneva, air pollution related to particulate matter (PM₁₀ and PM_{2.5}) reached dangerous levels for health in many cities. In Europe, the most exposed and most polluted cities in PM₁₀ are Plovdiv (70 µg/m³, Bulgaria), Sofia (68 µg/m³, Bulgaria), Pleven (57 µg/m³, Bulgaria), Thessaloniki (56 µg/m³, Greece), Nicosia (53 µg/m³, Cyprus), Torino (47 µg/m³, Italy), Zaragoza (45 µg/m³, Spain) (WHO, 2014d). In other countries in the European Union, the PM₁₀ concentrations far exceed the WHO standards (annual average of 20 µg/m³) and the EU standards (annual average 40 µg/m³). According to *AirBase V.7* of the European Environment Agency, the countries of Western Europe (Portugal, Spain, Italy, Greece, Belgium, Germany, and Turkey) emitted large amounts of PM₁₀ in the atmosphere in 2001 (EEA, 2012b). For PM_{2.5}, countries such as Poland (Zabrze, 40 µg/m³), Italy (Torino, 34 µg/m³), Greece (Athens, 27 µg/m³), Austria (Graz, 24 µg/m³), France (Paris, 23 µg/m³), Belgium (Liège, 21 µg/m³) and Germany (Berlin, 21 µg/m³) had high levels detected in 2008 beyond the threshold set by WHO (annual average of 10 µg/m³). The guidelines of the EU set the threshold at 25 µg/m³ to be achieved by 1 January 2015 (EEA, 2012a). In 2011, Poland, Bulgaria and Italy were

the biggest polluters of PM_{2.5}. Worldwide, high PM₁₀ concentrations were found in USA: Bakersfield (38 µg/m³), Fresno (35 µg/m³), and Canada: Hinton (36 µg/m³), Montreal (34 µg/m³). For PM_{2.5}, concentrations were 51 µg/m³ in Mexico, 34 µg/m³ in Peru, and 33 µg/m³ in Chile (WHO, 2014c).

1.3.3 The situation in developing countries

Exposure to environmental pollution remains a major source of health risk throughout the world, though risks are generally higher in developing countries, where poverty, lack of investment in modern technology and weak environmental legislation combine to cause high pollution levels (Briggs, 2003). While many developed countries have enforced emissions standards and regulations, this is not the case in many developing countries. In these countries, industrial activity continues to play a greater role in air pollution. PM is most concentrated in countries with low economic power, high poverty and large populations, such as Indonesia, Egypt and Sudan. The World Bank estimates that China alone has 16 of the world's 20 most air-polluted cities (Watts, 2005).

Air pollution in developing countries is derived not only from stack emission of pollutants from relatively large industries, like iron and steel, non-ferrous metals and petroleum products industries, but also from fugitive emission of pollutants from small-scale factories, such as cement mills, lead refineries, chemical fertilizer and pesticide factories and so on, where inadequate pollution control measures exist and pollutants are allowed to escape to the atmosphere. Since industrial activities always involve energy generation, the combustion of fossil fuels is a main source of air pollution in the developing countries, where coal is widely used not only for industrial, but also for domestic consumption. For instance, in China, more than 70% of total energy consumption relies on direct coal combustion, from which large amounts of pollutants (suspended particulates, sulphur dioxide...) are emitted under incomplete combustion and inadequate emission controls. In developed countries, modernization has been accompanied by a shift from biomass fuels such as wood to petroleum products and electricity. In developing countries, however, even where cleaner and more sophisticated fuels are available, households often continue to use simple biomass fuels (Bruce *et al.*, 2000). Around 50% of people, almost all in developing countries, rely on coal and biomass in the form of wood, dung and crop residues for domestic energy. Consequently, women and young children are exposed to high levels of indoor air pollution every day.

In addition, the countries of the Mediterranean, the Pacific and South-East Asia emits in the northern hemisphere important atmospheric particles. Cities like Ahwaz (372 µg/m³, Iran), Ulan Bator (279 µg/m³, Mongolia), Sanandaj (254 µg/m³, Iran), Ludhiana (India) and Quetta (251 µg/m³, Pakistan) are experiencing the highest annual PM₁₀ concentrations ever recorded in the world. WHO's data from 2008 and 2009 revealed that populations of countries like China (150 µg/m³, Lanzhou), South Korea (64 µg/m³, Seoul) and countries of West Africa such as Senegal (145 µg/m³, Dakar), Nigeria (122 µg/m³, Lagos), Ghana (98 µg/m³, Accra), Madagascar (68 µg/m³, Antananarivo), South Africa (66 µg/m³, Johannesburg) suffer from heavy PM₁₀ pollution (WHO, 2014d). 80 % of the world

population lives in areas where the annual average concentrations of $\text{PM}_{2.5}$ exceed $10 \mu\text{g}/\text{m}^3$ targets set by WHO (van Donkelaar *et al.*, 2010). Thus, developing countries that have higher exposure to fine particles are Mongolia ($63 \mu\text{g}/\text{m}^3$, Ulan Bator), China ($41 \mu\text{g}/\text{m}^3$, Hong Kong) and Kuwait ($51 \mu\text{g}/\text{m}^3$). In Africa, it was recorded as $38 \mu\text{g}/\text{m}^3$ in Dakar (Senegal), $50 \mu\text{g}/\text{m}^3$ in Accra (Ghana) and $59 \mu\text{g}/\text{m}^3$ in Antananarivo (Madagascar) (WHO, 2014d). Figure 1.3 highlights the high concentrations of fine particles from North Africa (Sahara desert) to East Asia compared to those of Europe, the Eastern United States and Western South America. WHO collected some data covering the period from 2003 till 2010, showing $\text{PM}_{2.5}$ concentrations (Figure 1.4). Taking all pollutants into account, Eastern China, Northern India, the Middle East, and North Africa are projected to have the world's poorest air quality in the future. In the latter locations this is due to a combination of natural desert dust and man-induced ozone. The effect of anthropogenic pollution emissions are predicted to be most harmful in East and South Asia, where air pollution is projected to triple compared to current levels (Pozzer *et al.*, 2012).

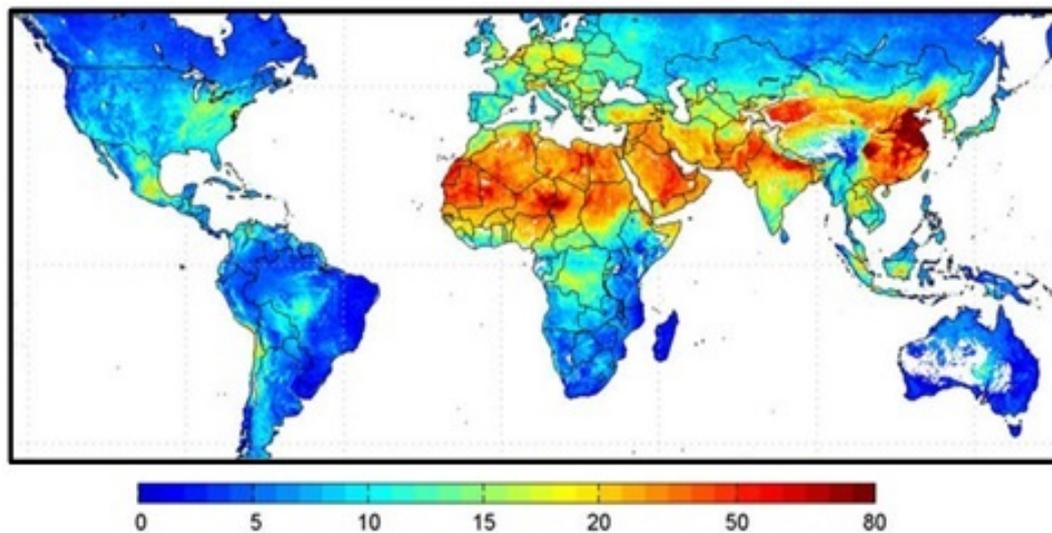


Figure 1.3: $\text{PM}_{2.5}$ annual average concentrations ($\mu\text{g}/\text{m}^3$) from 2001 to 2006 (van Donkelaar *et al.*, 2010)

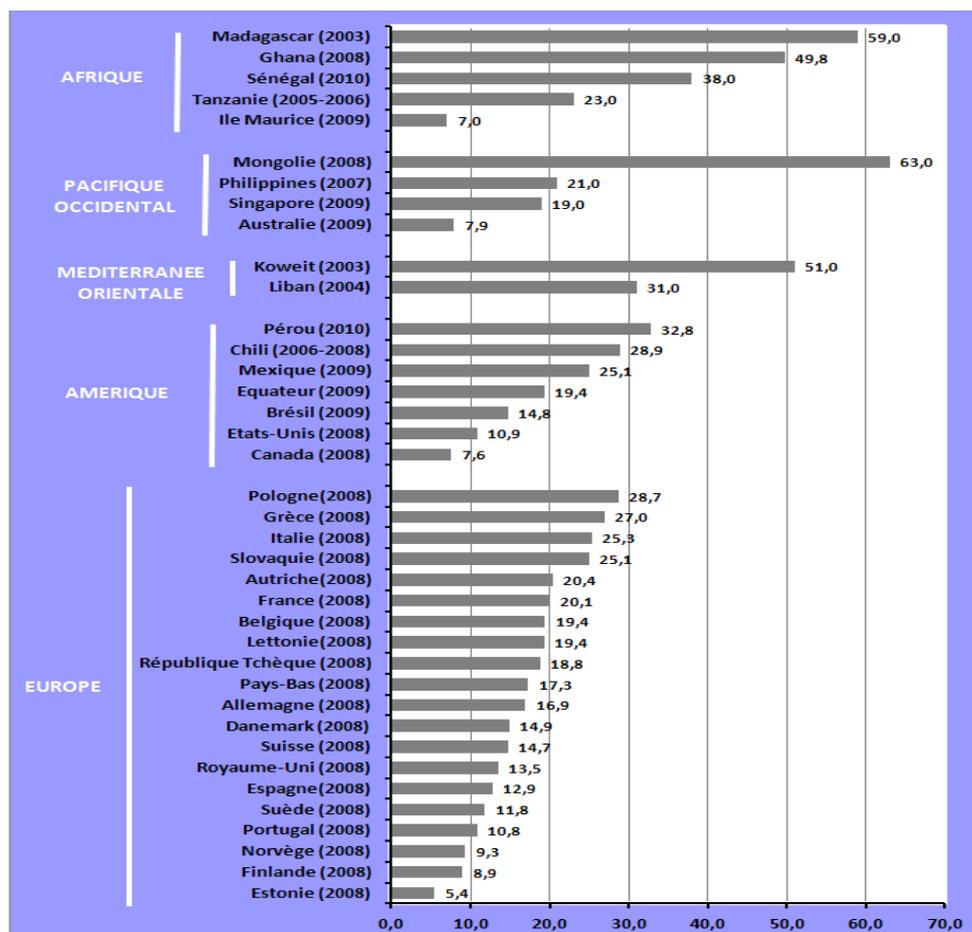


Figure 1.4: Annual average atmospheric concentrations of PM_{2.5} by country (WHO, 2014d)

1.3.4 General regulation of air pollution

Air pollution has become a global concern thus national and international bodies. WHO have established guideline values, exposition limit values and regulatory limits values on the basis of epidemiological and toxicological studies. The limits of PM₁₀ and PM_{2.5} set by WHO, the United States-Environmental Protection Agency (U.S. EPA) and European Environment Agency (EEA) are summarized in Table 1.3.

Table 1.3: Values recommended by WHO, the U.S. EPA and the EEA for atmospheric particles (µg/m³) (EEA, 2013a; Esworthy, 2012, 2013; WHO, 2005)

Particles	Exposure time	WHO (2005)	U.S EPA (2006)	EEA (2013)
PM _{2.5}	24- hour mean	25	35	-
	Annual mean	10	15	25
PM ₁₀	24- hour mean	50	150	50 (35 days of exceedance)
	Annual mean	20	50	40

According to WHO, air pollution killed about 7 million people in 2012, making it the world's single biggest environmental health risk. The 2014 report of the Ambient Air Pollution (AAP) data

base contains results of outdoor air pollution monitoring from almost 1600 cities in 91 countries (WHO, 2014d). WHO released a recent study mentioning that Delhi is the most polluted city in the world, with a $PM_{2.5}$ concentration of $153 \mu\text{g}/\text{m}^3$ and PM_{10} concentration of $286 \mu\text{g}/\text{m}^3$ much more than the permissible limits. Thirteen of the dirtiest 20 cities were Indian, with New Delhi, Patna, Gwalior and Raipur in the top four spots. Other cities with high levels of pollutants were located in Pakistan and Bangladesh (Park, 2014). Beijing's government notified that $PM_{2.5}$ concentrations stood at a daily average of $89.5 \mu\text{g}/\text{m}^3$ in 2013, 156% higher than the national standards. Such a reading would put Beijing 17th in the WHO database (WHO, 2014e). The biggest polluting countries are typically the states with the biggest industries, biggest production capabilities and the biggest populations. The top 10 polluted countries in the world are China, United States of America, Russia, India, Japan, Germany, Canada, United Kingdom, South Korea and Iran (AFOP, 2014). In order to safeguard our planet from environmental catastrophe these countries are either working towards or need to work towards implementing new regulations and control of their carbon dioxide emissions. This means reducing energy use and providing more forms of sustainable energy, improving transportation networks by offering improved public transport and looking for alternative fuels. For the cleanest cities, 32 cities reported a $PM_{2.5}$ reading of less than 5. Cities with the lowest level of pollution were located in Canada, the United States, Finland, Iceland and Sweden (Park, 2014).

Particulate matter (PM) toxicity occurs primarily by fine and ultrafine particles due to their physical and chemical characteristics. Although exposure to air pollution and especially to fine particulate matter ($PM_{2.5}$) is defined as carcinogenic to humans, toxicity mechanisms and particularly genotoxic pathways are not fully identified. The upcoming section presents the epidemiological and toxicological studies showing health impact of air pollution. It also describes the respiratory system, main target of atmospheric particles, and reveals the respiratory and cardiovascular effects of particulate matter.

1.4 Atmospheric particles and their impact on health

1.4.1 General health impacts of air pollution

Observational epidemiology, controlled human exposures to pollutants, animal toxicology and *in vitro* mechanistic studies can be performed to evaluate or explain the impact on health of air particles. Each of these approaches has its strengths and weaknesses. Epidemiology is valuable because it generally deals with the full spectrum of susceptibility in human populations. These studies provide the basis for experimental studies. The results obtained in epidemiological studies are relevant for morbidity criteria such as hospitalizations, medical visits, medication use and symptoms. By contrast, in controlled human exposures, exposure can be to a single agent that can be carefully generated and characterized, and the nature of the subjects can be rigorously selected and defined. Yet such studies are limited because they generally deal with short-term, mild, reversible alterations and a

small number of individuals exposed to single pollutants, and do not include those with severe disease who may be at most risk of adverse effects. Animal studies have the same strengths of well characterized exposures and more uniform subjects where invasive mechanistic studies can be carried out. More serious toxic effects can be produced in animals than in experimental human studies. Limitations can occur due to the possible interspecies differences especially in the concentrations of the tested toxin. For these reasons, the best synthesis incorporates different sources of information and should not rely solely on epidemiological evidence but it should also include findings from toxicological studies.

Most evidence linking the various human health effects to ambient levels of air pollution comes from the fields of epidemiology by short-term (time series studies, panel studies, meta-analysis, case-crossover analysis) and long-term studies (cohort studies) and toxicology. Exposure to air pollution has been associated with a variety of adverse health effects. Most of the evidence focuses on respiratory and cardiovascular effects attributed to short- and long-term exposures, as well as on the development of pregnancy-related outcomes (Analitis *et al.*, 2006; Cui *et al.*, 2016), premature mortality and reduced life expectancy (Brunekreef and Holgate, 2002). Research into the underlying biological mechanisms through which some air pollutants exert their effects on health has focused mainly on inflammatory and oxidative stress-related processes which could promote chronic diseases. WHO and the American Thoracic Society (ATS) have provided guidance on definitions of what constitutes an adverse effect of air pollution as following:

- The effects attributed to short-term exposure: daily mortality, respiratory and cardiovascular hospital admissions, respiratory and cardiovascular emergency department visits, respiratory and cardiovascular primary care visits, use of respiratory and cardiovascular medications, days of restricted activity and acute symptoms (wheezing, coughing, phlegm production, respiratory infections).

- The effects attributed to long-term exposure: mortality due to cardiovascular and respiratory disease, chronic respiratory disease incidence and prevalence (asthma, COPD, chronic pathological changes), lung cancer, chronic cardiovascular disease and intrauterine growth restriction (low birth weight at term, and intrauterine growth retardation).

Young children are among the most susceptible to effects of air pollution. Children have higher breathing rates than adults and therefore a higher intake of air pollutants per unit of body weight. They also spend more time outdoors than adults, thereby adding to their exposure potential. The developing lung may have a limited metabolic capacity to address toxic insults. Since 80% of alveoli are formed postnatally, and changes in the lung continue throughout adolescence, exposure to air pollutants poses a serious risk to this population group. For instance, increases in the use of asthma medication in children have been associated with ambient levels of air pollution (Favarato *et al.*, 2014). People with pre-existing cardiac or respiratory diseases are also more susceptible. Air pollution

may be responsible for increased rates of lung cancer (Nafstad *et al.*, 2003) and cardiovascular problems (Hassing *et al.*, 2009).

1.4.2 The respiratory system, main target of atmospheric particles

Air pollutants are in direct contact with the respiratory system. Human breathe an average of 15 m³ of air per day and the gas exchange area of the respiratory system has a surface area up to 100 m². Thus, the lung constitutes the most important interface between the external medium and the body, which gives it a particular interest in toxicology studies. Therefore, it is a prime target for various toxic agents and pollutants likely to be found in the air we breathe.

1.4.2.1 Physiology of the respiratory system

The respiratory and circulatory systems work together to deliver oxygen from the lungs to the cells, and return carbon dioxide to the lungs to be exhaled. The respiratory system contains the upper and the lower respiratory tracts (Figure 1.5). The upper respiratory tract contains the respiratory organs located outside the chest cavity: the nose and the nasal cavities, pharynx, larynx and upper trachea.

The lower respiratory tract consists of organs located in the chest cavity and is divided into conducting and respiratory zones. The conductive area includes regions that do not undergo gas exchange and begin with the trachea. The main functions of the trachea are to provide a clear airway for air to enter and exit the lungs and to trap dust and other contaminants to prevent them from reaching the lungs (mucus produced by the epithelium lining). The trachea is divided into two main bronchi that segregate into a series of intra-pulmonary bronchial and bronchiolar airways (16th generation). The diameters and length of each successive airway branch decrease progressively. This conductive region is followed by the respiratory bronchioles, a transition region having the characteristics of both the bronchioles and alveoli. The bronchioles' epithelium consists mainly of ciliated cells and Clara cells. The latter are particularly remarkable; they are non-ciliated secretory cells with intense metabolic activity. Their monooxygenase system of cytochrome P450 (CYP) allows the metabolism of a wide range of xenobiotics, and thus results in the release of potentially toxic reactive metabolites (Chang *et al.*, 2006). The bronchi and bronchioles also use the mucus and cilia of their epithelial lining to trap and move dust and other contaminants away from the lungs. The terminal parts of the bronchi are the alveoli; these are the functional units of the lungs since they form the site of gaseous exchange between the lung and pulmonary capillaries. As the airway progresses from the conducting to the respiratory zones, the cell types alter to accommodate the specific functions and defense mechanisms for each region (Proud, 2008).

The alveoli are lined with thin simple squamous epithelium and two pneumocytes types. Type I pneumocytes represent 95% of the alveolar surface (flattened and unable to divide) and act as thin

barrier through which gas exchange takes place between the blood and air. Alveolar type II cells (cubic shape) fulfill many known functions, including the regulation of the lung surfactant system, alveolar fluid content and are also the progenitor cells in this lung unit (Fehrenbach, 2001). There is also a resident population of alveolar macrophages which reside in the interstitial spaces and remove inhaled debris deposited in the air spaces.

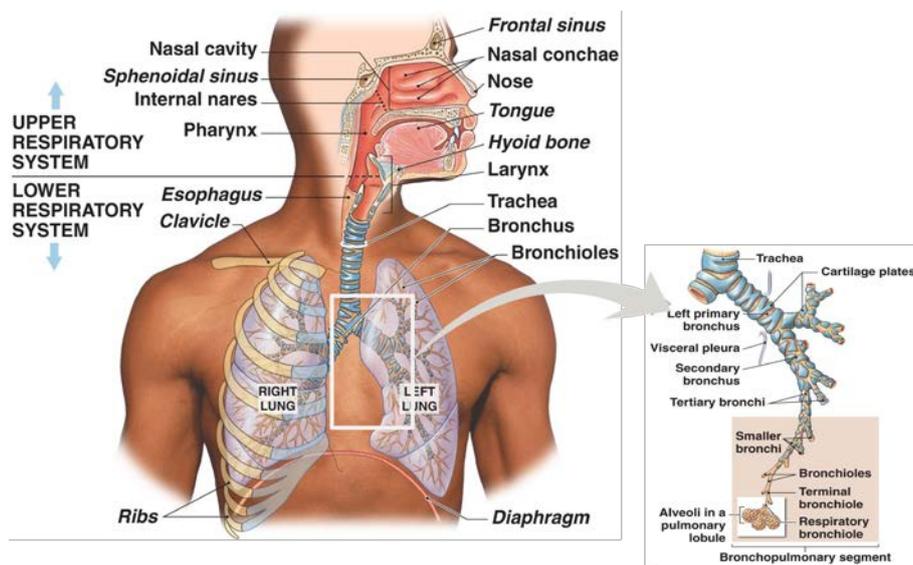


Figure 1.5: Human respiratory system

1.4.2.2 Penetration, deposition and clearance of airborne particles

The main route of entry for ambient PM to the body is via inhalation. In general, the particle deposition in the human respiratory tract is determined by biological factors such as lung morphology and breathing patterns, physical factors such as fluid dynamics, particle properties (shape, charge, density, hygroscopicity and size), and deposition mechanisms (Hofmann, 2011). These factors determine three deposit areas: the nasal-pharyngeal, tracheobronchial and alveolar regions. Depending on the particle size and mass, the deposition rate varies between these regions. Particles larger than $3\ \mu\text{m}$ and smaller than $0.003\ \mu\text{m}$ tend to deposit in the nasal-pharyngeal region. $\text{PM}_{0.1}$ are distributed throughout the respiratory tract and act as gases leading to an increase in the deposition rate at the extrathoracic level (Patton and Byron, 2007). Particles between $0.003\ \mu\text{m}$ and $3\ \mu\text{m}$ reach the alveolar region, with a maximum deposition for 20-30 nm particles. About 50% of $\text{PM}_{0.1-2.5}$ are able to reach the alveoli, carrying along the adsorbed potentially toxic compounds (Figure 1.6).

Indeed, particle deposition in the respiratory tract can be made by five mechanisms: inertial impaction, sedimentation, diffusion, interception and electrostatic interaction (Carvalho *et al.*, 2011). Inertial impaction takes place in the nasopharyngeal region and in the tracheobronchial tree where the air speed is high and the flow is turbulent. This mode characterizes the large particles having a diameter bigger than $10\ \mu\text{m}$. Sedimentation characterizes particles having a diameter between 1 and

5 μm and it occurs at the tracheobronchial tree and the alveolar region. Diffusion, also known as Brownian motion, is correlated to particle size and it is especially characteristic of the particles $< 0.5 \mu\text{m}$. Interception occurs when the particle, without deviating from its path, meets the wall of a duct pulmonary and deposits. Electrostatic interaction depends on the electrostatic charges of atmospheric particles and it is characteristic of particles smaller than $0.5 \mu\text{m}$.

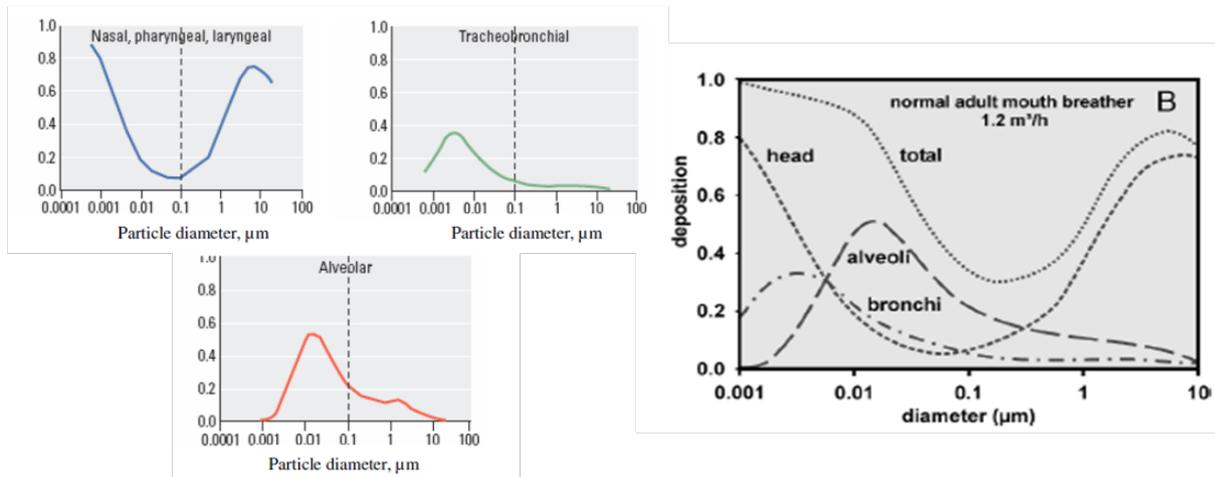


Figure 1.6: Grain size dependence of penetration of airborne PM into the respiratory system (Geiser and Kreyling, 2010; Wessels, 2009)

After deposition, the body tries to reduce the retention of inhaled particles by clearing them from the lung. The term “clearance” was introduced to describe the translocation, transformation and removal of deposited particles from the various regions of the respiratory tract. In the upper respiratory tract mainly PM_{10} are deposited and in this part of the lung the clearance is performed by coughing. $\text{PM}_{2.5-10}$ deposited onto ciliated surfaces are removed by the “mucociliary escalator” (rapid elimination in 24 hours). The fine and ultrafine fraction of PM ($\text{PM}_{2.5}$) mainly deposit in the alveolar region. After deposition, the particles could be phagocytosed by macrophages and transported to upper lung regions for mucociliary clearance or the particle-loaded phagocytes enter the lymphatic system (slow clearance). Depending on the inhaled concentrations of particles, a considerable part of the particles are taken up by alveolar epithelial cells, which act in addition to macrophages as initiators of inflammatory responses. Ultrafine particles taken up by epithelial cells are considered to escape from the clearance done by alveolar macrophages. A fraction of these particles is also able to reach the systemic circulation (Nemmar *et al.*, 2002; Oberdörster *et al.*, 2005; Semmler-Behnke *et al.*, 2007).

1.4.3 Pulmonary carcinoma

The term "cancer" refers to a wide range of diseases characterized by uncontrolled cell proliferation. Caused by many factors, these diseases can occur in different parts of the body and

involve several types of cells or tissues. Carcinogenesis includes 3 steps: the initiation where the DNA is altered by a genotoxic carcinogen that could be chemical, biological (viruses, parasites) or physical (ionizing radiation, UV), the promotion and the progression. This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasations), the formation of small nodules of cancer cells (micrometastases), and finally “colonization” or the growth of micrometastatic lesions into macroscopic tumors. Cancer cells share distinctive and complementary capabilities that enable tumor growth and metastatic dissemination such as (Figure 1.7): sustaining proliferative signaling (modification of extracellular signals of membrane receptors charged of signal transmission and of intracellular signal transduction), evading growth suppressors (inhibition of retinoblastoma-associated and TP53 proteins, entry of cells into quiescence (G₀), passage of cells at a terminal differentiation stage), resisting cell death (deregulation of the intracellular signaling pathway and loss of *TP53* tumor suppressor function, that eliminates the critical damage sensor from the apoptosis-inducing circuitry), inducing angiogenesis (activation of “angiogenic switch” by regulators such as VEGF causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths), enabling replicative immortality (overexpression of DNA polymerase weakly expressed in normal cells and correlated with a resistance to induction of both senescence and crisis/apoptosis), and activating invasion and metastasis or epithelial-mesenchymal transition (EMT) (loss of adherens junctions, expression of enzymes responsible of the extracellular matrix degradation, increased mobility, and resistance to increased apoptosis).



Figure 1.7: Cancerous cell properties (Hanahan and Weinberg, 2011)

In addition to these six characteristics, the cancer cells would have more properties such as: genome instability and mutation, tumor promoting inflammation, deregulating cellular energetics and

avoiding immune destruction (Hanahan and Weinberg, 2000, 2011). Lung cancers are classified into two broad categories: Non-Small Cell Lung Carcinomas (NSCLC) derived from epithelial cell of the bronchopulmonary mucosa and representing 80% of lung cancers, and Small Cell Lung Carcinoma (SCLC) which contains several types of cancers presenting morphological, histological and ultra-structural common properties, including particularly the presence of neuroreceptor granules and significant mitotic activity. These two types can be further subdivided into different subtypes.

The properties of cancerous cells are related to the activation of oncogenes and the inactivation of tumor suppressor genes resulting from genetic and epigenetic abnormalities (Sadikovic *et al.*, 2008; Sharma *et al.*, 2010). Genes involved in tumorigenesis include those whose products: i) directly regulate cell proliferation (either promoting or inhibiting); ii) control programmed cell death or apoptosis; iii) are involved in the repair of damaged DNA. Depending on how they affect each process, these genes can be grouped into two general categories: tumor suppressor genes (TSG) (growth inhibitory) and proto-oncogenes (growth promoting). Mutation in a single allele of a proto-oncogene can lead to cellular transformation and formation of oncogenes. In contrast, typically both alleles of a tumor suppressor gene must be altered for transformation to occur (Knudson, 2001). Some tumor suppressor genes like retinoblastoma gene *RBI* can be found by looking for loss-of-heterozygosity in a tumor. This phenomenon is frequently described in lung cancers. Genes that regulate apoptosis may be dominant, as with proto-oncogenes, or they may behave as tumor suppressor genes.

Oncogenes may be activated by different genetic and epigenetic mechanisms: i) mutation in the coding sequence of the gene leading to the production of an overactive protein in normal amounts; ii) gene amplification leading to the overproduction of a normal protein; iii) chromosomal rearrangement, leading to the overproduction of a normal protein under the influence of a strong promoter or production of an overactive or overexpressed fusion protein; iv) promoter hypomethylation, leading to the release of the inhibition of the transcription of the oncogene (Hur *et al.*, 2014); v) histone modifications leading to transcriptional activation in a normally inactive area (Füllgrabe *et al.*, 2011).

TSGs may be divided into two general groups: promoters and caretakers. Promoters are the traditional tumor suppressors, like *TP53* and *RB*. Mutation of these genes leads to transformation by directly releasing the brakes on cellular proliferation. Caretaker genes are responsible for processes that ensure the integrity of the genome, such as those involved in DNA repair. Although they do not directly control cell proliferation, cells with mutations in these genes are compromised in their ability to repair DNA damage and thus can acquire mutations in other genes, including proto-oncogenes, TSGs and genes that control apoptosis. A disability in DNA repair can predispose cells to widespread mutations in the genome, and thus to neoplastic transformation. Cells with mutations in caretaker genes are therefore said to have a “mutator phenotype”. These mutations can be due to different genetic and epigenetic mechanisms that are: i) mutation leading to the production of a non-functional

protein or whose activity is reduced; ii) deletion of the gene; iii) non-disjunction, resulting in the loss of an allele in one of the daughter cells and the presence of three copies of the gene into the other daughter cell; iv) mitotic recombination event which can lead to one of the daughter cells, if one of the alleles is non-functional (mutated or lost) before mitosis, to have two non-functional copies of the gene; v) hypermethylation of the promoter leads to epigenetic extinction (silencing) of the gene; vi) modifications of histones leading to loss of expression (Feng *et al.*, 2009).

1.4.4 Health outcomes of particulate matter and its respiratory and cardiovascular effects

Epidemiological and clinical studies have linked PM to a range of health outcomes (Figure 1.8). The lung is the main target organ of PM exposure. Acute health effects caused by PM include increased risk for chronic bronchitis and COPD as well as worsening of asthmatic symptoms (Atkinson *et al.*, 2001; Schikowski *et al.*, 2005). Epidemiological studies showed a correlation between PM and: mortality and hospital admission in COPD patients (MacNee and Donaldson, 2003), exacerbation of symptoms and increased use of therapy in asthma (Donaldson *et al.*, 2000), lung inflammation (Ghio and Devlin, 2001) and respiratory cancer (Cancer Research UK, 2015). The annual rate of cases of chronic bronchitis, cardiac problems, and respiratory diseases (all ages) increased in Europe with the increase of the rate of PM₁₀ and PM_{2.5} (World Health Organization, 2006). A number of studies indicate a relation between increased PM levels and emphysema, asthma and pneumonia (Peters *et al.*, 2001; Schwartz, 1994; Utell *et al.*, 1980). Long-term exposure to combustion-derived PM increases the risk for development of lung cancer (Engholm *et al.*, 1996; Vineis *et al.*, 2006; Wessels, 2009). A 10 µg/m³ increase in PM_{2.5} is associated with 8% increase of lung cancer mortality (Pope *et al.*, 2002). Walker *et al.* showed a significant correlation between the *Salmonella* mutagenicity of airborne particulate organics and lung cancer mortality rates, but not with mortality rates for other diseases (Walker *et al.*, 1982). Studies verified the association between lung cancer and diesel exhaust particles (DEP) and DEP was classified as carcinogenic to humans (Group 1) (IARC, 2012). Also increased allergic sensitizations against pollen were observed in areas with increased traffic (Wyler *et al.*, 2000). Pollen, which are bound to particles, are also involved in a higher rate of hospitalizations due to respiratory illness, including asthma (Namork *et al.*, 2006). Delfino *et al.* claimed that particle size and chemical composition are the most important factors for health risk of PM (Delfino *et al.*, 2005).

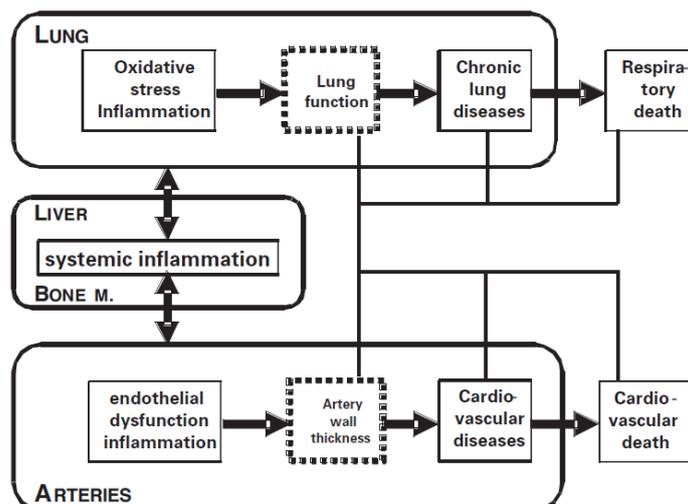


Figure 1.8: Model of the interrelated pulmonary, systemic, and vascular chronic inflammatory responses to particulate matter

Lung function and artery wall thickness are examples of markers of the continuous chronic process from health to disease. Thin lines denote correlations established in epidemiological studies (Künzli and Tager, 2005).

Additionally, studies have found associations between PM exposure and cardiovascular risk factors such as arteriosclerosis, thrombogenesis or decreased heart-rate (Dockery, 2001; Franchini and Mannucci, 2011; Pope *et al.*, 1999; Simkhovich *et al.*, 2008). Epidemiological studies showed the correlation between PM and mortality and hospital admission in cardiovascular disease patients (Donaldson *et al.*, 2005; Wellenius *et al.*, 2005). Exposure to PM_{2.5} was found to be a significant triggering factor among 772 patients presenting with acute myocardial infarction in the Boston, Massachusetts area (Peters *et al.*, 2001). It was also associated with increased frequency of cardiac arrhythmias (Peters *et al.*, 2000), increased risk for myocardial infarction (Peters *et al.*, 2001), endothelial and vascular dysfunction (Brook *et al.*, 2002; Mills *et al.*, 2005), reductions in HRV and increase in blood fibrinogen (Samet *et al.*, 2009). These studies suggest that exposure to ambient fine particles influences autonomic regulation of the heart, and is associated with increased arrhythmias and myocardial infarction (Massamba *et al.*, 2014; Zanobetti and Schwartz, 2005) in susceptible patients with heart disease. There is recent additional evidence, from both animal and human studies, indicating potential mechanisms by which PM may worsen cardiovascular disease: instigation of systemic inflammation that leads to atherosclerosis (Suwa *et al.*, 2002), activation of hemostasis factors, production of reactive oxygen species through the oxidative stress pathway, alterations in vascular tone, decreased heart rate variability (a marker of cardiac autonomic dysfunction and a predictor of sudden cardiac death and arrhythmias) (Franchini and Mannucci, 2011), constriction of the brachial artery of the forearm (Brook *et al.*, 2002), alteration of the blood leukocyte expression adhesion molecules and the lung diffusing capacity (Frampton *et al.*, 2006). PM exposure has been associated with an increase in blood C-reactive protein (Peters *et al.*, 2001), a reduction in red blood cells (Seaton *et al.*, 1999) and an increase in plasma viscosity (Peters *et al.*, 1997). All of these

changes involve potential mechanisms for adverse cardiovascular effects of PM exposure. Pope *et al.* and Dominici *et al.* certified that PM_{2.5} increases the risk for hospital admission for cardiovascular and respiratory diseases and it is a risk factor for cause-specific cardiovascular disease mortality via mechanisms that likely include pulmonary and systemic inflammation, accelerated atherosclerosis, and altered cardiac autonomic function (Dominici *et al.*, 2006; Pope *et al.*, 2004). The three main hypotheses of how inhaled particles could cause cardiovascular effects are: PM induce an inflammatory response in the lungs, leading to the release of cytokines and other mediators that reach the systemic circulation; ultrafine particles may translocate across the alveolar wall and directly interact with the cardiovascular system (this process may be aided by inflammatory cells) and/or particles may activate the autonomic nervous system through sensory receptors on the alveolar surface.

According to different epidemiological studies, fine particles have an important effect on the mortality rate. PM_{2.5} pollution is responsible for 3% of mortality from cardiopulmonary diseases, about 5% of deaths by cancer of the trachea, bronchus and lung, and 1% mortality due to acute respiratory infections in children under 5 years (Cohen *et al.*, 2005). Studies showed that a reduction in exposure to ambient fine-particulate air pollution contributed to significant and measurable improvements in life expectancy in the United States (Pope *et al.*, 2009). PM air pollution imparts a tremendous burden to the global public health, ranking it as the 13th leading cause of mortality (Brook, 2008).

The International Agency for Research on Cancer (IARC) has classified outdoor air pollution and the PM in outdoor air pollution as carcinogenic to humans, as based on sufficient evidence of carcinogenicity in humans and experimental animals and strong support by mechanistic studies (Loomis et al., 2014). Biomarkers can enhance research on the health effects of air pollution by improving exposure assessment, increasing the understanding of mechanisms, and enabling the investigation of individual susceptibility. With regard to the specific health outcomes of particulate matter listed above in relation to human studies, relevant toxicological evidence is briefly reviewed below.

1.5 Toxicity cellular mechanisms induced by PM_{2.5}

PM_{2.5} can induce direct and indirect genotoxic DNA damages and epigenetic modifications (Figure 1.9). The direct effect is generally associated with an electrophilic genotoxic agent that interacts with the nucleophilic DNA leading to DNA damage. Direct genotoxicity is mainly caused by the oxidative stress mechanisms and the metabolic activation of the organic compounds adsorbed on the particulate matter. Indirect genotoxicity is mainly due to the alteration of intracellular systems such as DNA repair systems, telomerase, or the mitotic spindle. The epigenetic machinery includes DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically

microRNA expression. The most commonly accepted definition of these mechanisms is that they lead to a change in the expression of genes without changing the sequence.

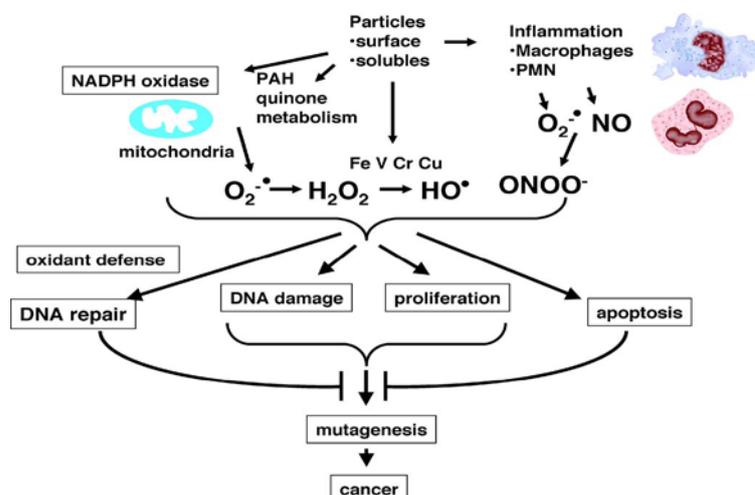


Figure 1.9: Induction of oxidative stress and DNA damage by PM and the roles in carcinogenesis (Risom *et al.*, 2005)

1.5.1 Cellular oxidative stress and $PM_{2.5}$

1.5.1.1 Oxidative stress, reactive oxygen species and anti-oxidant defense in the lung

The term “oxidative stress” is the disturbance in the oxidant/antioxidant balance, leading to potential damage. Oxidative stress is based on an increased availability of reactive oxygen/nitrogen species (ROS/RNS) (Table 1.4).

Table 1.4: Overview of ROS and RNS (Wessels, 2009)

	ROS	Non-radicals in <i>italic</i>	RNS
Superoxide	$\cdot O_2^-$	Nitric oxide	$\cdot NO$
Hydroxyl radical	$\cdot OH$	Nitrogen dioxide	$\cdot NO_2$
<i>Hydrogen peroxide</i>	H_2O_2	<i>Nitrite</i>	NO_2
<i>Singlet oxygen</i>	1O_2	<i>Peroxynitrite</i>	$ONOO^-$
<i>Hypochlorous acid</i>	$HOCl$	Nitrous acid	HNO_2
<i>Ozone</i>	O_3		

Reactive oxygen species (ROS) are species, having an unpaired electron in their valence shell, react with other molecules close to their place of production. Some are free radicals such as superoxide anion O_2^- and hydroxyl radical OH , while others are pro-oxidant non-radical species, such as hydrogen peroxide H_2O_2 . These species are produced during normal cell functioning, mainly through the mitochondrial respiratory chain. This can lead to membrane compounds (lipids, carbohydrates and proteins) and nucleic acid oxidation. Hence ROS are involved in multiple biological processes such as aging and inflammation as well as carcinogenesis and atherosclerosis

(Stadtman, 2004). The generation of these reactive O₂ metabolites in a biological environment exposes most living organisms to the so-called “oxygen paradox”: Oxygen is necessary for life but it is also potentially hazardous since ROS may easily become a source of cell and tissue injury. The concentration of ROS influences the cellular response, causing the activation of different signaling pathway: NRF2 pathway at low dose, NF-κB and AP-1 pathways at intermediate concentration, and cell death at high doses (Novo and Parola, 2008) (Figure 1.10). Relating to this, the lifespan of these species is an important issue and ranges from minutes (H₂O₂) to seconds (peroxide radical) to about a nanosecond, in the case of the hydroxyl radical.

The extent of damage is related to the availability of neutralizing enzymatic and non-enzymatic antioxidant defenses, as these specialized molecules preferentially react with free radicals, while the products of these reactions often have low toxicity. Furthermore, antioxidant mechanisms can be divided in extracellular and intracellular defense systems.

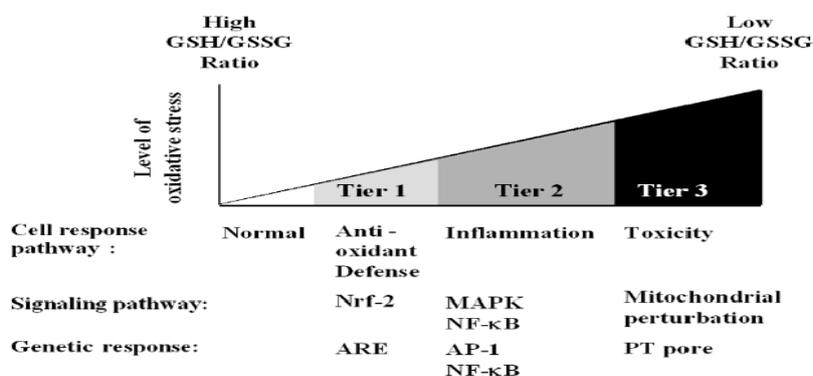


Figure 1.10: Hierarchical oxidative stress responses (Li *et al.*, 2008)

At a low level of oxidative stress (Tier 1), antioxidant enzymes are induced to restore cellular redox homeostasis. At an intermediate level of oxidative stress (Tier 2), activation of MAPK and NF-κB cascades induces pro-inflammatory responses (cytokines and chemokines). At a high level of oxidative stress (Tier 3), perturbation of the mitochondrial permeability transition pore and disruption of electron transfer result in cellular apoptosis or necrosis.

After entering the lung, particles first face the extracellular antioxidant mechanisms present in the respiratory tract lining fluid (RTLF). It contains enzymatic antioxidants such as superoxide dismutase (SOD) (it catalyses the dismutation of O₂⁻ to H₂O₂ and O₂), catalase (CAT) (it decomposes H₂O₂ to H₂O) and glutathione peroxidase (GPx) (reduces H₂O₂ by oxidizing glutathione (GSH to GSSG)). Also low molecular mass nonenzymatic antioxidants, including ascorbate, uric acid, glutathione (GSH) and α-tocopherol are constituent parts of the fluid. In the alveolar region of the lung, GSH is the major antioxidant and it depends on the activity of gamma-glutamyl-cysteine synthetase (γ-GCS) which is the rate-limiting step. Metal-chelating proteins such as ferritin or transferrin are also present in the RTLF and are part of the protection against oxidative stress by binding of free transition metals. If cellular antioxidant defense systems are overwhelmed, one consequence of oxidative stress is the oxidation of DNA.

Oxidative stress can arise for many reasons, including consumption of alcohol, medications (including birth control pills), trauma, cold, air pollutants, toxins, and radiation and it is involved in many diseases including inflammation, autoimmune diseases, brain disorder, cancer, diabetes, neurodegenerative diseases, heart attack and stroke (Araujo, 2010; Imrich *et al.*, 2007; Li *et al.*, 2008).

1.5.1.2 Cellular effects of oxidative stress

ROS overwhelming the antioxidant systems are then capable of interacting with different intracellular macromolecules, resulting in DNA damage, lipid peroxidation, or protein oxidation (Figure 1.11). The DNA damage induced by ROS cover several aspects: i) formation of adducts between the lipid derivatives from the peroxidation of polyunsaturated fatty acids and DNA; ii) formation of adducts between proteins affected by oxidative stress and DNA; iii) alteration of the deoxyribose-phosphate pool; iv) hydroxylation of purine and pyrimidine bases of DNA; v) single and double strand breaks of DNA; vi) induction of chromosomal aberrations; vii) abasic sites; viii) distortion of the double helix of DNA.

The alteration of the membrane unsaturated lipids by lipid peroxidation generally results in the formation of many compounds, particularly including alkanes and carbonyls. Some of them, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are inherently toxic and may serve as intermediates in the formation of free radical damage, generating adducts with proteins and DNA (Forman *et al.*, 2008; Valavanidis *et al.*, 2008). The evaluation of MDA in pathological conditions was described as a biological marker of membrane alterations induced by lipid peroxidation (Shirali *et al.*, 1994). ROS can lead to many different types of post-translational modifications of proteins including electrophilic modifications, protein carbonylation, S-glutathionylation, oxidation of methionine residues, nitration, and hydroxylation of amino acids, oxidation of sulfhydryl groups, etc. These effects have functional implications including inactivation of proteins, and downstream effects such as epigenetic changes, including changes in the methylation of DNA and post-translational modifications of histones (methylation, tyrosine nitration, phosphorylation, ubiquitination, and SUMOylation), which may then affect gene expression and thus protein expression (Delfino *et al.*, 2011). Protein oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino acid side chains, reactive oxygen-mediated peptide cleavage and from reactions with lipid and carbohydrate oxidation products (in particular MDA). It is now becoming clear that the presence of carbonyl groups in proteins may indicate that the proteins have been subjected to oxidative free radical damage. An increase in protein carbonyl content of tissues is associated with a number of pathological disorders: including rheumatoid arthritis, Alzheimer's disease, respiratory distress syndrome, Parkinson's disease and atherosclerosis (de Zwart *et al.*, 1999). The altered proteins have a heightened sensitivity to the proteases action. In addition, the

formation of adducts of the compounds resulting from lipid peroxidation with the protein results in the formation of auto-antibodies or lipofuschines characteristic of aging.

The nature of the damage depends on the nucleotide site affected by the radical attack. Thus, an abasic site may be formed if the attack affects the bond between the base and sugar, while a deoxyribose attack can lead to the formation of a single-strand break (Dizdaroglu and Jaruga, 2012). The base oxidation mainly occurs at the oxygen and nitrogen atoms, and may result in the formation of many modified bases (Cooke *et al.*, 2003). The radical attack can be direct or indirect. The direct radical attack can mostly lead to modified bases such as 8-oxoguanine, 8-nitroguanine, formamidopyrimidine, 8-oxoadenine, formimido uracil, 5-hydroxy-cytosine, 5-hydroxymethyl uracil, and thymine-diol oxazolone. Indirect attack can cause DNA oxidative damage caused by reactive aldehydes resulting from lipid peroxidation that can be added to the amino group of the DNA bases (Sedelnikova *et al.*, 2010). These reactions are potentially mutagenic and may cause point mutations in the genome or inhibition of the DNA replication leading to apoptosis. In particular, the deoxyguanosine is very sensitive to the action of the hydroxyl radical; 8-hydroxy-2'-deoxyguanosine (8-OHdG), formed by the covalent bonding of the hydroxyl radical with deoxyguanosine, is a specific biological marker of oxidative stress and has deleterious effects on nucleic acid (Floyd *et al.*, 1986; Klaunig and Kamendulis, 2004). 8-OHdG, if not repaired, can induce G:C > T:A transversions. The accumulation of 8-OHdG in nuclear DNA caused poly-ADP-ribose polymerase (PARP)-dependent nuclear translocation of apoptosis-inducing factor, whereas that in mitochondrial DNA caused mitochondrial dysfunction and Ca^{2+} release, thereby activating calpain (Oka *et al.*, 2008).

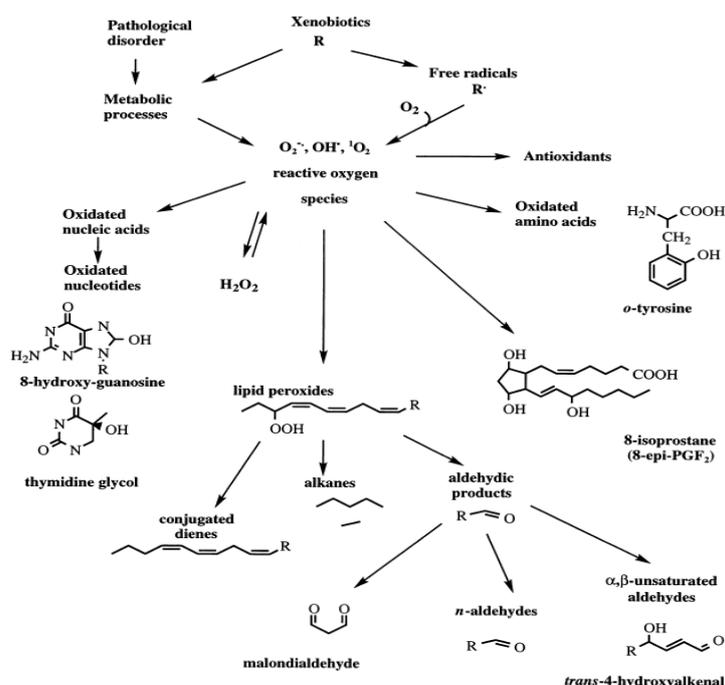


Figure 1.11: Products of free radical damage (de Zwart *et al.*, 1999)

1.5.1.3 ROS production due to PM_{2.5}

The exposure of lung cells to PM_{2.5} led to the production of ROS. The induced oxidative stress plays an important role in particle toxicity and carcinogenicity (Weichenthal *et al.*, 2016). The relationship between oxidative stress and PM is characterized by ROS production, induction of 8-OHdG, DNA strand breaks, MDA, CAT, SOD and decrease of the cellular level of GSH (Table 1.5).

Different factors can be involved in the ROS formation such as the PM size (Møller *et al.*, 2014), the transition metals, the organic compounds, the altered mitochondrial function, or the activation of inflammatory cells capable of producing ROS (Billet *et al.*, 2007; Dagher *et al.*, 2006; Risom *et al.*, 2005). The ultrafine particles have an increased interacting surface reactivity, leading to increased production of ROS (Singh *et al.*, 2007). Some transition metals in the particles, such as iron, copper, chromium or vanadium, can produce highly reactive hydroxyl radicals by catalyzing the Haber-Weiss and Fenton reactions (Novo and Parola, 2008). It was also shown that cadmium, although not being capable of catalyzing the Fenton reaction, was able to induce oxidative stress *in vitro* and *in vivo* (Waisberg *et al.*, 2003). Cadmium decreased intracellular concentrations of glutathione or reduced the activity of SOD, catalase or GPx (Del Carmen *et al.*, 2002; El-Maraghy *et al.*, 2001). Direct evidence of the generation of ROS, activation of redox sensitive transcription factors (NF-kappaB, AP-1 and Nrf2) and alteration of ROS-related gene expression have been proved experimentally with various metals (Cd, As, Ni, Cr(VI)) (Valavanidis *et al.*, 2013). ROS can be produced during the incomplete catalytic cycles of organic compounds metabolism by CYP450. The activity of CYP1A1 is thus associated with the production of H₂O₂ intracellularly (Barouki and Morel, 2001). In addition, the quinones, contained in the organic fraction or formed by the metabolism of other compounds such as PAHs and VOCs, are capable of generating ROS via a phenomenon of “redox cycle” (Dellinger *et al.*, 2001; Li *et al.*, 2008). Several *in vitro* and *in vivo* studies have shown a relation between concentrations of PAHs, oxy-PAHs, nitro-PAHs and polar organic chemicals in ultrafine PM, and oxidative stress and inflammatory responses (Li *et al.*, 2009; Shinyashiki *et al.*, 2009). Studies suggest that organic constituents and transition metals (Fe, Cu, Ni, and Zn) in PM are capable of generating ROS directly or as a result of their capacity to activate neutrophils or other leukocytes, alveolar macrophages, respiratory epithelial cells, and endothelial cells (Delfino *et al.*, 2013, 2011; Ntziachristos *et al.*, 2007). The cell-based ROS activity induced by PM has been correlated with transition metal and OC concentration in several studies (Shafer *et al.*, 2010; Verma *et al.*, 2009; Zhang *et al.*, 2008).

Table 1.5: Studies showing the effects of PM on the occurrence of oxidative stress

BEAS-2B: Bronchial epithelial cells, A549: Alveolar epithelial cells, HUVEC: Human umbilical veins epithelial cells, AM: Alveolar macrophages; BEC: Bronchial epithelial cells, L132 = Human embryonic lung epithelial cells, NHBE: Normal human bronchial epithelial, RAW264.7: Murine macrophage Cells, DEP: Diesel exhaust particles, #: no change.

In vitro studies				
Particles	Cell types	Doses	Effects	References
PM _{2.5}	BEAS-2B	3 and 12 µg/cm ² (24-48-72h)	↑ GSSG/GSH, SOD, MDA	(Cachon <i>et al.</i> , 2014)
DEP	A549	25–100 µg/mL (24h)	↑ROS, 8-OHDG	(Vattanasit <i>et al.</i> , 2014)
PM _{2.5}	A549	25, 50, 100, or 200 µg/mL	↑ROS ↓CAT and SOD	(Deng <i>et al.</i> , 2013)
PM _{2.5}	BEAS-2B	3.75 and 15 µg/cm ² (24-48-72h)	↑ROS, 8-OHDG and GSSG/GSH	(Dergham <i>et al.</i> , 2012)
PM _{2.5}	BEAS-2B	3 and 12 µg/cm ² (24-48-72h)	# MDA # GSH/GSSG	(Dieme <i>et al.</i> , 2012)
PM _{2.5} and PM ₁₀	HUVEC	25 µg/cm ²	↑ROS and NER	(Montiel-Dávalos <i>et al.</i> , 2010)
PM _{2.5}	AM and BEC	5–15 µg/mice (24h)	# H ₂ O ₂	(Huang <i>et al.</i> , 2009)
PM _{2.5}	Lung cells	1.5 mg/kg (body weight) - 7.5 mg/kg (7 and 14 days)	↓MDA ↓ GSH/GSSG	(Lin <i>et al.</i> , 2009)
PM _{2.5} and PM ₁₀	A549	20 µg/cm ²	↑ 8-OHDG + DNA strand breaks	(Shi <i>et al.</i> , 2006)
PM _{2.5}	L132	18.84 and 75.36 µg/mL	↑ 8-OHDG	(Dagher <i>et al.</i> , 2006)
PM _{2.5-10} , PM _{2.5} and PM _{0.1}	NHBE and AM	NHBE: 11 µg/mL MA : 50 µg/mL (18h)	↑ ROS	(Becker <i>et al.</i> , 2005)
DEP	RAW 264.7	10-100 µg/mL (6h)	↓ GSH/GSSG	(Xiao <i>et al.</i> , 2003)

Different proinflammatory genes (*TNF-α* and *IL-8*, among others) induced upon exposure to PM are regulated by redox sensitive transcription factors such as NF-κB, activator protein 1 (AP-1) and CAATT/enhancer binding protein (C/EBP). In the presence of high ROS formation, mitochondrial damage with induction of NADPH oxidase isoform 4 (NOX4) occurs. In addition, inflammatory cells (neutrophils, eosinophils, and monocytes) are activated and macrophages increased which are capable of ROS and reactive nitrogen species generation (Lodovici and Bigagli, 2011). Soberanes *et al.* showed that in lung epithelial cells, exposure to PM_{2.5} resulted in the generation of ROS from site III in the mitochondrial electron transport chain (Soberanes *et al.*, 2012). ROS then activated apoptosis signaling kinase 1 (ASK1), a MAP kinase kinase kinase (MAPKKK) that is activated when freed from its normal binding partner thioredoxin upon its oxidation to activate the JNK and p38 MAPKs31. The ASK1-mediated activation of JNK, resulted in the phosphorylation and activation of p53, increased the transcription of the proapoptotic protein NOXA to activate BAX/BAK and induce cell death (Urich *et al.*, 2009). Miller *et al.* suggest the different pathways by which PM can induce oxidative stress (Figure 1.12).

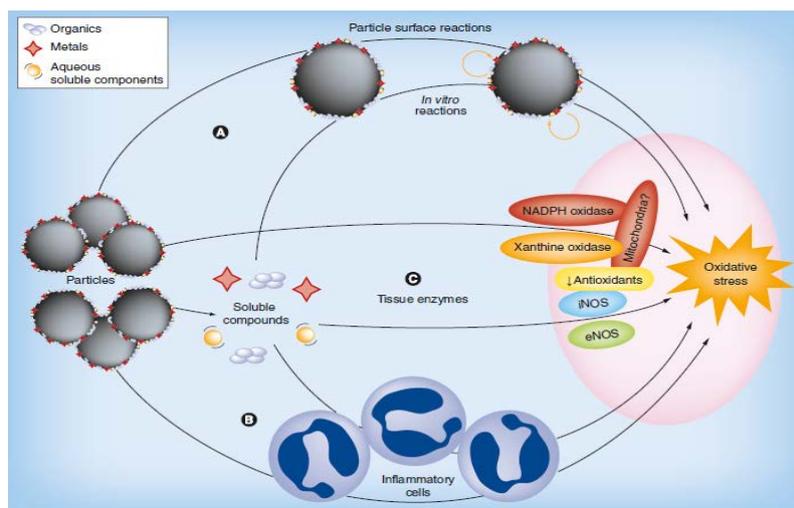


Figure 1.12: Different pathways through which contact between particulate matter and cells can induce cellular oxidative stress (Miller *et al.*, 2012)

eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase.

Free radicals may be produced by redox reactions between different chemicals on the surface of the particulate (A). PM can activate inflammatory cells that release cytokines, chemokines and oxidative mediators (B). After the interaction between PM and cells, free radicals can be generated (from stimulation of enzymes, such as NADPH oxidase, xanthine oxidase, uncoupling of eNOS, iNOS, exacerbation of free radicals from mitochondrial inefficiency or depletion of antioxidant defenses) (C). These pathways may be stimulated by particulate itself or through release of soluble constituents of PM in biological fluids.

1.5.2 Biotransformation of the organic compounds adsorbed on the PM

One of the most important determinants of xenobiotic persistence in the body and subsequent toxicity to the organism is the extent to which they can be metabolized and excreted. The bioactivation of pro-toxicants is the biological process through which some chemicals are metabolized into reactive metabolites. 75% of carcinogenic molecules would indeed result from the activation of pro-carcinogenic molecules by intracellular enzyme systems (Nebert and Dalton, 2006).

1.5.2.1 Biotransformation principle and mechanisms

Most xenobiotics that enter the body are lipophilic, a property that enables them to bind to lipid membranes and be transported by lipoproteins in the blood. The transformation or the metabolism of xenobiotics and metabolites lead to the formation of water-soluble substances that are more easily removed by aqueous media such as urine, bile, saliva or sweat. Therefore, it is usually a sequence that increases water solubility and hence decreases the biological half life of the xenobiotic *in vivo* (Hodgson, 2004). The liver is the main organ involved in this process and to a lesser extent certain organs such as the skin, kidney, intestinal mucosa and lung. Several families of metabolic enzymes, often with wide arrays of substrate specificity, are involved in xenobiotic metabolism. Xenobiotics may undergo one or two phases of metabolism, in phase I a polar reactive group is introduced into the molecule rendering it a suitable substrate for phase II enzymes. Following the addition of a polar group, conjugating enzymes typically add much more bulky substituents, such as

sugars, sulfates, or amino acids that result in a substantially increased water solubility of the xenobiotic, making it easily excreted.

Although this process is generally a detoxication sequence, reactive intermediates may be formed that are much more toxic than the parent compound, this phenomenon is called metabolic activation. So the metabolism of a given compound can generate more reactive metabolites, and often more toxic. Indeed, the lung tissue is known to activate pro-carcinogens, such as PAHs, and nitrosamines and more reactive intermediates that can easily bind to DNA and form adducts (Castell *et al.*, 2005) (Figure 1.13). The toxic potential reflects the balance between activation reactions and those of detoxification (Ding and Kaminsky, 2003).

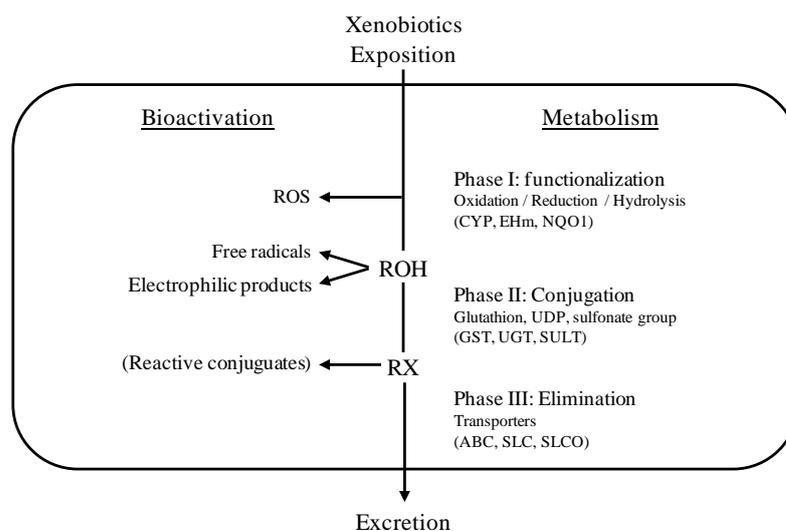


Figure 1.13: Metabolism and metabolic activation of xenobiotics (Xu *et al.*, 2005)

CYP: Cytochrome P450, EHm: microsomal epoxide hydrolase, NQO1: NADPH quinone oxidoreductase 1, GST: Glutathione-S-transferase, UGT: UDP-glucuronosyltransferase, SULT: sulphotransferase, ABC: ATP-Binding Cassette, SLC: solute carrier, SLCO: solute carrier organic anion.

Three phases are usually required to complete the processing. This classification is essentially functional; some phase II enzymes may thus act before other phase I enzymes, or even be the only acting enzymes. Phase III systems allow the expulsion of these xenobiotics or their metabolites out of the cell and organism. Detection of the presence of xenobiotics in the cell and the coordination between the expression of genes encoding enzymes of metabolism and the most appropriate carriers to deactivate and/or remove these compounds, involve nuclear receptors also called “xenosensors”, including the aryl Hydrocarbon Receptor (AhR), the Pregnane X Receptor (PXR), the constitutive Androstane Receptor (CAR) and the retinoid X receptor (RXR) (Xu *et al.*, 2005).

1.5.2.1.a Phase I reactions or functionalization

Phase I reactions involve introducing or unmasking a polar functional group on the xenobiotic molecule by oxidation, reduction or hydrolysis reactions. Phase I reactions involve a group of enzymes including mainly mono-oxygenase cytochrome P450, the flavin monooxygenases (FMO),

alcohol and aldehyde dehydrogenases, the monoamine oxidase (MAO), the epoxide hydrolases, NADPH quinone oxidoreductase (NQO1) (Table 1.6). These reactions release or introduce a functional group (-OH, -NH₂, -SH, -COOH), thereby increasing the polarity of the parent product. The oxidation of xenobiotics is mainly carried out by the CYP, a superfamily of heme proteins comprising 57 active genes in humans and one of the functional classes (FC-X) is specialized in the detoxification of xenobiotics and in the metabolism of many endogenous compounds such as arachidonic acid, eicosanoids, cholesterol, etc. (Gotoh, 2012; Guéguen *et al.*, 2006). The CYP catalyzes oxidation reactions requiring oxygen and NADPH (nicotinamide adenine dinucleotide phosphate) as a source electrons. They are mainly localized in the endoplasmic reticulum membrane but they also exist in the inner membrane of mitochondria. The families 1, 2 and 3 of CYP are generally involved in phase I metabolism of drugs and xenobiotics, while other CYPs have endogenous functions. In human lungs, CYPs are mainly expressed in Clara cells, alveolar type I and II pneumocytes, endothelial cells, alveolar macrophages, as well as bronchial hair cells (Hukkanen *et al.*, 2002). The main isoform in human are: 1A1, 1B1, 2B1, 2B6, 2E1, 2F1, 2F2, 2J2, 3A4, 3A5, 3A7 et 4B1 (Dahl and Lewis, 1993).

Table 1.6: Enzymes implied in catalyzing metabolic activation reactions (Hodgson, 2004)

Type of Reaction	Enzyme
Oxidation	Cytochromes P450, Prostaglandin synthetase, Flavin-containing monooxygenases, Alcohol and aldehyde dehydrogenases
Reduction	Reductases, Cytochromes P450, Gut microflora
Conjugation	Glutathione transferases, Sulfotransferases, Glucuronidases
Deconjugation	Cysteine S-conjugate β -lyase
Hydrolysis	Gut microflora, hydrolyses

1.5.2.1.b Phase II reactions or conjugation

Products of the phase I metabolism and other xenobiotics possessing functional groups such as hydroxyl, amine, carboxyl, epoxy, or a halogen atom may undergo conjugation reactions with endogenous metabolites (Table 1.7). These include sugars, amino acids, glutathione, sulfate, etc (Table 1.6). The conjugation products, with rare exceptions, are more polar, less toxic and more easily excreted than are their parent compounds (Hodgson, 2004).

Table 1.7: Main reactions of phase II metabolism

UDP: Uridine Diphosphate, PAH: Polycyclic Aromatic Hydrocarbon

Enzymes	Reactions	Transferred group	Examples of substrates
UDP-Glucuronosyl-Transferases (UGT)	Glucuro- conjugation	Glucuronic acid	Hydroxylamines, arylamines
Sulfo-Transferases (ST)	Sulfo- conjugation	Sulfate	Steroids, phenols, amines, hydroxylamines
Methyltransferases	Methylation	Methyl	Amines, catecholamines, imidazoles, thiols
Glutathion-S-Transferases (GST)	Glutathion conjugation	Glutathion	PAH Epoxydes, Arylamines
Acetyl-transferases	Acetylation	Acetyl	Arylamines, hydrazines
Glycine-transferases	Glycine conjugation	Glycine	Aromatic and aliphatic carboxylic acids

1.5.2.1.c Phase III reactions or elimination

The essential step in detoxification is based on the transport of the xenobiotics out of the cell. This transport is done via protein carriers using adenosine triphosphate (ATP), such as MRP (Multidrug Resistance Proteins) belonging to the superfamily of ABC (ATP Binding Cassette) and via Solute Carriers (SLC) (Lecomte *et al.*, 2006).

1.5.2.2 Organic compounds metabolic activation

As mentioned before, different organic compounds can be in the composition of PM, such as PAHs, VOCs, PCDDs, PCDFs and PCBs. These compounds are inducers and substrates of some enzymes of phase I and phase II metabolism, with the exception of PCDD and PCDF which are resistant to biodegradation and accumulate in the liver and adipose tissues. A bioactivation phenomenon can take place mainly under the action of phase I enzymes. These compounds induce the expression of metabolic enzymes that activate other compounds such as endogenous estrogens, like the CYP that leads to the formation of catechol derivatives with carcinogenic activity. The reactive compounds formed (quinones, epoxides, aldehydes) exert their toxicity via oxidative stress, DNA adduct, or proteins or lipids modifications. The induction done by the organic compounds is carried out by two mechanisms.

The first mechanism is controlled by the AhR, a transcription factor specific to aromatic compounds. The unliganded AhR resides in the cytoplasm, forming a complex with a heat shock protein 90 (HSP90) dimer and the co-chaperone protein X-associated protein 2 (XAP2). The organic compounds bind directly to the AhR and the complex then translocate to the nucleus, where the bound proteins dissociate and AhR dimerizes with ARNT (AhR Nuclear Translocator). The complex AhR/ARNT activates the expression of a gene cluster containing a xenobiotic response element (XRE) such as CYP1A1, CYP1B1, NQO1, AhRR (AhR repressor), GST, UGT and ABCB6 (Figure 1.14A) (Chavan and Krishnamurthy, 2012; Mimura and Fujii-Kuriyama, 2003; Ramadoss *et al.*, 2005). The AhR target gene *CYP1A1* is almost totally dependent on AhR activity for expression and is highly induced by AhR activation through multiple XREs. *CYP1A1* metabolizes a number of pro-carcinogens, such as benzo[*a*]pyrene (B[*a*]P), to intermediates that can react with DNA to form adducts, resulting in mutagenesis. Moreover, it seems that B[*a*]P causes the inhibition of the expression of the repressor AhRR, reinforcing its inducer effect (Gualtieri *et al.*, 2012).

The second mechanism consists in the activation by electrophilic metabolites of PAHs of the antioxidant response elements (ARE) located in the promoter regions of the genes of phase II enzymes. These enzymes regulated by AhR receptor are connected to a second battery, the NF-E2-related factor 2 gene (Nrf2). Electrophilic metabolites (such as PAHs o-quinones) stimulate the cytosolic protein KEAP1, resulting in the activation and release of the transcription factor Nrf2 of the KEAP1-Nrf2 complex. In the nucleus, the activated Nrf2 forms a complex with the Maf proteins, inducing thereafter the battery genes having ARE in their promoter sequences such as Phase II

metabolism genes (*NQO1*, *GST*, *UGT*) (Figure 1.14B) (Miao *et al.*, 2005). Different studies showed that VOC and PAH-coated onto PM induced significant increases in CYP1A1, CYP2E1, CYP2F1, NQO1, and GSTP1 (Billet *et al.*, 2007, 2008; Gualtieri *et al.*, 2012).

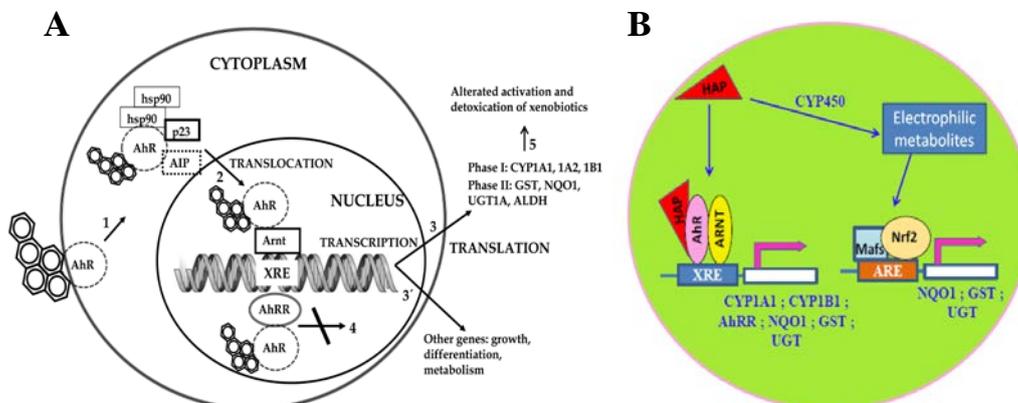


Figure 1.14: Induction of the genes of phase I and II metabolism (A) and AhR and Nrf2 signaling pathways (B) (Arenas-Huerta *et al.*, 2011)

PAHs can have genotoxic effects by ROS generated during their metabolism. PAH can also be converted to electrophilic metabolites capable of forming bulky DNA adducts, which can cause mutations which distort the double helix structure (Pakotiprapha *et al.*, 2012). B[a]P is often used as a model for studying the metabolic activation of organic compounds. There are three major pathways for its activation, which result in the formation of radical cations, diol epoxides, and electrophilic and redox-active *o*-quinones (Zhang *et al.*, 2012) (Figure 1.15). Several enzymes can thus be involved in the metabolic activation of B[a]P, such as CYP1A1, CYP1B1 and CYP2S1. NQO1 prevents the "redox cycle" phenomenon of quinones, transforming them into hydroquinones (Joseph *et al.*, 1998). The Diol epoxide pathway is considered as the major way and consists of the oxidation of B[a]P catalyzed by CYP which can generate the B[a]P-7,8-diol-epoxide (BPDE), extremely reactive with DNA. BPDE can then be hydrolyzed into inactive tetraols (Xue and Warshawsky, 2005) or lead to DNA damage by DNA-adduct formation (Chen *et al.*, 1996), or by oxidative stress (Douki *et al.*, 2008). Quinones formed during the metabolism of PAHs are also capable of forming bulky DNA adducts, mainly on guanine, but also on adenine and cytosine (Balu *et al.*, 2006). Same as the lesions formed by the ROS, damages caused by PAH adducts interfere with repair mechanisms or DNA replication, potentially leading to the formation of double-strand breaks (Sedelnikova *et al.*, 2010).

The metabolism of VOCs leads also to reactive metabolite production. CYP2E1, CYP2F1 and ADH (Alcohol Dehydrogenase) are the main enzymes involved in the metabolic activation of VOCs leading to the formation of aldehyde, hydroquinone or toxic quinones. SULTs, GST, UGT and NQO1 are involved in the detoxification of these metabolites (Bauer *et al.*, 2003).

The biotransformation of PCBs is carried out by CYP (1A1, 1A2, 2B1, 2B2, 3A) and leads to the formation of epoxides which are then primarily converted to phenolic compounds or dihydrodiols.

Epoxides can react with nucleic acids or proteins and form adducts. Furthermore, the metabolism of PCBs may give rise to quinones capable of reacting with the nucleophilic sites of proteins or DNA (Pereg *et al.*, 2002). When the degree of PCBs chlorination increases, the metabolic rate decreases and the accumulation in adipose tissue increases (Song *et al.*, 2013).

The toxicokinetic behaviour of PCDD/PCDF depends on three major properties: lipophilicity, metabolism and binding to the CYP1A2 in the liver (Pereira, 2004). Lipophilicity controls the absorption and partitioning of PCDD/F which are very resistant to metabolism. Their metabolism leads to the substitution of chlorine by OH groups and formation of dichlorocatechol from 2,3,7,8-TCDD. Toxicokinetic analysis of human data indicates that the elimination half-life is approximately 8.5 years for occupational cohorts and 15.5 years for the general population.

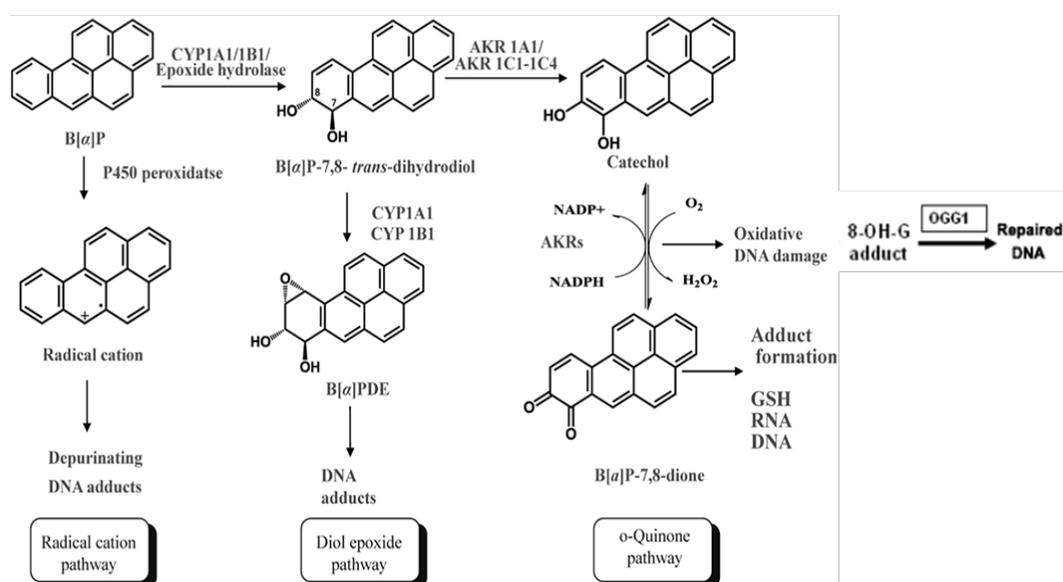


Figure 1.15: Metabolic activation of B[a]P (Zhang *et al.*, 2012)

1.5.3 DNA methylation

1.5.3.1 Definition

DNA methylation is the most frequently studied epigenetic modification. It has been known for more than three decades that DNA methylation in regulatory regions such as promoters and enhancers could silence gene expression (Szyf, 2011). It provides a stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in association with histone modifications and other chromatin associated proteins. DNA methylation occurs mainly on specific cytosine residues in CpG dinucleotides. Methylated cytosines represent 3-6% of total cytosines of a normal human DNA. CpG dinucleotides are not evenly distributed across the human genome but are instead concentrated in: short CpG-rich DNA stretches called ‘CpG islands’ and regions of large repetitive sequences (centromeric repeats, retrotransposon elements, ribosomal DNA, etc.) (Sharma *et al.*, 2010; Stirzaker *et al.*, 2015). The CpG island is, so far, the only feature known to

play a well-established role in epigenetic-mediated transcriptional repression, though the search for other CpG-based genomic sequences involved in methylation-controlled transcriptional modulation is in progress. The role of isolated CpG sites and the extension of non-CpG methylation are topics that have only recently received more attention (Fuso *et al.*, 2010). DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which can act *de novo* (mainly DNMT3a and DNMT3b) or maintain the existing methylation patterns (mainly DNMT1) assisted by accessory proteins such as UHRF1 (Liloglou *et al.*, 2015).

1.5.3.1.a Methylation of CpG islands

CpG islands are located at the 5' end of many genes, at the level of the promoters. While most of the CpG sites in the genome are methylated, the majority of CpG islands usually remain unmethylated during development and in differentiated tissues (Suzuki and Bird, 2008). Some CpG island promoters become methylated during development, which results in long-term transcriptional silencing, like X-chromosome inactivation. The methylation profile of many crucial genes promoters is altered during tumorigenesis. Hypermethylation of many tumor suppressor genes leads to loss of their expression (Esteller, 2011; Ng and Yu, 2015; Schwartz *et al.*, 2007) (Table 1.8) and hypomethylation of many oncogenes leads to activation of their transcription (Hur *et al.*, 2014). Many TSGs are repressed in lung cancer, such as *P16^{INK4A}* or O⁶-methylguanine-DNA methyltransferase gene (*MGMT*). P16 protein inhibits the cyclin D-dependent phosphorylation of RB, thereby prevents the G1-S transition; a loss of activity of P16 therefore leads to a deregulation of cell proliferation (Agarwal *et al.*, 2013). The protein O⁶-alkylguanine DNA alkyltransferase (AGT), encoded by *MGMT*, is a DNA repair enzyme ensuring its protection against alkylating agents (Shiraishi *et al.*, 2000). Other than *P16^{INK4A}* and *MGMT*, hypermethylation of CpG island promoters is the most recognized epigenetic disruption in human tumors. The methylation of promoters of genes involved in central cellular pathways such as DNA repair (*hMLH1* and *BRCA1*) and cell cycle control (*P15^{INK4b}* and *RB*) results in the silencing of the expression. Taking into account results of many individual studies, meta-analyses confirmed that the methylation status of *MGMT*, *CDKN2A*, *APC*, and *RARβ* promoters is strongly related to lung cancer development (Exner *et al.*, 2015; Liloglou *et al.*, 2015; Warton and Samimi, 2015). Zhang *et al.* showed a significantly higher frequency of methylation of *APC*, *CDH13*, *KLK10*, *DLEC1*, *RASSF1A*, *EFEMP1*, *SFRP1*, *RARβ* and *P16^{INK4A}* in NSCLC compared with the normal tissues (Zhang *et al.*, 2011).

While the role of CpG island promoter methylation in gene silencing is well established, much less is known about the role of methylation of non-CpG island promoters. DNA methylation is also important for the regulation of non-CpG island promoters which influences its expression level. For example, tissue-specific expression of maspin, which does not contain a CpG island within its promoter, is regulated by DNA methylation (Futscher *et al.*, 2002).

Table 1.8: Hypermethylated tumor suppressor in lung cancers (Liloglou *et al.*, 2015)

Function	Gene	Methylation frequency	Cancer type
Adhesion and extracellular matrix	<i>T-cadherin</i>	28 - 30 %	NSCLC
	<i>E-cadherin</i>	12 - 34 %	NSCLC
	<i>TIMP3</i>	13 %	NSCLC
	<i>TIMP4</i>	94 %	NSCLC
Proliferation and cell cycle regulation	<i>P16^{INK4A}</i>	22 - 47 %	NSCLC
	<i>P14^{ARF}</i>	30 %	NSCLC
	<i>FHIT</i>	34 - 47 %	NSCLC
	<i>RARβ2</i>	26 - 45 %	NSCLC
	<i>APC</i>	30 - 53 %	NSCLC
Apoptosis	<i>Fas</i>	40 %	SCLC
	<i>CASP8</i>	52 %	SCLC
	<i>DAPK</i>	26 - 45 %	NSCLC
DNA repair	<i>MGMT</i>	20 %	NSCLC
	<i>BRCA1</i>	30 %	NSCLC
	<i>BRCA2</i>	42 %	NSCLC
Signalization	<i>RASSF1A</i>	25 - 45 %	NSCLC
	<i>PTEN</i>	26 %	NSCLC

1.5.3.1.b Global DNA methylation

The repetitive genomic sequences that are scattered all over the human genome are heavily methylated, preventing chromosomal instability by silencing non-coding DNA and transposable DNA elements in normal cells, in contrary to cancer cells (Figure 1.16). DNA methylation can lead to gene silencing by either preventing the recruitment of regulatory proteins to DNA (inhibit transcriptional activation by blocking transcription factors from accessing target-binding sites like c-myc and MLTF) or promoting this recruitment (provide binding sites for methyl-binding domain proteins, which can mediate gene repression through interactions with histone deacetylases, HDACs) (Sharma *et al.*, 2010). In cancer cells, DNA is generally hypomethylated at long repetitive sequences such as centromeric repeats, elements or ribosomal DNA (Esteller, 2011). Long interspersed nucleotide element 1 (LINE 1), constituting about 17% of the human genome, is used as an indicator of global DNA methylation which is hypomethylated in many NSCLC cases (Suzuki *et al.*, 2013). The hypomethylation of *LINE1* and *Alu* is associated with the reactivation of these elements leading to genetic instability, involved in carcinogenesis (Park *et al.*, 2014). The loss of methylation of these repetitive sequences could be related to depletion in *DNMT3A* responsible of the protection of the active areas of chromosomes against hypomethylation (Raddatz *et al.*, 2012).

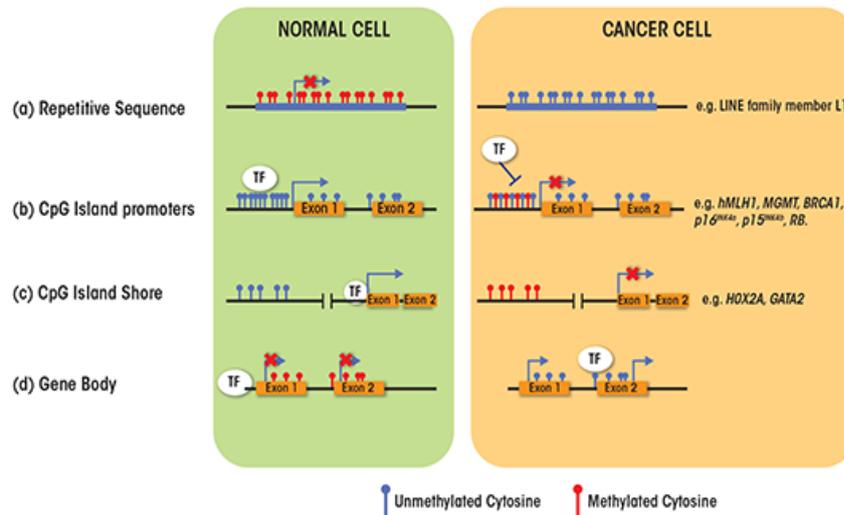


Figure 1.16: DNA methylation patterns in normal and cancer cells (Varela-Rey *et al.*, 2015)

1.5.3.2 Effect of environmental chemicals and PM_{2.5}

Environmental chemicals can modify DNA methylation machinery by different mechanisms of action (Figure 1.17) (Ruiz-Hernandez *et al.*, 2015). Metals, persistent organic pollutants (POPs) and PAHs increase ROS formation. Under chronic consumption of GSH for conjugation with ROS, chemicals, and their metabolites, homocysteine is employed into GSH rather than methionine synthesis pathways, leading to a reduced synthesis of S-adenosylmethionine (SAM). SAM is a substrate for DNMTs which catalyze the addition of the methyl group onto the 5-carbon cytosine (5C) to become 5-methylcytosine (5mC). SAM depletion induced by oxidative stress, thus, potentially inhibits DNA methylation and results in subsequent DNA hypomethylation (Lee *et al.*, 2009). Short-term Cd, PAHs, Pb, and Hg exposures can reduce the enzymatic activity and concentrations of DNMTs (Cheng *et al.*, 2012). In addition, oxidative stress is proposed to stimulate the alpha-ketoglutarate (α -KG) production from isocitrate. α -KG activates ten-eleven translocation (TET) proteins that catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC) in the presence of cofactors, iron and oxygen. 5hmC, 5fC, and 5caC could act as an intermediate in both passive and active DNA demethylation pathways involving DNA repair enzymes like AID, APOEC, and TDG (Dao *et al.*, 2014).

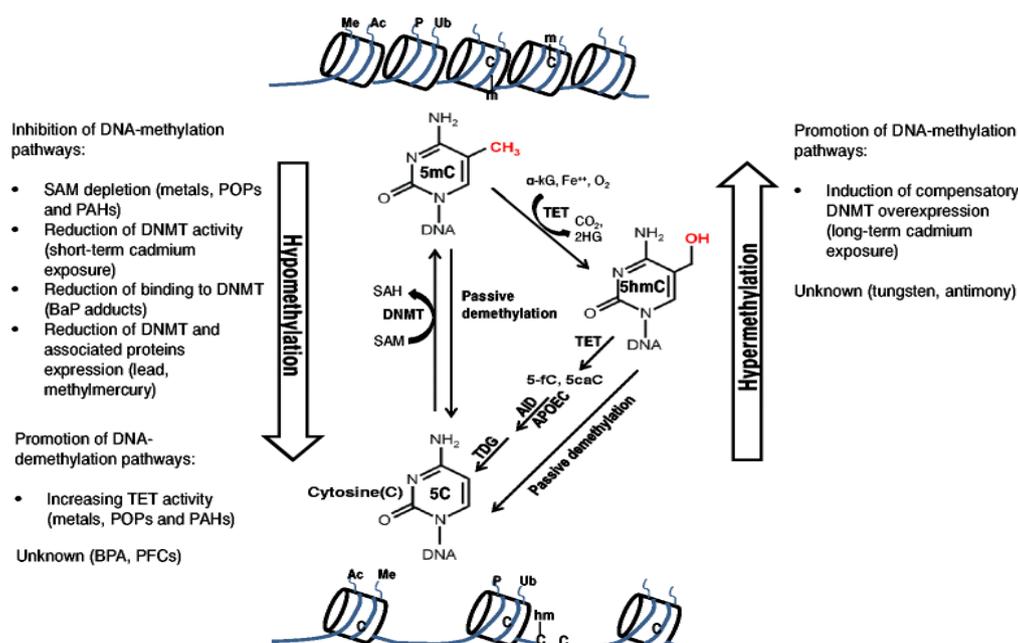


Figure 1.17: Possible mechanisms of action for environmental chemicals on DNA methylation (Ruiz-Hernandez *et al.*, 2015)

POPs: persistent organic pollutants, SAM: S-adenosylmethionine, TET: ten-eleven translocation, α -KG: alpha-ketoglutarate, 5C: 5-carbon cytosine, 5caC: 5-carboxycytosine, 5hmC: 5-hydroxymethylcytosine, 5mC: 5-methylcytosine.

Environmental chemicals can modulate the enzymes involved in acetylation, methylation, phosphorylation and ubiquitination at the histone tails that can interact with the DNA methylation or demethylation machinery. *In vitro* and *in vivo* studies have demonstrated that PM or the adsorbed chemicals on these particles are able to alter the methylation profile of the DNA.

Some studies showed the effect of PM and especially PM_{2.5} on DNA methylation. As shown in Table 1.9, most of studies showed that long-term and short-term exposures to PM_{2.5} were associated with the hypomethylation of both *Alu* and *LINE-1*, the hypermethylation of tumor suppressor genes such as *APC*, *P16*, *TP53* and *RASSF1A* and the hypermethylation of *iNOS* gene (inducible nitric oxide synthase) in blood DNA samples. Methylation of repair genes is caused by the presence of PM_{2.5} such as oxoguanine glycosylase1 (*OGG1*) involved in base excision repair of 8-OHdG (Bind *et al.*, 2016; Soberanes *et al.*, 2012).

Cd exposure leads to alteration in DNA methylation status (Rodríguez and Berrios, 2015) and to compensatory DNMT overexpression that could lead to increased DNA methylation (Takiguchi *et al.*, 2003). The effect of Cd was not consistent in different studies. Epigenome-wide association studies found a trend towards hypermethylation with elevated Cd exposure (Sanders *et al.*, 2014). In rat liver cells, short-term Cd exposure induced DNA global hypomethylation, however, prolonged exposure resulted in global DNA hypermethylation (Benbrahim-Tallaa *et al.*, 2007). Most *in vitro* and *in vivo* studies showed increased gene-specific DNA methylation after exposure to Cd like the hypermethylation of *P16* promoter in lymphocytes (Yuan *et al.*, 2013), global CpG hypermethylation

in eels (Pierron *et al.*, 2014), and hypermethylation in *LINE-1* elements (Hossain *et al.*, 2012). When evaluating global or candidate gene methylation (5-mC), significant associations with Cd biomarkers were observed (Tellez-Plaza *et al.*, 2014). In *in vivo* and *in vitro* studies, Pb exposure was associated with changes in DNA methylation and expression of specific genes (Senut *et al.*, 2014). Studies reported that Pb exposure can lead to hypermethylation (Zhang *et al.*, 2013) especially of *LINE-1* (Wright *et al.*, 2010). In the only epigenome-wide association study (N= 24), CpG sites showed DNA hypomethylation with elevated blood Pb exposure (Sanders *et al.*, 2014). Pb has been related with transcription-active histone modifications associated to DNA hypomethylation, while Hg and Ni have been related with transcription-repressive histone modifications associated to DNA hypermethylation (Chervona and Costa, 2012). In *in vitro* and *in vivo* studies, Ni resulted in promoter hypermethylation, increased global DNA methylation and hypermethylation of *P16* (Govindarajan *et al.*, 2002; Tajuddin *et al.*, 2013). DNA methylation was reduced to about 60% of control levels in zinc-deficient oocytes (Tian and Diaz, 2013). Arsenic (As) exposure causes changes in the DNA methylation maintenance machinery (Rager *et al.*, 2015). It induced the global hypomethylation in rat liver epithelial cells, or the appearance of hypo- or hyper-methylated area, as well as the hypermethylation of *TP53* promoter in A549 cells (Zhong and Mass, 2001). *In vivo* studies highlighted hypomethylation of the promoter ER- α and global hypomethylation of DNA after mice exposure to As (Chen *et al.*, 2004). Hypermethylation of *P16* was shown in As-induced lung adenomas in mice however, they contrast with the finding that As induced hypomethylation of *P16* and *RASSF1A* in human liver tumor cells resulting in increased expression of these genes (Cui *et al.*, 2006a, 2006b; Reichard and Puga, 2010). Studies explained that a high As dose was associated with global hypermethylation (Pilsner *et al.*, 2007), but the highest levels of As exposure demonstrated a trend towards hypomethylation proposing that low dose As might induce DNMT3A leading to subsequent DNA hypermethylation, whereas the high-dose exposures cause SAM depletion and DNMT inhibition (Majumdar *et al.*, 2010; Reichard *et al.*, 2007). Paired case-control studies showed that high exposure of As in human is positively linked to DNA hypermethylation of *P16* (Lu *et al.*, 2014). As is associated with global methylation of peripheral blood mononuclear cell DNA (Niedzwiecki, 2014), gene-specific differential white blood cell DNA methylation (Argos *et al.*, 2014), changes in global 5-methyl-2'-deoxycytidine in blood DNA (Niedzwiecki *et al.*, 2015). Studies showed an association between As exposure and DNA methylation in *Alu* repetitive elements (Lambrou *et al.*, 2012).

For most POPs, studies evaluating DNA methylation globally showed a trend towards hypomethylation (*Alu*) with increasing levels of exposure in plasma (Rusiecki *et al.*, 2008) and in serum (Itoh *et al.*, 2014). In breast cancer cell lines and in mouse cells, B[a]P treatment was related to DNA hypomethylation (Sadikovic and Rodenhiser, 2006). Binding of B[a]P adducts to DNA decreased methylation by reducing binding and activity of DNMTs (Subach *et al.*, 2007) suggesting that PAH-DNA adduct formation may preferentially target methylated genomic regions that may interfere their DNA methylation status (Chen *et al.*, 1998). DNA hypermethylation in *P16* promoter

(Yang *et al.*, 2012) and hypomethylation of *LINE-1* and *MGMT* promoter (Duan *et al.*, 2013) were associated with PAH exposure.

PM exhibits a strong interference on DNA methylation, evidenced not only by modulated global DNA methylation but also by regulated DNMT activities (Liu *et al.*, 2010; Song *et al.*, 2011). DNMT not only plays a pivotal role in lung tumorigenesis, but also is a promising molecular biomarker for early lung cancer diagnosis and therapy. Therefore the elucidation of the DNMT and its related epigenetic regulation in lung cancer is of great importance, which may expedite the overcome of lung cancer (Tang *et al.*, 2009). DNMT1 has a role in the establishment and regulation of tissue-specific patterns of methylated residues. Oxidative DNA damage can interfere with the ability of DNMT to interact with DNA, thus resulting in a generalized altered methylation of cytosine residues at CpG sites (Baccarelli and Bollati, 2009). PM-induced up-regulation of the catechol-O-methyltransferase (COMT) depletes the SAM intracellular pool, therefore indirectly inhibiting DNMT (Fiskin *et al.*, 2006). PM is related to modulated activities of DNMT (Wang *et al.*, 2013). Blind *et al.* and Soberanes *et al.* showed that PM_{2.5} increased ROS production and lead to the methylation of repair genes such as DNMT1 and OGG1 and the overexpression of DNMT1 (Bind *et al.*, 2016; Soberanes *et al.*, 2012). Georgia *et al.* explained that DNMT1 is bound to the p53 regulatory region and that loss of DNMT1 results in derepression of the p53 locus (Georgia *et al.*, 2013). In alveolar epithelial cells, increased transcription of DNMT1 and methylation of the p16 promoter were inhibited by a mitochondrially targeted antioxidant and a JNK inhibitor. These findings provide a potential mechanism by which PM exposure increases the risk of lung cancer (Soberanes *et al.*, 2012). Heavy metals adsorbed on PM_{2.5} can upregulate DNMT expression. Inhibition of DNMT activity by arsenic appears to cause whole-genome and localized gene specific demethylation (Reichard and Puga, 2010). Binding of B[a]P adducts to DNA decreased methylation by reducing binding and activity of DNMTs (Subach *et al.*, 2007) suggesting that PAH-DNA adduct formation may preferentially target methylated genomic regions that may interfere their DNA methylation status (Chen *et al.*, 1998; Weisenberger and Romano, 1999).

Duan *et al.* demonstrated hypomethylation of *MGMT* after exposure to PAHs (Duan *et al.*, 2013). In addition, expression of *MGMT* protein varies in normal and tumoral tissue (Sharma *et al.*, 2009). The study done by Miousse *et al.* showed the effect of PM on DNMT1 mRNA and protein expression, suggesting that epigenetic alterations, in concert with cytotoxicity, oxidative stress, and inflammation, might contribute to the pathogenesis of PM-associated respiratory diseases (Miousse and Chalbot, 2014).

Table 1.9: Studies showing the effects of PM on DNA methylation
iNOS: inducible nitric oxide synthase, 5mdC: 5-methyl-2'-deoxycytidine, #: no change.

Particles	Sample	Exposure	Effects	References
PM ₁₀	Human blood	long-term exposure	↓ methylation in <i>Alu</i> and <i>LINE-1</i>	(Tarantini <i>et al.</i> , 2009)
PM _{2.5}	- mice - primary murine alveolar epithelial cells	- short-term exposure - daily doses of 5 mg/cm ²	↑ <i>P16</i> ^{<i>INK4A</i>} promoter	(Soberanes <i>et al.</i> , 2012)
PM ₁₀ , PM _{2.5}	Human blood	long-term exposure	↓5mdC	(De Prins <i>et al.</i> , 2013)
PM _{2.5}	Human blood	long-term exposure	↓5mdC	(Madrigano <i>et al.</i> , 2012)
PM _{2.5}	Human blood	short-term exposure	↓ methylation in <i>LINE-1</i> and <i>Alu</i>	(Bellavia <i>et al.</i> , 2013)
PM _{2.5}	Human blood	short-term exposure	↑ methylation in <i>LINE-1</i> # in <i>Alu</i>	(Fan <i>et al.</i> , 2014)
PM _{2.5}	Human blood	short-term exposure	↓ methylation in <i>LINE-1</i> and <i>Alu</i>	(Guo <i>et al.</i> , 2014)
PM _{2.5}	Human blood	long-term exposure	↓ methylation in <i>LINE-1</i>	(Baccarelli <i>et al.</i> , 2009)
PM _{2.5}	Human blood	long-term exposure	↑ methylation in tumor suppressor genes: <i>APC</i> , <i>P16</i> , <i>TP53</i> and <i>RASSF1A</i>	(Hou <i>et al.</i> , 2011)
PM _{2.5}	Human blood	acute and chronic exposure	↑ methylation in <i>iNOS</i> gene	(Kile <i>et al.</i> , 2013)

1.5.4 DNA repair

1.5.4.1 DNA repair system

The damages described above can have serious consequences on the genome. The replication without repair of the “microlesions”, such as the oxidation adducts, abasic sites and single-strand breaks can lead to substitutions of base pairs, as well as the insertion or deletion short DNA fragments. The “macrolesion”, such as bulky DNA adducts, bypass, or double-strand breaks, can cause the gain, loss or rearrangement of large fragments chromosomes. So the repair of these lesions is essential to the integrity of the genome and is ensured by various DNA repair mechanisms. The heart of the cellular defense against DNA injuries is formed by a variety of DNA repair mechanisms, each with their own damage specificity. DNA repair is a phenomenal multi-enzyme-pathway system that ensures the integrity of the cellular genome. These cellular mechanisms must cope with the plethora of DNA base pair adducts that arise. DNA damage can arise spontaneously in the cellular milieu through chemical alteration of base nucleotides or as a consequence of errors during DNA replication. The basic mechanisms underlying distinct DNA repair pathways include direct reversal of DNA damage, base excision repair (BER), nucleotide excision repair (NER), DNA strand break repair (DSBR), mismatch repair (MMR) and the replication past DNA lesions by specialized DNA bypass polymerases (bypass replication). The repair of damaged DNA is intimately associated with a number of other distinct cellular processes such as DNA replication, DNA recombination, cell cycle checkpoint arrest, and other basic cellular mechanisms as outlined herein. A failure to repair DNA produces a mutation. The publication of the human genome has already revealed 130 genes participating in DNA repair.

1.5.4.1.a Direct reversal of DNA damage

The simplest and most accurate repair mechanism is the direct reversal of damage in a single-step reaction. The most frequent cause of point mutations in humans is the spontaneous addition of a methyl group (CH_3^-) to cytosines, followed by deamination to a thymine. Most of these changes are repaired by enzymes, called glycosylases, which remove the mismatched T restoring the correct C. Some of the methyl groups can be removed by a protein encoded by the *MGMT* gene that transfers the methyl group from DNA by covalently coupling it to an internal cysteine residue of the MGMT protein (Yi and He, 2013). Therefore, the protein can only do it once, so the removal of each methyl group requires another molecule of protein.

1.5.4.1.b Base excision repair (BER)

Bases with small chemical alterations that do not strongly disturb the DNA double-helix structure are substrates for BER. BER is responsible for repairing most endogenous lesions like oxidized bases and apurinic/apyrimidinic sites (AP) (known as abasic sites), as well as DNA single-strand breaks by eliminating several dozen damaged (oxidized or alkylated) or inappropriate bases that are generated endogenously or induced by genotoxicants, predominantly, ROS (Figure 1.18). BER involves 4-5 steps: i) starting with base excision by a DNA glycosylase forming an AP site; ii) followed by a common pathway usually involving an AP-endonuclease (APE) to generate 3'OH terminus at the damage site; iii) followed by repair synthesis with a DNA polymerase; iv) nick sealing by a DNA ligase. This pathway is also responsible for repairing DNA single-strand breaks with blocked termini directly generated by ROS.

Abasic sites and single-strand breaks are repaired by one of the two pathways:

- The "short-patch "or" single nucleotide" pathway is set up to fix a modified base or an abasic site, caused by PAH adduct for example. The modified base is excised by a DNA glycosylase (OGG1, NTH1, NEIL1 or NEIL2) generating 3'PUA by β -elimination of deoxyribose phosphate and forming an abasic site supported by the endonuclease APE1 (Apyriminic/apurinic endonuclease 1) eliminating deoxyribose, allowing the DNA polymerase β to fill the sequence, and then the complex ligase III/XRCC1 restore the connections (Sedelnikova *et al.*, 2010).

- The "long-patch" pathway allows the reparation of single-strand breaks. It involves initially PARP1 which is rapidly activated by SSBs and causes auto-poly-ADP-ribosylation, then the DNA polymerase β , δ and ϵ together with the PCNA protein for the synthesis of a new sequence of 2 to 12 nucleotides. Former nucleotides are moved progressively to the synthesis, and then excised by FEN1 protein. Finally, the ligase I allows the reformation of the bonds.

Some studies showed that 8-oxoG is a poor substrate of NEILs on duplex DNA substrate, others have shown NEIL1's robust 8-oxoG excision activity (Morland *et al.*, 2002). NEILs have $\beta\delta$ lyase activity and thus generate 3' phosphate, a poor substrate of APE1. However, mammals, unlike *E. coli*, express high level of polynucleotide kinase (PNK) with dual 5'kinase/3'phosphatase activities.

PNK has already been implicated in repair of ROS-induced single-strand breaks (Meijer *et al.*, 2002). OGG1 binds to its repair product 8-oxoG and activates canonical Ras family GTPases, causing gene activation via MAPK signaling (Boldogh *et al.*, 2012). The choice of LP-BER vs. SN-BER may thus depend on, among other factors, the state of the 5'-deoxyribose phosphate terminal (5'-dRP) moiety. With unaltered aldehyde group in deoxyribose, Pol β could carry out SN-BER by excising the 5'-dRP. On the other hand, LP-BER is necessary when the AP sites are further oxidized by ROS and after cleavage of the DNA strand by APE, the 5' blocking groups could not be removed by Pol β 's 5' end cleaning lyase activity. The common blocks at ROS induced strand break are 3'phosphoglycolate and 3'phosphoglycolaldehyde, which are processed by APE1 or 3'phosphate which is removed by PNK. However, the 'end cleaning' is perhaps the most diverse enzymatic step in strand break repair, due to the variety of termini generated.

X-ray repair cross-complementing group 1 (XRCC1) and PARP1 serve as single-strand break sensor proteins (recruiting and activating BER proteins). XRCC1 interacts with NEILs, APE1, PNK (Vidal *et al.*, 2001). Its interaction with Pol β and Lig III α suggests that XRCC1 is involved primarily in SN-BER. Genotoxic agents that generate SSB in the genome directly or indirectly activate PARP1 and PARP-2 leading to depletion of NAD and indirectly of ATP and induce necrosis. PARP1 is recruited very early to the site of DNA strand-break and the activated enzyme may help recruit other repair proteins involved in BER. Studies showed the direct relation between PARP inhibitor and BER deficiency (Horton *et al.*, 2014).

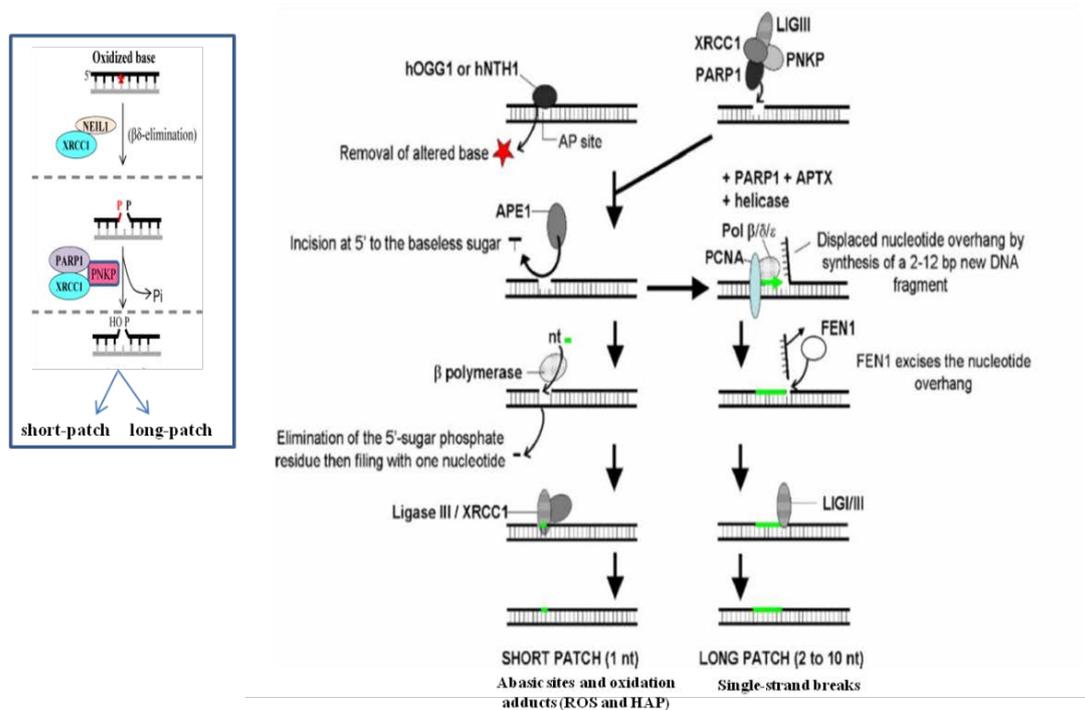


Figure 1.18: A Schematic illustration of BER pathways for damaged bases and DNA strand breaks (Hegde *et al.*, 2012; Sedelnikova *et al.*, 2010)

1.5.4.1.c Nucleotide excision repair (NER)

NER removes a broad spectrum of single-strand lesions that cause local helix-destabilization. NER is a complex multi-step process, involving the concerted action of at least 25 different polypeptides. In higher eukaryotic cells, NER excises 24-32-nt DNA fragments containing a damaged link with extreme accuracy. Two different modes of damage detection are operational in NER: global genome NER (GG-NER) detects and eliminates bulky damages in the entire genome including the untranscribed regions and silent chromatin, while transcription-coupled nucleotide excision repair (TC-NER) operates when damage to a transcribed DNA strand limits transcription activity and efficiently removes transcription-blocking lesions such as bulky adducts allowing thus quick resumption of transcription, thereby preventing cell death (Petruševa *et al.*, 2014). Consequently, defects in GG-NER result in cancer predisposition, whereas defects in TC-NER cause a variety of diseases ranging from ultraviolet radiation-sensitive syndrome to severe premature ageing conditions such as Cockayne syndrome (Marteijn *et al.*, 2014) (Figure 1.19). The steps of the NER are: i) damage is recognized by one or more protein factors; ii) DNA is unwound producing a “bubble” by Transcription Factor IIIH (TFIIH, functions in normal transcription); iii) cuts are made on both the 3' side and the 5' side of the damaged area so the tract containing the damage can be removed; iv) fresh burst of DNA synthesis (using the intact (opposite) strand as a template) fills in the correct nucleotides; v) DNA ligase covalently inserts the fresh piece into the backbone.

In GG-NER subpathway, the damage sensor XPC (Xeroderma pigmentosum, complementation group C), in complex with RAD23 homologue B (RAD23B) and centrin 2 (CETN2), constantly probes the DNA for helix-distorting lesions, which are recognized with the help of the ultraviolet radiation–DNA damage-binding protein (UV–DDB) complex (DDB1 and DDB2/XPE). Upon binding of the XPC complex to the damage, RAD23B dissociates from the complex. In TC-NER subpathway, damage is indirectly recognized during transcript elongation by the stalling of RNA polymerase II (RNA Pol II) at a lesion (the enzyme responsible for synthesizing messenger RNA). During transcript elongation UV-stimulated scaffold protein A (UVSSA), ubiquitin-specific-processing protease 7 (USP7) and Cockayne syndrome protein, complementation group B (CSB) transiently interact with RNA Pol II. Upon stalling at a lesion, the affinity of CSB for RNA Pol II increases and the Cockayne syndrome WD repeat protein CSA–CSB complex is formed, which probably results in reverse translocation (backtracking) of RNA Pol II that renders the DNA lesion accessible for repair. RNA Pol II and the nascent mRNA transcript are not depicted further.

TC-NER and GG-NER pathways are similar after this step in the recruitment of TFIIH complex. The bi-directional helicase of TFIIH opens the damaged DNA segment over a stretch of approximately 30 nucleotides (Sugasawa *et al.*, 2009). Upon binding of TFIIH, the CDK-activating kinase (CAK) subcomplex dissociates from the core TFIIH complex. TFIIH complex contains helicases (XPB and XPD) which open the double helix DNA. The unwound DNA is stabilized by

XPA, that binds the damaged site and helps assemble the other proteins needed for NER, and RPA (Replication Protein A) that also orient the two structure-specific endonucleases XPG (3') and the ERCC1-XPF (5') complex, which respectively incise the damaged strand 30 and 50 with respect to the lesion (Postel-Vinay *et al.*, 2012; Rass *et al.*, 2012; Sollier *et al.*, 2014). The resulting 25-30 nucleotide single strand gap is filled in by normal DNA replication proteins, including Replication factor C (RFC), trimeric Proliferating cell nuclear antigen (PCNA), RPA, and the DNA polymerases δ , κ and ϵ (Petrușeva *et al.*, 2014). Finally, the gap is sealed by DNA ligases I or III. Some mutations in XPB and XPD also produce signs of premature aging.

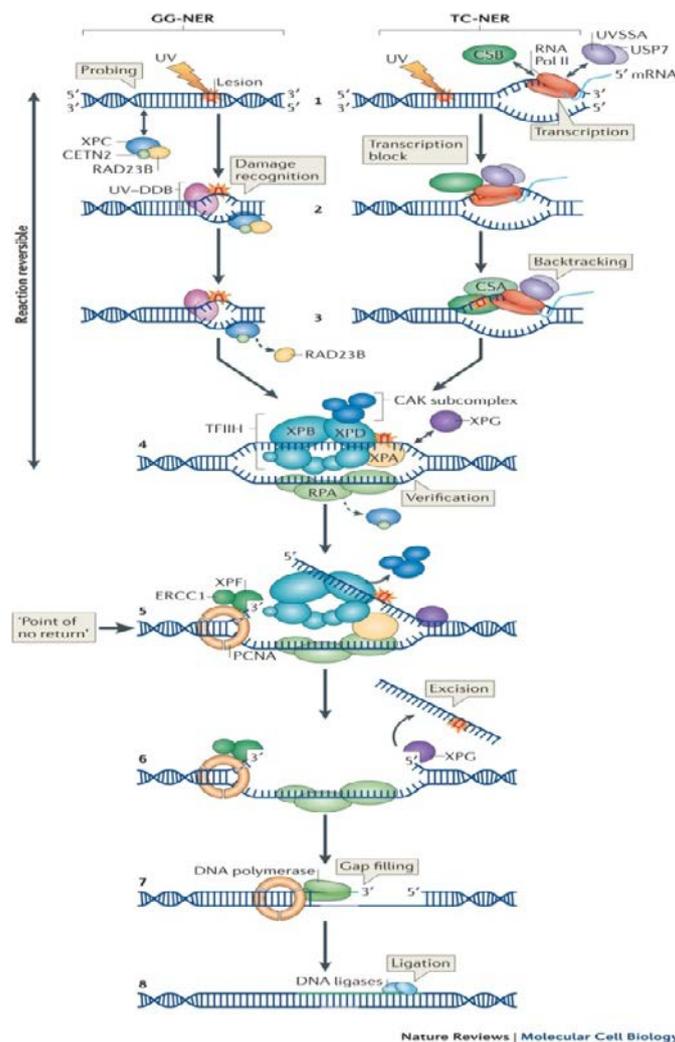


Figure 1.19: Nucleotide excision repair (NER) (Marteiijn *et al.*, 2014)

1.5.4.1.d DNA double-strand break repair (DSBR) and γ H2AX

Lesions that are substrates for NER and BER are located in one of the strands of DNA and are removed in a “cut-and-patch” mechanism. In both cases, the undamaged complementary strand serves as a faithful template for the repair of the damaged strand. Some damaging agents, however, affect both strands that induces DNA double-strand breaks (DSBs) and agents that produce inter-strand

cross-links (ISCLs). The factors leading to the formation of DSB include endogenous factors such as ROS production, and exogenous factors such as ionizing radiation (Tanaka *et al.*, 2007). These lesions are extremely cytotoxic because they are more difficult to repair as the cell cannot rely on simply copying the information from the undamaged strand. DSBs induce phosphorylation of the serine at position 139 of the histone variant H2A (H2AX). DSBs can be caused by a variety of factors: direct interaction with an agent, ROS, metabolic processes, deficient repair, telomere erosion... (Bonner *et al.*, 2008). DNA topoisomerases are enzymes that regulate DNA over- and underwinding and remove knots and tangles from the genetic material by creating transient breaks in DNA double-helix (Nitiss, 2009; Wang, 2002).

In eukaryotic cells, the efficient repair of DSB is essential for survival and two major pathways deal with these lesions, homologous recombination repair (HRR), and non homologous end-joining (NHEJ), according to the phase of the cell cycle. HR requires a homologous sister chromatid, so it acts exclusively in S and G2 phases (Figure 1.20A). HR is required for the restart of blocked or collapsed replication forks, as well as during the repair of ISCLs (Petermann *et al.*, 2010). Although there is evidence that HR events may be initiated by a SSB (Metzger *et al.*, 2011). HR is initiated by binding of the MRE-Rad50-Nbs1 (MRN) complex to a DSB and functions to hold the broken pieces together and provides the structural bases for the CtIP nuclease (C-terminal binding protein 1 interacting protein). Two of the proteins used in HR are encoded by the genes *BRCA1* and *BRCA2*. The MRN-CtIP complex catalyzes end resection at the break in concert with Exonuclease I (EXO1) (Leslie, 2013). Subsequently, RPA binds to the newly created single strand region and through a complicated handoff mechanism; the RPA-filament is exchanged into a RAD51 nucleo-protein filament. This RAD51-filament is crucial for strand invasion into the homologous sister, creating a temporarily triplex-DNA structure in which strand exchange occurs (Karpenshif and Bernstein, 2012; Rass *et al.*, 2012). DNA polymerases δ and ϵ , and DNA ligase I are involved in the synthesis and the DNA rebinding (Postel-Vinay *et al.*, 2012).

Post-mitotic cells and cycling cells in G1 phase have to seal DSBs by NHEJ (Figure 1.20B). DSBs are quickly recognized by the Ku70/Ku80 heterodimer that activates the PI3-kinase DNA-PK and sets the scene for subsequent recruitment of the Artemis nuclease and the MRE11/Rad50/NBS1 (MRN) protein complex (Rojowska *et al.*, 2014). These proteins are involved in DNA end-processing, preceding ligation performed by the XRCC4/Ligase IV complex (Rass *et al.*, 2012). During DNA end-processing, loss or changes of a few nucleotides may occur. For this reason, NHEJ, although it very rapidly seals DSBs, is an errorprone repair process (Chapman *et al.*, 2012).

New studies showed that in absence of NHEJ and HRR, several enzymatic activities have been implicated in repair, which is named Backup NHEJ (B-NHEJ) and considered to be distinct from the other NHEJ (DNA-PKcs dependent NHEJ, D-NHEJ) (Lee-Theilen *et al.*, 2011; Mladenov and Iliakis, 2011) (Figure 1.20). A major protein implicated in B-NHEJ is PARP1, which plays a main role in the repair of SSBs and which may effectively compete with KU heterodimer for DNA end-

binding (Robert *et al.*, 2009; Wang *et al.*, 2006). Indeed, MRE11 and CtIP, both involved in DNA end-resection during HRR, were found to facilitate B-NHEJ (Lee-Theilen *et al.*, 2011; Mansour *et al.*, 2010). Backup NHEJ, like D-NHEJ, is active in all phases of the cell-cycle, but its activity is significantly potentiated during S and G2, probably due to the increased activity of DNA end-resection enzymes in these cell-cycle phases. A ligation activity finalizing B-NHEJ is DNA Ligase 3 which in complex with XRCC1 also participates in the repair of SSBs and DNA base damages and Ligase 1 (Della-Maria *et al.*, 2011; Paul *et al.*, 2013). B-NHEJ is much slower than D-NHEJ and is highly error-prone which will cause translocations and other genomic rearrangements with high probability. A high number of B-NHEJ associated genetic rearrangements were observed in chromosomal translocations associated with both spontaneous and therapy-related cancers. Thus, B-NHEJ-derived mutations appear to be associated with cancer development and may support tumor especially when classical NHEJ or HRR are compromised (Bennardo *et al.*, 2008). Therefore, the activation of B-NHEJ fuels the evolution of cancer and it might serve as target in specialized cancer therapies (Mladenov *et al.*, 2013). Figure 1.21 shows the causes and repair of double-strand DNA breaks.

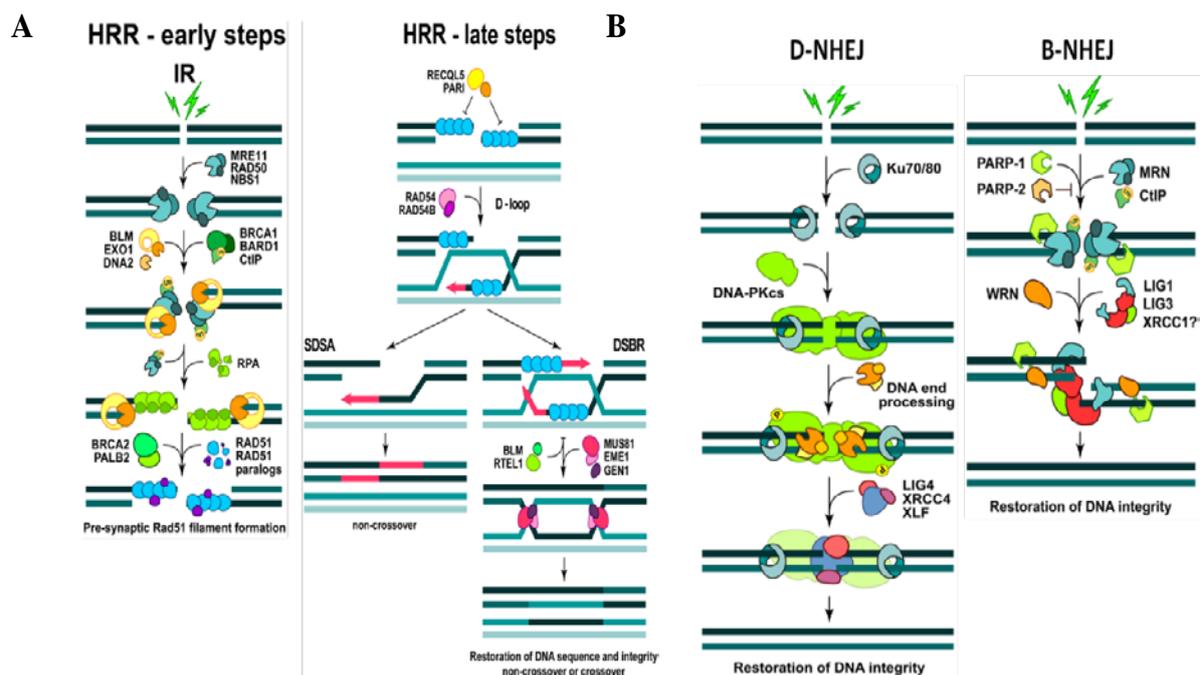


Figure 1.20: Schematic representations of DSB repair by homologous recombination repair (A) and non-homologous end-joining (B) (Mladenov *et al.*, 2013)

D-NHEJ: DNA-PKcs dependent NHEJ; B-NHEJ: Backup NHEJ, IR: ionizing radiation.

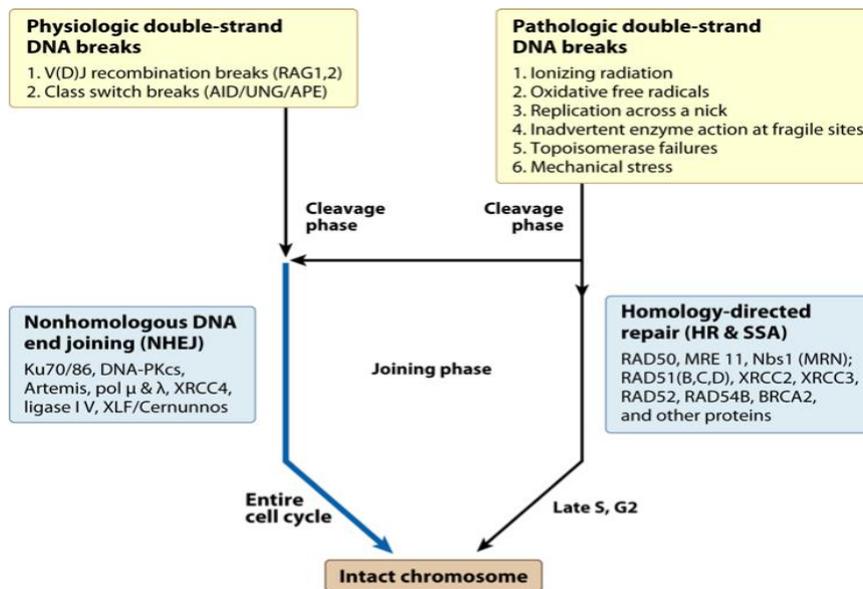


Figure 1.21: Causes and repair of DNA double-strand breaks (Lieber, 2010)

The DNA damage response (DDR) represents a signal transduction pathway that senses DNA damage and replication stress and regulates physiological processes that involve critical cellular decisions whether to undergo DNA repair, enter terminal differentiation through senescence, or if the damage is more severe, undergo apoptosis. It is primarily mediated by proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), and DNA dependent protein kinase (DNA-PK) and by members of the PARP family (Turinetto and Giachino, 2015) (Figure 1.22). ATM and DNA-PK are activated by DNA-damaging agents such as irradiation that create DSBs. ATR in complex with its partner protein ATRIP is activated following recruitment to RPA-coated single-stranded (SS) DNA regions that are generated primarily in S-phase at stalled DNA replication forks (Elledge, 2015). PARP1 and PARP2 are activated by SSBs and DSBs and catalyze the addition of poly (ADP-ribose) chains on proteins that recruit DDR factors to chromatin at DNA breaks. PARP1 is thought to mediate the initial accumulation of the MRE-Rad50-Nbs1 (MRN) complex at DSBs. Recruitment of ATM by MRN and PARP1 could then contribute to the activation of the γ H2AX complexes and stabilization of DDR factors at sites of DNA damage (Figure 1.23).

The phosphoepitope of γ H2AX directly binds Mediator of DNA Damage Checkpoint protein 1 (MDC1) on its BRCT site, which further enhances MRN-ATM binding and contributes to a positive feedback loop that spreads γ H2AX over mega-base domains around the DSBs. These initial signaling events activate one of the two DNA DSB repair pathways (Chowdhury *et al.*, 2005). γ H2AX dephosphorylation by protein phosphatase 2A facilitates DSB repair. While ATM is the primary kinase that forms γ H2AX at DSBs, ATR and DNA-PKcs, which are recruited to chromatin by ATRIP and Ku70/80 respectively, are also involved (Sharma *et al.*, 2012). Apart from H2AX, the target substrates phosphorylated by ATM are BRCA1, 53BP1, and MDC1 as well as checkpoint proteins,

Chk1 and Chk2. These processes are aimed to stop the progression of the cell cycle and to activate proteins responsible for DNA repair (Podhorecka *et al.*, 2010).

Histone H2AX phosphorylation in response to DNA damage on serine 139 within its SQEY motif at the site of DSBs allows the DNA damage signal to spread along the chromatin covering large regions of the chromosome surrounding each DSB lesion, thus providing a platform for recruitment of other proteins that participate in DDR (Kinner *et al.*, 2008; Mariotti *et al.*, 2013). Formation of foci of different nuclear proteins is a widely used method for assessing formation of DSBs (formed within seconds after induction) and their repair; among these are 53BP1, Nbs1, Rad51, and BRCA1, with γ H2AX being the most frequently used in the cell because technically the other proteins are more challenging to detect because of their limiting sensitivity or more restricted occurrence (Polo and Jackson, 2011; Tsamou *et al.*, 2012).

The normal function of PARP1 is the routine repair of DNA damage by adding poly (ADP ribose) polymers in response to a variety of cellular stresses. Recently, it has become widely appreciated that PARP1 also participates in diverse physiological and pathological functions from cell survival to several forms of cell death and has been implicated in gene transcription, immune responses, inflammation, learning, memory, synaptic functions, angiogenesis and aging. PARP1 is also a preferred substrate for several 'suicidal' proteases (caspases, calpains, cathepsins, granzymes and matrix metalloproteinases) and the proteolytic action of suicidal proteases on PARP1 produces several specific proteolytic cleavage fragments with different molecular weights. These PARP1 signature fragments are recognized biomarkers for specific patterns of protease activity in unique cell death programs. Endogenous and low levels of exogenous DNA lesions are usually repaired efficiently by BER (which involves PARP1 activity). PARP1 is needed to repair ROS-induced DNA lesions, PARP expression may lead to increased inflammation via upregulation of NF κ B signaling, and to more DNA damage, including the formation of oxidative clustered DNA lesions (OCDLs). The formation of OCDLs has been shown to be elevated in numerous tumor types. PARP1 activity could potentially be beneficial or harmful in the repair of ROS-induced DNA lesions (Swindall *et al.*, 2013) (Figure 1.24). PARP1 plays a role in several nuclear processes including chromatin modification, transcriptional regulation, and DNA damage repair (Hassa and Hottiger, 2008). Kraus *et al.* showed that PARP1 binds to DNA not only at sites of damage (SSB and DSB), but also DNA crossovers, supercoils and cruciform (Kraus and Lis, 2003). Several proteins involved in the BER/SSB repair process, that serve to correct ROS damage or subjection to methylating reagents and irradiation, have been identified to interact with PARP1 including XRCC1, DNA polymerase β , PCNA and others (Jackson and Bartek, 2009; Masson *et al.*, 1998; Smith *et al.*, 2000; Srivastava *et al.*, 1998). Interestingly, PARP1 is highly expressed in a variety of cancers like breast and hepatocellular carcinoma, and is correlated with poor prognosis in early breast cancers (Domagala *et al.*, 2011; Shimizu *et al.*, 2004). Many observations showed that components of the HRR pathway are aberrantly

expressed in many tumors (Mladenov *et al.*, 2013) and that genetic variations within DSB repair pathway could influence the risk of developing NSCLC (Zhao *et al.*, 2016).

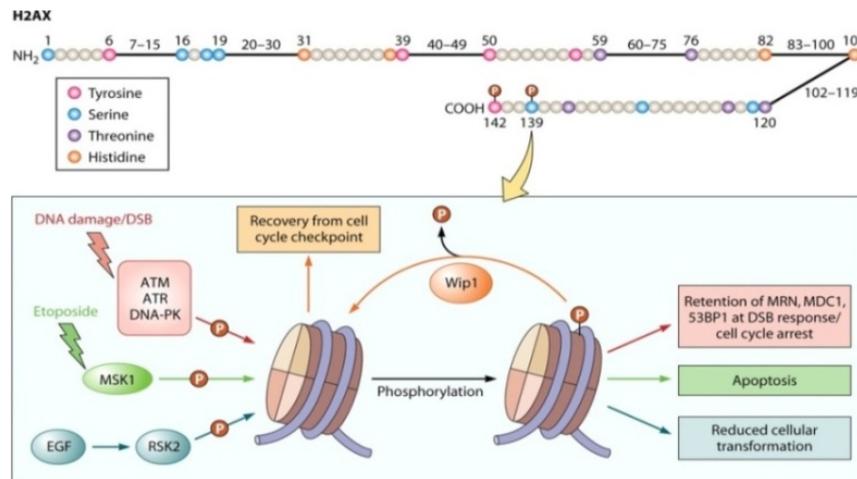


Figure 1.22: Histone H2AX phosphorylation (Banerjee and Chakravarti, 2011)

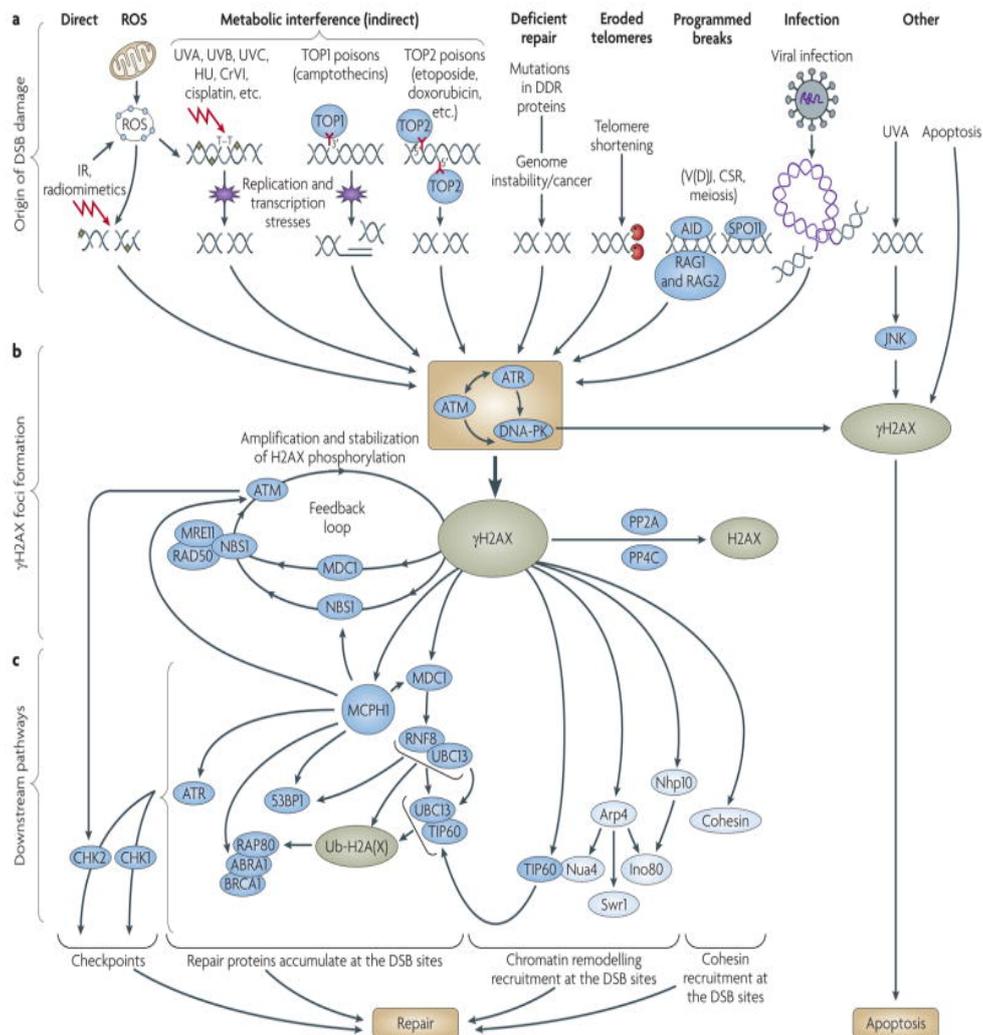


Figure 1.23: H2AX is a central component of numerous signalling pathways in response to DNA double-strand breaks (DSBs) (Bonner *et al.*, 2008)

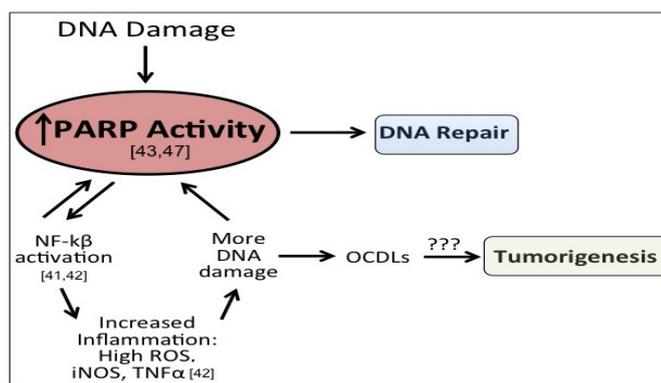


Figure 1.24: Potential role of elevated PARP1 in tumorigenesis (Swindall *et al.*, 2013)

iNOS: Inducible nitric oxide synthase, OCDLs: Oxidative clustered DNA lesions, PARP1: Poly-ADP-Ribose-Polymerase1.

1.5.4.2 Effect of PM_{2.5} on the DNA repair system

PM_{2.5} and its components such as PAHs, transition metals, and fatty acids can contribute to PM-related lung carcinogenesis. These components are able to activate inflammatory cells, alter the function of mitochondria or NADPH-oxidase, generate ROS and RNS, mediate oxidative stress and oxidative modifications in DNA including strand breaks and base oxidations (8-oxodG), lead to PAHs bulky DNA adducts, damage DNA, alter the methylation profile of the DNA, induce mutations and initiate carcinogenesis. Furthermore, PM_{2.5}, its components and oxidative stress perturb the repair of DNA lesions (Birnboim, 1986; Pero *et al.*, 1990). Lesions formed by ROS and damages caused by PAH adducts interfere with repair mechanisms or DNA replication, potentially leading to the formation of double-strand breaks (Sedelnikova *et al.*, 2010). Mehta *et al.* showed that the carcinogenicity of PM may act through its combined effect on suppression of DNA repair and enhancement of DNA replication errors (Mehta *et al.*, 2008).

1.5.4.2.a BER repair system

Several findings demonstrate that the change in expression and activity as well as genetic variability of APE1 caused by environmental chemical (heavy metals and cigarette smoke) and physical carcinogens (ultraviolet and ionizing radiation) is likely associated with various cancers. APE1 is used as a vital marker for the prediction of environmental carcinogenesis risk, it is involved not only as key element of base excision repair and as a redox factor for regulation of transcription factors, but also as an RNA modulator and transcriptional repressor (Park *et al.*, 2014; Wang *et al.*, 2004). Puglisi *et al.* suggested a potential role of Ape1/ref-1 sub-cellular localization as a prognostic indicator in patients with non-small cell lung carcinomas (Puglisi *et al.*, 2001). Traversi *et al.* showed a marked and significant increase in APE1 expression levels in exposed to PM_{2.5} (Traversi *et al.*, 2015). Studies demonstrated that an excess of iron or zinc in nervous tissue could disrupt the BER system by altering the activity of APE1, FEN1 and DNA polymerase β , and the ligation step (Li *et al.*, 2009). Altered expression of antioxidant systems leads to enhanced production of superoxide

production and lipid peroxidation, which can induce APE1/ref-1 in the tumor regions of NSCLS (Lin *et al.*, 2012; Yoo *et al.*, 2008). Different studies showed the effect of As, Pb and Cd on the expression of APE1 and therefore on the DNA repair system. It is known that As can affect both the endonuclease and the redox functions of APE1 to increase oxidative stress and inhibit DNA repair. In particular, APE1 activity is affected indirectly by As through the changes in transcription levels (Hamadeh *et al.*, 2002; Hu *et al.*, 2002). Cd inhibits the initial steps of BER, including the removal of AP site by APE1 endonuclease activity (Giaginis *et al.*, 2006). The induction of oxidative stress, the inhibition of DNA repair, and the formation of DNA/protein crosslink have been suggested as main toxic mechanisms of Pb exposure (Méndez-Gómez *et al.*, 2008). In addition, McNeill *et al.* have demonstrated a dose-dependent accumulation of AP sites as well as inhibition of APE1 activity in AP site incision under Pb exposure, implying an underlying mechanism by which Pb promotes carcinogenesis (McNeill *et al.*, 2007). *APE1* overexpression is often observed in tumor cells (Wang *et al.*, 2009; Zaky *et al.*, 2008).

Base excision repair deals with oxidative DNA lesions which are recognized by OGG1, NTH1 and NEIL1 DNA glycosylases. This is the primary DNA repair pathway that corrects base lesions that arise due to oxidative, alkylation, deamination, and depurination/depyrimidination damage, such as 8-OHdG (Cai *et al.*, 2012). Many studies showed the ROS-mediated activation of NEIL1, APE1, NEIL2 complex formation. ROS also enhances modification of OGG1, the major repair enzyme for 8-oxodG base (Aburatani *et al.*, 1997; Arai *et al.*, 2002; Hegde *et al.*, 2008; Hill *et al.*, 2001; Klungland *et al.*, 1999; Nishimura, 2002; Vidal *et al.*, 2001).

Overexpression of *OGG1* in pulmonary artery endothelial cells reduces xanthine oxidase-induced mitochondrial DNA damage and cell apoptosis (Ruchko *et al.*, 2005; 2011). $PM_{2.5}$ leads to mitochondrial dysfunction and the up-regulation of IL-1 β could be reversed by overexpression of *OGG1* in BEAS-2B cells (Yang *et al.*, 2015). Blind *et al.* showed that $PM_{2.5}$ decreases *OGG1* methylation that may lead to *OGG1* expression and to a decrease of 8-OHdG. Despite this repairing mechanism, air pollution is still associated with increased 8-OHdG, suggesting that the oxidative defense machinery may not be sufficient (Bind *et al.*, 2016). Studies confirmed the contribution of the *TP53* and *OGG1* genes to the risk for lung squamous cell carcinoma (Kohno *et al.*, 2011; Miller-Pinsler and Wells, 2014). A study done in China showed that *OGG1* polymorphism play an important role in the risk of lung cancer related to indoor exposure to smoky coal. Moreover, *OGG1* cause differences in DNA damage after short term exposures to particles and long term exposures, where *OGG1* may be upregulated (Lan *et al.*, 2004). Similarly, other defense genes, such as *HO-1* may be upregulated and modify effects. Increased expression of *OGG1* was associated with the oxidative DNA damage in lung epithelial cells during early-stage bronchopulmonary dysplasia (Jin *et al.*, 2015).

The MutT Homolog 1 (*MTH1*) gene or *NUDT1* encodes an 8-oxodGTPase that hydrolyzes 8-oxodGTP into 8-oxo dGMP and PP_i (Cai *et al.*, 2012). This gene is involved in large number of

tumors in MTH1-deficient mice (Sakumi *et al.*, 2003; Tsuzuki *et al.*, 2001). Choi *et al.* showed that after 24 hours of exposition, PM_{2.5} induced the overexpression of *MTH1* in rat lung epithelial cell (Choi *et al.*, 2004). Giribaldi *et al.* certified *MTH1* overexpression in BEAS-2B immortalized epithelial cells due to the presence of ROS (Giribaldi *et al.*, 2015). MTH1 not only enables evasion of oxidative DNA damage and its consequences, but can also function as a molecular rheostat for maintaining oncogene expression at optimal levels (Patel *et al.*, 2015).

NTH1 is required for the repair of both oxidative DNA damage and spontaneous mutagenic lesions (Hazra *et al.*, 2002). Dumax-Vorzet showed that NTH1 and OGG1 protect mammalian cells against PM-induced cell death while NEIL1 did not have any effect (Dumax-Vorzet, 2010). The expression pattern of this DNA repair protein for oxidative DNA damage showed a significant correlation with aggressive features of colorectal cancer such as the depth of tumor, and lymph node metastasis, as well as with Dukes' classification and disease-free survival (Koketsu *et al.*, 2004). Goto *et al.* showed the altered expression of the human base excision repair gene *NTH1* in gastric cancer (Goto *et al.*, 2009).

NEIL1 is one of the DNA repair genes most frequently hypermethylated in cancer causing NEIL1 messenger RNA and NEIL1 protein to be repressed (Bjelland and Seeberg, 2003). Data strongly suggest that decreased repair of oxidative DNA base lesions due to an impaired *NEIL2* expression in non-smokers exposed to sidestream smoke (SSS) would lead to accumulation of mutations in genomic DNA of lung cells over time, thus contributing to the onset of SSS induced lung cancer (Sarker *et al.*, 2014). *NEIL1* promoter is methylated in NSCLC at a high frequency (42%) (Do *et al.*, 2014).

1.5.4.2.b NER repair system

Mehta *et al.* demonstrated that in A549 cells exposed to PM_{2.5}, the NER repair capacity decreased proportionally to the dose of particles (Mehta *et al.*, 2008). The NER system can be disrupted by various metals and metalloid elements, such as cadmium II (Schwerdtle *et al.*, 2010), nickel II (Hu *et al.*, 2004b), chromium VI (Hu *et al.*, 2004a), antimony (Grosskopf *et al.*, 2010), or arsenic as monomethylarsonic acid (Shen *et al.*, 2008). It seems that the malfunction is related to the ability of metals to interact with zinc ions or magnesium content in certain proteins (Mateuca *et al.*, 2006). Recent studies showed that transition metals, such as nickel and chromium, and oxidative stress induced lipid peroxidation metabolites, such as MDA and 4-hydroxy-2-nonenal, can inhibit NER and enhance carcinogen-induced mutations (Feng *et al.*, 2006). The functionality of the P53 protein for the detection of DNA damage is affected, resulting in a decrease in the transcription of the XPC protein after exposure to cadmium, necessary for the proper functioning of the NER system (Schwerdtle *et al.*, 2010). The zinc finger domain of the XPA protein can be altered by certain metals and metalloids, disrupting its assembly and dissociation with the injured DNA (Grosskopf *et al.*, 2010). Hartwig *et al.* showed the inhibition of XPA and PARP by Cd(II), Cu(II), Co(II) and Ni(II)

(Hartwig *et al.*, 2002). Another study on A549 cells showed that cadmium and oxidative stress strongly disturb the assembly of XPA and XPC (Schwerdtle *et al.*, 2010). Other studies showed that *Xpc*^{-/-} mice, in contrary to *Xpa*^{-/-} and wild type mice, have an increased mutational load upon induction of oxidative stress (Melis *et al.*, 2013).

1.5.4.2.c Strand breaks repair and γ H2AX

XRCC1 was discovered to have four functional polymorphisms: T-77C, Arg194Trp, Arg280His and Arg399Gln, associated with alteration in repair capacity of DNA damage induced by PAH adducts (Ji *et al.*, 2010). In a Cd-exposed group, 3 DNA repair genes (*XRCC1*, *OGG1*, and *ERCC1*) significantly decreased in the rat liver, kidney, heart, and lung according to the β -actin internal standard ($P < 0.01$). Western blotting indicated the same trend for the different tissues (Lei *et al.*, 2015).

γ H2AX has also been observed in humans pre-cancerous lesions and in different tumors, including melanoma, colon carcinoma, osteosarcoma, fibrosarcoma and cervical cancer (Dickey *et al.*, 2009). Thus, in addition to serving as specific marker of genotoxicity, γ H2AX could be used for early detection of cancer because it is involved in the pathways of apoptosis and senescence (Gire *et al.*, 2004). Some studies showed the effect of PM on the induction of H2AX phosphorylation after the presence of DNA DSBs. Sánchez-Pérez *et al.* showed an increase level of γ H2AX following the formation of DSBs in A549 cells after the exposure to PM₁₀ and demonstrated the important role of ROS as mediator of PM₁₀-induced genotoxicity (Sánchez-Pérez *et al.*, 2009). Gualtieri *et al.* showed that PM_{2.5} induced DNA strands breaks and triggered a DNA-damage response characterized by increased phosphorylation of ATM, Chk2 and H2AX in BEAS-2B cells (Gualtieri *et al.*, 2011). Longhin *et al.* showed that DNA double-strand breaks, assessed by measuring the levels of γ H2AX, were increased in cells exposed for 3 hours to PM_{2.5} and organic extract (Longhin *et al.*, 2013). Lepers *et al.* showed high γ H2AX levels observed following cells exposure to PM_{2.5} suggesting that PAHs, or even their derivatives such as nitro-PAHs, could be involved in DNA DSBs (Lepers *et al.*, 2014). A study done by Borgie *et al.* certified that a significant increase in γ H2AX formation compared to non-exposed cells was observed after BEAS-2B cells exposure to urban PM_{2.5-0.3} due to the formation of DSBs that may be due to ROS formation and/or reactive electrophilic metabolites of PAHs formed via CYP-dependent reactions (Borgie, 2013). Sun *et al.* provided evidence that the genetic variants of H2AX gene may modulate the susceptibility to PM_{2.5}-modulated DNA damage (Sun *et al.*, 2015). Many studies showed the effects of the heavy metals (nickel, arsenic and chromium), B[a]P and PM on the post-translational histone modifications of different histones (Chervona *et al.*, 2012; Wang *et al.*, 2012; Zhou *et al.*, 2009). In *in vitro* studies, increased levels of γ H2AX was observed after exposure of cells to DNA-damaging compounds such as heavy metals (Bailey *et al.*, 2015; DeLoughery *et al.*, 2015), cigarette smoke (Ishida *et al.*, 2014), the dinitrobenzo[e]pyrene (Kawanishi *et al.*, 2009), PAHs and nitro-PAHs (Audebert *et al.*, 2012; Oya *et al.*, 2011).

Lebanon is one of the developing countries, where exposures to environmental pollution are a major source of health risk. Studies showed that the levels of air pollutants in Lebanon and Northern Lebanon have reached alarming levels. Furthermore, this region has been the subject of few studies on atmospheric aerosols. Therefore, the following part focuses on Lebanon and Northern Lebanon, where our study is conducted.

1.6 Local context: Lebanon

1.6.1 Geographic, demographic and meteorological Data

The Mediterranean region is an enclosed area where stagnant winds from Eastern Europe and intense sunshine contribute to trapping pollutants. In the East and Southern Mediterranean parts, where the emissions are mainly due to road transport and limited industrial activities, few data are available to characterize medium- to long-term environmental issues (Afif, 2009). Lebanon is a small and mountainous country in Western Asia located on the eastern edge of the Mediterranean Sea. Lebanon covers an area of 10452 km², making it approximately 88 km at its widest point and 32 km at its narrowest, with an average length of 220 km. The country is located between Latitudes 33° 03' 20" N and 34° 41' 35" N and Longitudes 35° 06' 15" E and 36° 37' 21" E. The Eastern Mediterranean is considered one of the most controversial for aerosol transport regions, due to its location at the intersection of circulating air masses between the three continents: Europe, Asia and Africa (Lelieveld *et al.*, 2002). It is a semi-enclosed area surrounded by the Mediterranean Sea and the deserts of Arabia and the Sahara and situated in the south of the densely populated and highly industrialized European continent (Figure 1.25).

Lebanon is bordered by Syria to the north and east sides, and occupied Palestine on the south side. It contains 210 km of coast line along the Mediterranean Sea. The physiographic regions consist of fertile coastal plains, the Lebanese western mountain range, the Bekaa valley (Lebanon's chief agricultural area) and the Lebanese eastern mountain range (Figure 1.26). Lebanon contains many rivers and streams, most of which having their origin in springs. The climate is generally Mediterranean with hot dry summers and cold rainy winters. Average annual rainfall is estimated at 840 mm, most precipitations fall between November and March. The highest mountain peaks are covered with snow for most of the year. The estimated population in 2009 was 3875000; the population density in Lebanon is considered high (about 400 persons/ km²); 80% of the Lebanese population live in urban areas. Beirut and Mount Lebanon have about 50% of the population. This explains the rapid degradation of the environment in these regions (Administration centrale de la Statistique, 2006).



Figure 1.25: Lebanon in the Eastern Mediterranean



Figure 1.26: Lebanon map (Asmar, 2011)

1.4.1.1 Topography

Lebanon has a complex geomorphology with a narrow coastal strip bordered by steep hills and mountains. It is characterized by the abrupt change in elevation within a distance of less than 20 km between sea level and 1750 m above mean sea level and by the presence of two mountain chains. The average altitude of the mountains is 400 m while the western mountain chain “Mount Lebanon” rises to 3088 m. Accordingly, Lebanon can be divide into four topographic components (Counsel for Development and Reconstruction, 2004; Walley, 2001). The level coastal plain, parallel to the sea, reaches a maximum of 9 km in the Akkar plain in the North, a minimum of 1 km near Nakoura in the South and nil in Chekka and Jounieh, where the mountain dips directly into the sea. The “Mount Lebanon” chain stretching from North to South with the Kornet Es-Saouda (3088 m) as the highest crest in the country is located near the Cedar area and covered by snow between November and September. The Bekaa Valley reaches 900 m in its western and central parts and less

than 600 m in the north-east. The “Anti-Lebanon” mountain chain in the east runs along the Syrian border which has a lower elevation than “Mount Lebanon”. In the south-east, the Hermon summits rise above the south Bekaa and the Syrian Golan and reaches an altitude of 2820 m.

1.6.1.2 Precipitation, temperature and wind

Lebanon’s climate conditions vary from a Mediterranean climate along the coastal plain and in the middle mountain range, to reach sub-alpine or mountain Mediterranean climates on the highest slopes, covered by snow during most of the year; it becomes sub-desertic and almost too dry for agriculture in some of the northern plains.

The amount of rain varies with topography and wind patterns. The precipitation in Lebanon is caused by the wet winds coming from the West or Southwest bringing large masses of humid air over the littoral. As the warm and humid air climbs the western mountain range, the relative humidity increases until it reaches saturation. At that point, rain caused by the relief occurs. On the other hand, dryer air caused by winds coming from the Caucasus contributes to slightly lower rainfall in the northern part of the littoral as compared to the central littoral. As in most countries with a Mediterranean climate, the majority of precipitation falls between November and March, in the form of heavy showers.

The spatial distribution of mean annual temperature depends on the presence of mountains. Over the course of a year, the temperature typically varies from 11°C to 31°C and is rarely below 8°C or above 31°C. As altitude increases, a decrease in temperature is observed. The temperature curves at 5°C and 10°C are located above 1800 m altitude except for a small area in the Bekaa plateau. A slight portion of the littoral benefits from the dampening effect of the sea and has a yearly average temperature above 20°C.

Wind speed and wind direction generally depend on the general barometric situation. In general, we can say that in Lebanon, maritime air is blown from the sea nine months a year from September to May. It is a humid wind and most precipitations in Lebanon are caused by the humid air mass movement caused by these winds. Due to its geographical location, Lebanon has two types of wind: the synoptic wind and the local wind. The synoptic wind is in winter, Lebanon receives continental air masses from Europe and Asia (the Caucasus and the Balkans). This air is usually dry and cold, and snow sometimes occurs when mixed with warmer and humid maritime air. In summer, air masses come only from the littoral, passing by Cyprus. They are warmer and slightly humid after their passage over the sea but are not sufficiently saturated to bring rain. Some hot and dry desert air is also blown from Africa through Egypt but in a much lower quantity. This wind, called the Khamsin, blows into Lebanon from North Africa during spring and occasionally during autumn (UNDP/GEF/MOPWT/DGU, 2005; United Nations Development Program, 2011). According to the weather station of the Beirut Rafic Hariri International Airport, the prevailing local wind observed in 2005 and 2014 was marked by a southwesterly direction as shown in Figure 1.27 (Chelala *et al.*,

2007). The average wind speed recorded was about 2.9 m/s. Seasonal wind shows a dominant southwest in summer and northwest and southwest winds in the winter wind. Due to the topography of the country, Lebanon experiences local breezes, sea breezes, land breezes and valley wind (Figure 1.28). The speed of these winds is less than 3 m/s.

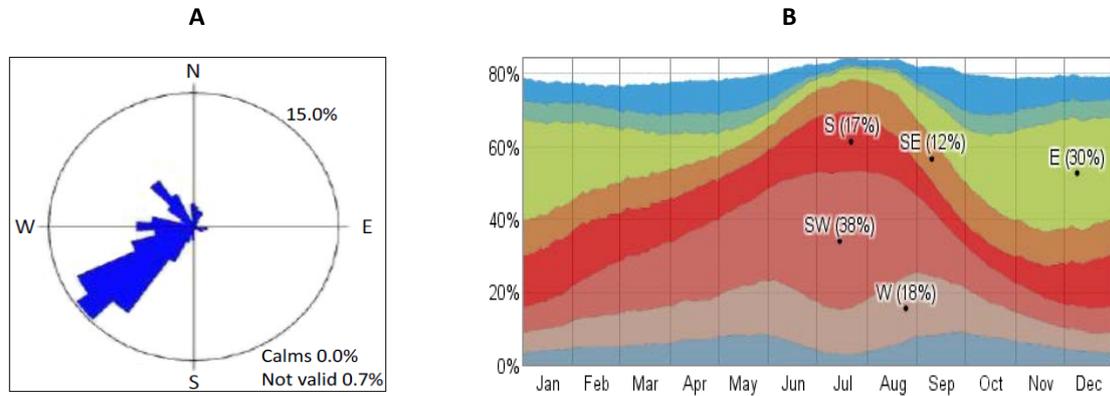


Figure 1.27: Annual average of the wind direction recorded by Rafik Hariri airport in 2005 (A) and 2014 (B) (Chelala *et al.*, 2007; WeatherSpark, 2014)

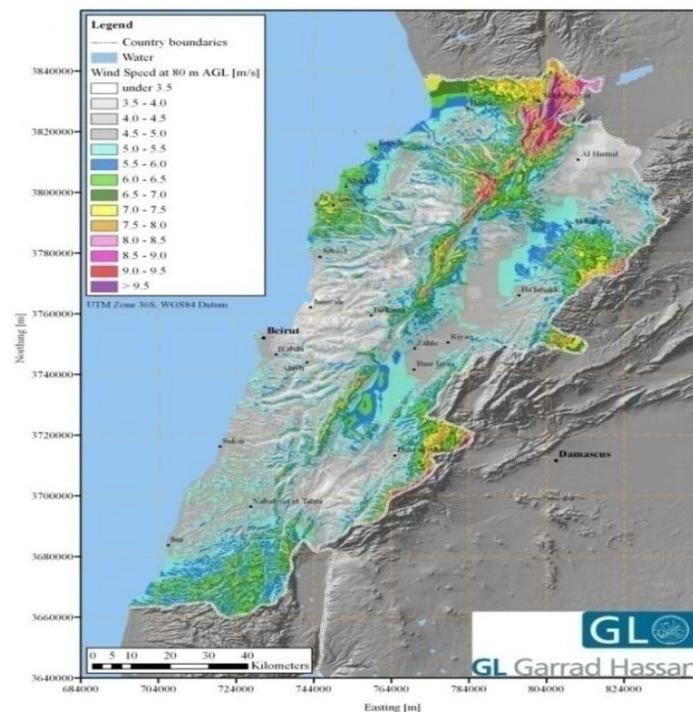


Figure 1.28: Wind map of Lebanon at 80 m above ground (United Nations Development Program, 2011)

1.6.2 The Cadastre of emissions in Lebanon

In Lebanon, air quality studies began from the 90s, and most of the research has been concentrated on the evaluation of air pollution due to the transport system in urban areas especially in Beirut (El-Fadel *et al.*, 2000; 2009; El-Fadel and Bou-Zeid, 1999, 2000; El-Fadel and Massoud, 2000; Sbayti *et al.*, 2002). Most of these studies have focused on the emissions of atmospheric particles, greenhouse gases (GHG), NO₂, SO₂ and O₃.

Many forces, acting together or in isolation, are impacting the air quality in Lebanon. These forces may affect ambient and/or indoor air, and may stem from natural phenomena or anthropogenic activities generated from several regional and local sources (Figure 1.29).

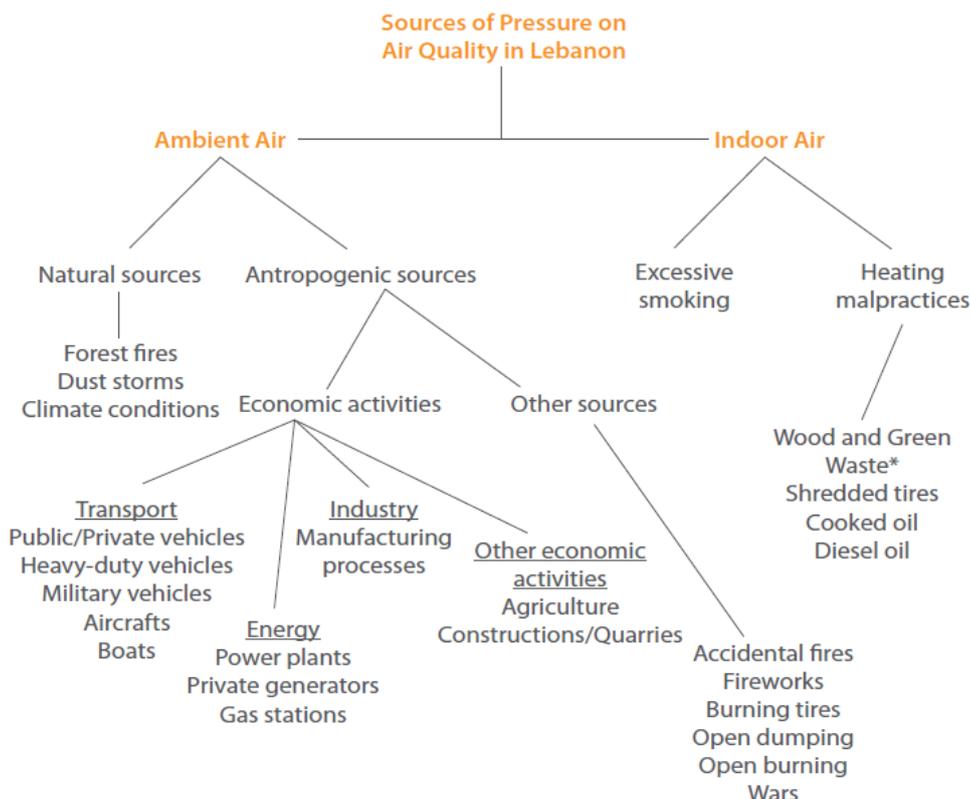


Figure 1.29: Driving forces affecting air quality in Lebanon (MoE/UNDP/ECODIT, 2011)

In Lebanon, the following natural phenomena may impact air quality:

- Seasonal dust storms, in the fall and spring mild seasons coming from the Arabian (South-East) and Saharan Deserts (South-West), constitute the major source of mineral elements. These hot air masses are loaded with high levels of PM₁₀ and PM_{2.5} rich in crustal elements (Borgie *et al.*, 2016; Kubilay *et al.*, 2000).
- Long-range transport of pollutants coming from central Europe enrich the air with SO₂ (Sciare *et al.*, 2003).
- Marine aerosols coming from the Mediterranean sea enrich the atmospheric particles in sea-salt especially in the coastal areas (Saliba *et al.*, 2007).
- Intense solar radiation in a hot and humid summer (June, July and August) accompanied by low wind speed enhance the photochemical reactions and thus the formation of secondary pollutants and other reactive species, and an increase in their lifetime in the atmosphere occurs in the absence of wet deposition and dispersion of the pollutants (Kouvarakis *et al.*, 2000; Kouyoumdjian and Saliba, 2006).

- Forest fires are caused by natural drought, accumulation of dead and highly flammable wood, or by arson (MoE, 2007). These fires produce smoke plumes charged with fine particles rich in carbon (VOCs, PAHs, PCDD, PCDF...) and potassium which are spread over vast areas. Recorded fires in the country are devastating around 0.4% of the forest cover per year (NNA, 2015).

- Temperature, humidity, atmospheric pressure, wind speed, wind direction and height of the mixing layer (height: 1 km) have an effect on the concentration of air pollutants in the atmosphere. Under normal conditions, temperature decreases with height but in case of temperature inversion (an increase in temperature with height) with low wind speeds, pollutants are trapped in the mixing layer and move horizontally since their dispersion is blocked vertically.

The most significant sources of pollution from economic activities are the transport, energy and industry sectors:

- The transport sector in Lebanon includes road transport, air transport (Beirut International Airport) and maritime transport (the main ports of Beirut, Tripoli, Saida and Sour). In terms of emissions, it accounted for nearly 21.4% of Lebanon's GHG emissions for the year 2000, and it is the main source of CO, NO_x and NMVOC emissions, with 94%, 59% and 66% respectively. This sector is considered as the main source of air pollution in the country and it is the main contributor to the poor quality of urban air (MoE/UNDP/ECODIT, 2011; MoE/UNEP/GEF, 2012). In the absence of an efficient public transport system covering the whole territory, the current land transport system mainly relies on private cars accounting for about 80% of Lebanese car fleet in 2007. The rate of car ownership is estimated to be 3 persons per car in 2002 and the trend is to increase in the next decade with an annual rate of 1.5%. The passenger cars fleet is old with an average age exceeding 13 years. 63% of the fleet is older than 20 years and 90% older than 10 years. SO₂ and lead emissions are directly correlated to the sulfur and lead content in fuels. Lead emissions in the country have gradually decreased since the introduction of unleaded gasoline in 1993 and the ban on leaded gasoline in 2001 (Law341/2001) (Hashisho and El-Fadel, 2001). However, the 2001 ban on diesel oil in vehicles (not to be confused with Diesel oil in Europe with physical and chemical properties described under EN590) and the 1995 emission standards on trucks, buses and motor vehicles (Decree 6603/1995) did not lead to significant emission reductions as trucks and buses continue to run on diesel oil without any inspections generating significantly more pollutants (PM, soot, NO_x and CO) than gasoline (MoE/UNDP/ECODIT, 2011). Vehicle emissions are also influenced by a number of factors including age, maintenance, speed, traffic, and road conditions. This sector highly affects the composition of PM in Lebanon especially in Beirut and Tripoli (Borgie *et al.*, 2016; Saliba *et al.*, 2010; TEDO, 2009).

- The thermal power plants are one of the largest contributors to air pollution in Lebanon emitting black plumes of HC, CO, CO₂, SO₂, NO_x, soot, PM, and other pollutants (MoE/EU/NEAP, 2005). The plants located in the coastal zones, Zouk, Jiyeh, Hrayche, Sour, Baalbeck, Beddawi and

Zahrani are the largest producers of CO₂, representing 39% of the national CO₂ emissions in 2005 (MoE/UNDP/ECODIT, 2011). The impact of energy industries on air quality is further aggravated by the sulfur content of burning High Emission Factor Fuel (HEFF) such as Heavy Fuel Oil (HFO); containing typically around 2.5% sulfur by weight. Thermal power plants accounted for 68% of national SO₂ emissions in 2005 (MoE/GEF/UNDP, 2010). Their stacks are not equipped with effective treatment units such as Dust Collection Units, and/or Flue Gas Desulfurization to reduce SO_x. Therefore, SO₂ is the major energy pollutant causing the formation of H₂SO₄ as well as sulfate particulates (NH₄)₂SO₄ and (NH₄)HSO₄. Non-Methane Volatile Organic Compounds (NMVOC) are also generated from thermal power plants during fuel storage, loading and unloading operations. Gas stations also affect air quality, they are a major source of NMVOC emissions during fuel loading and unloading. Because Lebanon's formal energy production currently does not meet demand, private backup generators produce an estimated 500MW, equivalent to 20% of the total production (Chelala *et al.*, 2007; MoEW, 2010b). These private diesel generators are found in industries and other establishments, and may be located on balconies, in basements, empty lots, and curbsides. They usually have short stacks, they are not properly maintained, generate significant noise (especially if not cased or equipped with noise mufflers) and release soot and PM inside cities and between buildings.

- Industries in Lebanon are spread all over the country and most of them have a restricted production and employ less than five workers. *Details in paragraph 1.6.3.*

- Other factors: Other economic sectors and other factors also affect air quality including: agriculture, construction, quarrying, open dumping and/or burning of municipal solid waste, burning of tires, fire and explosion accidents in poorly regulated warehouses, fireworks and wars (July 2006 war). The burning of 60,000 m³ of fuel oil at the Jiyeh Power Plant and 5,000 m³ of kerosene at the Beirut Rafiq Hariri International Airport generated large plumes of SO₂, NO₂, CO, soot, PM, VOC, dioxins, furans, and other compounds from the incomplete combustion of oil and oil products. Following site clearing and removal, disposal of demolition wastes and construction activities, Total Suspended Particles (TSP) were estimated to reach 860 µg/m³ under worst-case scenario and 190 µg/m³ under typical scenario in the ambient air of Beirut Southern Suburbs, exceeding the Lebanese (120 µg/m³), EPA (75 µg/m³) and WHO (150 µg/m³) 24-hr exposure standards for TSP in ambient air. Table 1.10 shows that other anthropogenic activities are able to generate a large variety of pollutants such as SO₂, NO_x, pesticides, PAH, metals, PCB, etc.

Indoor air quality can be affected by combustion sources (oil, gas, coal, and wood), tobacco smoking, building materials and furnishings, asbestos-containing insulation, pressed wood products; household cleaning and maintenance products, personal care, or hobbies; central heating and cooling systems and humidification devices; and outdoor sources such as radon, pesticides, and outdoor air pollution. In Lebanon, the main sources of indoor air pollution are smoking and heating malpractices (MoE/UNDP/ECODIT, 2011).

The spatial distribution of the emissions shows that emissions of CO, NO_x and NMVOCs are mainly in Beirut and its suburbs, Tripoli and Sidon, which are characterized by dense population, heavy traffic and small industries. Large power plants and industrial facilities in the regions of Zouk Mikhael, Jiyeh, Chekka and Selaata are the main contributors to the concentrations of SO₂, PM₁₀ and PM_{2.5} (Waked A. *et al.*, 2012). The inventory done by El-Fadel *et al.* indicated that the industrial sector is the second largest GHG emitter after the energy sector in Lebanon, and that cement plants, industry asphalt and iron are responsible for about 99% of emissions industrial (El-Fadel *et al.*, 2001). According to the Lebanese Ministry of Environment, CO₂ (emitted by energy sector especially power plants) is 94% of total GHG emissions in Lebanon for the years 2000 to 2006 and the remaining emissions consist mainly of CH₄ and N₂O (agriculture and forestry) (MoE, 2015). Table 1.11 indicates air quality indicators in the Great Beirut Area (GBA) and outside the GBA (MoE/UNDP/ECODIT, 2011). In 2009, Al Chaarani *et al.* showed that concentrations of heavy metals in nonwashed vegetables were slightly higher than levels in washed vegetables. Levels of Cr and As in non-washed cucumber and lettuce were considerably higher than in washed vegetables (Al-Chaarani *et al.*, 2009). Heavy metals in ambient air may deposit on the surface of vegetables by adsorption and eliminated by washing whereas heavy metals taken up by the roots from contaminated water or from the soil will enter plant tissue through absorption, are difficult to remove and therefore pose a major health concern.

Table 1.10: Summary of pollutants from other anthropogenic activities (MoE, 2001)

	Activity Generated air pollutants
Farming- Agriculture	Sprayed pesticides, NH ₃ , odors, GHGs (CH ₄ and CO ₂)
Open dumping	GHGs (CH ₄), bacteria, viruses
Open burning	Products from incomplete combustion: CO, NO _x , SO _x , HC, PM and other hazardous substances including dioxins and furans (POPs)
Burning tires	CO _x , SO _x , NO _x , NMVOCs, PAHs, dioxins, furans, HCl, benzene (C ₆ H ₆), PCBs; Metals: As, Cd, Ni, Zn, Hg, Cr, and V
Quarrying, construction, open-air storage sites	Large dust plumes comprising PM ₁₀ and PM _{2.5}
Fireworks	CO ₂ , K ₂ S and N ₂ – For every 270 grams of black powder (gunpowder, propellant) used, 132 grams of CO ₂ are created. Colors are generated by oxidized metals (e.g., the color red derives from strontium, blue from copper, gold from charcoal and iron).

Table 1.11: Summary of air quality indicators in Lebanon (MoE/UNDP/ECODIT, 2011)

<i>Pollutants</i>	<i>GBA</i>	<i>Source</i>	<i>Outside GBA</i>	<i>Source</i>
NO ₂	58µg/m ³	AQRU Conference 2011	6.4-10.11 ppm	MOE–ECODIT
SO ₂	3.1 ppb	Afif <i>et al.</i> 2008	0.45-0.7 ppm	2002
O ₃	-	-	115.5 µg/m ³	
PM ₁₀	63.38 µg/m ³	Saliba and co-researchers	81.4 µg/m ³	TEDO
PM _{2.5}	20.4 µg/m ³	Saliba and co-researchers	29.1µg/m ³	2009

GBA: Great Beirut Area

Monitoring stations:

GBA 6 automatic stations (PM and gaseous pollutants) and 66 passive sampling stations (NO₂ and SO₂)

Outside GBA 3 Impactors (PM and TSP) and 13 passive sampling stations (NO₂, SO₂ and O₃)

In 2013, the MoE under the Environmental Resources Monitoring in Lebanon (ERML) project with the support of the United Nations Environment Programme (UNEP) and United Nations Development Programme (UNDP) launched real time air quality monitoring in five sites in Lebanon of which two are also equipped with meteorological stations. These stations use online analysers connected to a supervisory control and Data Acquisition System (DAS) located at MoE. In 2016, the ministry of environment set a national strategy for air quality management in Lebanon. Besides addressing air quality issues, this strategy highlighted the need for forecasting wildfire danger in Lebanon as a means to prevent large forest fires and avoid or mitigate the associated health and environmental impacts while taking full advantage of technical resources related to Air Quality Management (meteorological stations) (MoE/UNDP/GEF, 2016). A third national communication (TNC) presented an improved methodology of national Greenhouse Gas (GHG) emissions for 2012 with a trend series for 1994-2012, and analysis of reduction potentials, climate change vulnerability, impacts and adaptation capacity (MoE/EU/GFA, 2016). This study showed that the main contributor to greenhouse gas emissions is the energy production sector with 53% of GHG emissions, followed by the transport sector (23%), waste sector (10.7%) and industrial processes (9.7%) (MoE/EU/GFA, 2016).

1.6.3 Industries in Lebanon

1.6.3.1 Industrialization and geographic distribution

The last comprehensive survey conducted by the Ministry of Industry (MOI) in 2000 showed that there were 22,026 industrial establishments in the country (MoE, 2001). According to CAS, in 1996-1997, there were 29,282 industrial establishments. The majority has a restricted output and employs less than five workers. Both surveys showed that half of these industries are located in Mount Lebanon and 18% in the North. The MoI statistics for 1998-1999 represent a 2% increase compared to 1994 and more than a 50% increase compared to the early 1990s (more than 11,600 new establishments were registered between 1990 and 2000) (MoE, 2001). Although there are 72 decreed industrial zones, almost 82% of all industrial establishments in Lebanon are located outside industrial

zones, in cities and villages. These industries are mostly located in urban areas where two thirds of Lebanon's population resides.

Class I industries are considered environmental high-risk facilities. They include tanneries, cement, paper (from pulp), fertilizer, ammunition production plants, tanneries, and gas products. There are about 973 Class I industries employing 4,650 people and generating an estimated 104 million US\$ in added value per year (Table 1.12). Industries that manufacture cement lime and plaster account for 68% of the total added value of Class I industries. There is no information concerning the geographic distribution of Class I industries (MoI/ ALI/ UNIDO, 2010). Class II and III industries are considered high to medium-risk facilities and potentially pose significant risks to human health and the environment, because they are numerous (several thousand), scattered, and located often in residential areas. Unlike Class I industries, Class II and III industries generally do not have the awareness and resources required to prevent or control pollution.

Table 1.12: Overview of class I industries based on decree 5243/2001 (MoE, 2001)

ISIC code and industrial branch	Number of Establishments	Number of permanent employees	Value added (US\$'000)
19 Leather and Leather Products	47	339	7,843
21 Pulp, Paper and Paper Products	73	732	7981
24 Chemical Products and Man Made Fibres			
- Production of gas products	10	132	8,528
- Manufacture of fertilizers and Nit. compounds	2	37	2,463
26 Other non-metallic mineral products			
- Manufacture of cement, lime and plaster	812	3,304	71,542
29 Machinery and Equipment			
- Manufacture of weapons and ammunition	29	107	5,462
Total	973	4,651	103,819

1.6.3.2 Industries emitted pollutants

Industries in Lebanon are blamed for many environmental ills. Industries generate industrial effluents, solid waste and potentially toxic air emissions. It should be noted that our study is located in the Northern Lebanon near cement and fertilizer industries and thereby more details will be given in *paragraph 1.6.3.2.d* about these industries and their emissions. Currently, most of the liquid, solid and gaseous emissions are discharged into the environment without any form of treatment. Figure 1.30 shows the location of domestic and industrial sewer outfalls, dumpsites and river mouths, which are discharged along the Lebanese coastline without any treatment.

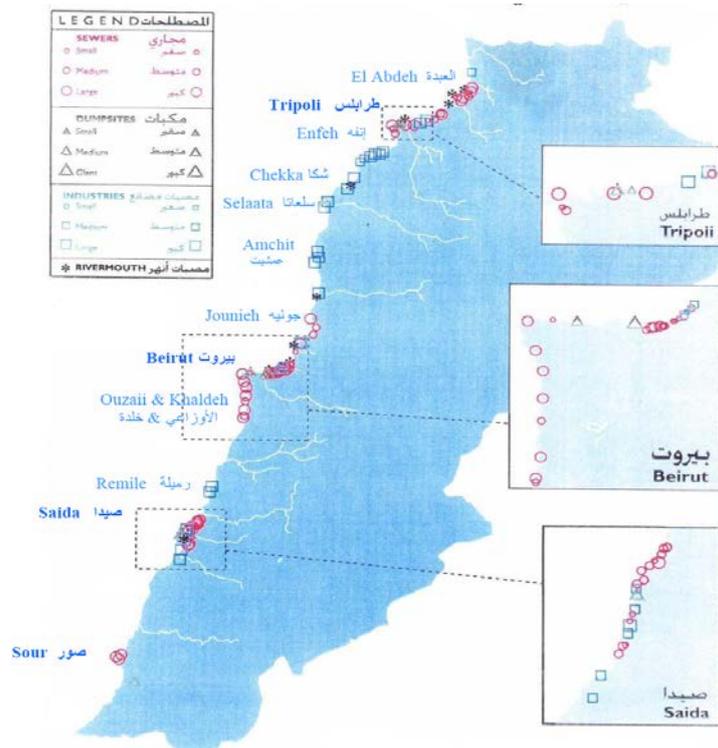


Figure 1.30: Location of domestic and industrial sewer outfalls, dumpsites and river mouths along the Lebanese coastline as identified in 2001 (UNEP/GEF/MOE, 2005)

1.6.3.2.a Industrial emissions impacting air quality

Industries generate combustion and process emissions. Combustion emissions are similar to those of the energy and transport sector and include HC, NMVOC, PM, Soot, CO_x, SO_x and NO_x, produced from burning oil and fuel to generate on-site electricity. However those of the manufacturing process are different depending on the process itself and the efficiency of industrial equipments, as well as the loading and unloading operations of raw materials before entering into process (MoE/UNDP/ECODIT, 2011).

Industries release primary air pollutants (particulate matter, lead, CO, SO_x, NO_x), some of which are greenhouse gases (CO, SO_x, NO_x), as well as other greenhouse gases (CO₂, methane). Air pollution loads for different gases vary from one industrial branch to another. No information is available on the contribution of different industry branches to air pollution loads for different gases. In terms of total air emissions, the potentially most polluting sectors are the cement industries, the fertilizer industry, the asphalt mixing plants and the thermal power plants. The First National Communication on Climate Change estimated greenhouse gases emissions from all sectors, including the industrial sector, for the year 1994 (MoE-UNDP, 1999a). That year, industries contributed 14% of total CO₂ emissions by all sectors, 4% of SO₂, and 76 % of non-methane volatile organic compounds (NMVOC). About 77% of CO₂ emissions from the industrial sector came from the cement industry alone, and 22% from the steel and iron industry. Carbon emissions from industries result mainly from

the combustion of fuel oil in furnaces and boilers (Table 1.13) and there is no quantitative data related to PM. Figure 1.31 shows the estimated air pollutant loads from industrial activities in Lebanon in 2011. PM₁₀ concentrations were higher in the North (Chekka and Selaata) and in Kesserwan (Zouk Mikhael and Mosbeh) than the others industrial places.

NMVOC emissions are mainly produced during road asphaltting; the production of sulfuric acid is the biggest source of SO₂; iron; steel mills are the major source of CO emissions; and the cement industry is the greatest producer of CO₂ (MoE/GEF/UNDP, 2010). Cement production accounts for 91.58% of total industrial processes GHG emissions (MoE, 2011). Table 1.14 presents the major sources of heavy metal air emissions (UNEP/GEF/MOE, 2005).

Table 1.13: Major greenhouse emissions from industrial sector from 1994 (in K Tonnes) (MoE, 2001)

Greenhouse gas sources	CO ₂	NO _x	CO	NMVOC	SO ₂
Thermal power plants	3,615.1	9.5	0.7	0.2	45.0
Manufacturing industries and construction:	4,698.2	7.7	1.1	274.1	28
- Energy combustion	2,774.1	7.7	1.1	0.3	24.7
- Industrial processes	1,924.1	0.01	Negligible	273.9	3.4
Total	8,313.2	17.1	1.8	274.4	73.1

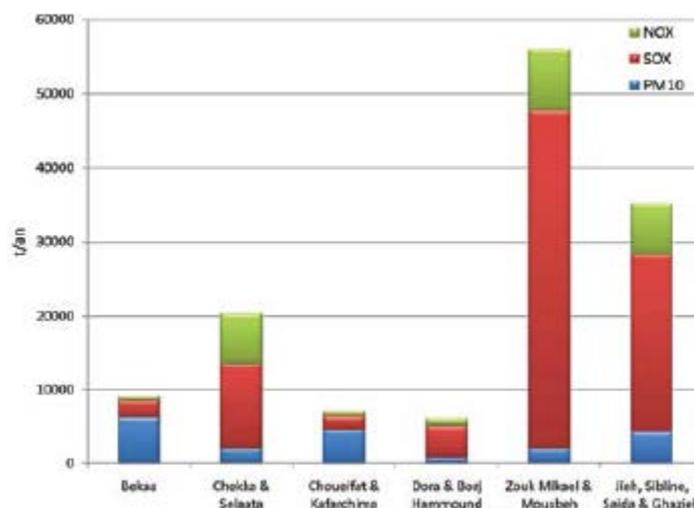


Figure 1.31: Estimated air pollutant loads from industrial activities in Lebanon (MoE/UNDP/ECODIT, 2011)

Table 1.14: Highest sources of heavy metals air emission in Lebanon (UNEP/GEF/MOE, 2005)

Pollutant	Total national annual release (Kg/year)	Main source industry	Main source industry % of total emissions
Cadmium	2,065	Fertilizer	95.5
Chromium	169.5	Power generation	94.4
Copper	336	Power generation	100
Lead	339,883	Lead smelting	99.7
Mercury	290	Cement	92.4
Nickel	16,000	Power generation	100

* Based on the Baseline Budget of pollutant releases

1.6.3.2.b Solid waste generated by industry

There are no comprehensive waste surveys or industrial production statistics nor dedicated landfills to dispose those wastes. The most recent effort to develop a management strategy for industrial and hazardous waste has estimated that Lebanon generates about 188,850 tonnes of industrial solid waste annually (MoE, 2001). This estimate includes hazardous waste (pesticides, heavy metals, waste oil, resins, paints and PCBs), non-hazardous waste, construction and demolition waste, and putrescible waste. To date, Lebanon has no industrial hazardous waste landfill or treatment facility.

1.6.3.2.c Industrial wastewaters

Industries generated about 61,000 m³ of wastewater per day in 1994, or about 12% of the total (domestic and industrial) wastewater stream generated in Lebanon (MoE, 2001). Mount Lebanon generates over two-thirds of the national industrial wastewater flow (about 44,000 m³/day) and has the highest proportion of industrial wastewaters in its overall wastewater stream (21%).

1.6.3.2.d Cement factories and phosphate fertilizer industries

- Cement industry:

This type of industry is flourishing in the Middle East and North African countries due to the rapid population growth, the change in living style, and the availability of raw material in the region (Abu-Allaban, 2011). Lebanon has five cement plants (Holcim Lebanon, Cimenterie Nationale SAL, Ciment de Sibline, Cimenterie du Moyen Orient, Société Libanaise des Ciments Blancs) of which four are located in North Lebanon. Raw materials include silica, aluminum, iron and lime which is obtained from calcium carbonate. Other raw materials are introduced as sand, clay, shale, iron ore and blast furnace slag. The cement industry includes many processes such as mining/quarrying, crushing, grinding, and calcining, all of which generate pollutants: particulates, CO, SO₂, NO_x, HC from extraction, crushing and grinding of raw materials, grinding and bagging of cement, and kiln operation and cooling.

The production process for cement consists of drying, grinding and mixing limestone and additives like bauxite and iron ore into a powder known as “raw meal”. The raw meal is then heated and burned in a pre-heater and kiln and then cooled in an air cooling system to form a semi-finished product, known as a clinker. Clinker (95%) is cooled by air and subsequently ground with gypsum (5%) to form the cement. Cement is produced from geological materials that contain CaO, SiO₂, Al₂O₃ and FeO in certain proportions to define the main properties of cement (Smadi *et al.*, 2009). The predominant constituent of the cement raw material mix is calcium carbonate in one form or another. Most often, the calcareous component is limestone but it can be marl, chalk, or marine deposits of shell or aragonite. Because 48% of the weight of the calcium carbonate is carbon and

oxygen, the calcareous component of the raw material mix is a significant source of CO₂ emissions through calcination (decarbonization). The non-calcareous components of the raw mix may be natural in origin, e.g., sand and shale, or be derived from the wastes of other industries, e.g., steel mill scale or power plants fly ash. These materials can contain sulfates, sulfides (metallic and organic), and elemental sulfur that have the potential to generate SO₂ which can be also generated from the combustion of sulfur-bearing compounds in coal, oil, and petroleum coke, and from the processing of pyrite in raw materials. Limestone also can contain petroleum and/or kerogens that can be partially volatilized or pyrolyzed at temperatures present at the feed end of the pyroprocesses to result in organic emissions. These organic constituents or their nonvolatile residues can result in CO emissions when burned in an oxygen-deficient section of the pyroprocessing system. Organic, CO, and CO₂ emissions also may result from kerogens, crude petroleum, or refined petroleum products in these components of the raw mix. The amount of CO₂ emitted by the cement industry is nearly 900 kg of CO₂ for every 1000 kg of cement produced (Mehraj and Bhat, 2013). The nitrogenous constituents in cement raw materials and combustion of fuels at high temperatures in cement kilns result in the release of NO_x emissions (Mousavi *et al.*, 2014). These nitrogenous constituents may also contribute to emissions of NH₃. These raw materials may contain chlorine in trace amounts that could contribute to the formation of HCl or the precursors of D/Fs. Combustible wastes of industry and consumers can be used in cement kilns to supplement traditional carbonaceous fuels. The four most common wastes burned in cement kilns are used or rejected automobile and truck tires, blended liquid and solid hazardous wastes, used oil, and combustible nonhazardous solid wastes. These wastes may contain sulfur, nitrogen, and/or chlorine that could contribute to the formation of SO₂, NO_x, NH₃, HCl, or D/Fs (Greer, 2003). On the influence of the cement industry, Al, Si, Ca, SO₄²⁻, K, Mg, Sr and can be considered as highly associated in atmospheric PM originated from cement factories (Santacatalina *et al.*, 2010). In addition, Cd, Pb, As, Cu, Sb, Ni, Cr, Co and Tl were found mostly enriched, related to solid fuel conversion processes and cement plant activities (Chaurasia and Karwariya, 2013; Shukla and Nagpure, 2008; Soussia *et al.*, 2015). In 2011, a report done by the ministry of environment in Lebanon showed that the cement industry is the most important industrial source of PM, CO₂, SO₂ and NO_x while road paving and food production are the main emitters of NMVOCs (Mishra and Siddiqui, 2014; MOE, 2011).

Studies have shown adverse respiratory health effects in the people exposed to cement dust, exemplified in increased frequency of respiratory problems (Al-Neaimi *et al.*, 2001), chronic impairment of lung function, gastro intestinal diseases (Adak *et al.*, 2007), skin irritation (Isikli *et al.*, 2003), eye disorders (Soussia *et al.*, 2014), cardiovascular or respiratory hospital admission in children (Bertoldi *et al.*, 2012), risk of liver abnormalities, and pulmonary carcinogenesis. Its deposition in the respiratory tract causes a basic reaction leading to increased pH values that irritates the exposed mucous membranes (Zelege *et al.*, 2010). Mehraj *et al.* showed that there was high level of air pollution in the area, adverse health impacts, over production of nitrogen species as well as ROS

in subjects residing around cement pollution affected area (Mehraj *et al.*, 2013). Decreased antioxidant capacity and increased plasma lipid per-oxidation have been posed as possible causal mechanisms of disease (Darweesh and El-Sayed, 2014).

- Phosphate fertilizer industries:

Phosphate fertilizers are produced by adding acid to ground or pulverized phosphate rock. If sulfuric acid is used, single or normal, phosphate (SSP) is produced, with a phosphorus content of 16-21% as phosphorous pentoxide (P_2O_5). If phosphoric acid is used to acidify the phosphate rock, triple phosphate is the result. TSP has a phosphorus content of 43-48% as P_2O_5 . Phosphate fertilizer complexes often have sulfuric and phosphoric acid production facilities. Sulfuric acid is produced by burning molten sulfur in air to produce sulfur dioxide, which is then catalytically converted to sulfur trioxide for absorption in oleum. Sulfur dioxide can also be produced by roasting pyrite ore. Phosphoric acid is manufactured by adding sulfuric acid to phosphate rock. The reaction mixture is filtered to remove phosphogypsum (main causes of radioactive pollution), which is discharged to settling ponds or waste heaps. As a matter of fact phosphorus, potassium and nitrogen are essential elements for plants growth. Phosphorus can be found in nature in a combined state in fluorapatite $Ca_5(PO_4)_3F$, hydroxylapatite $Ca_5(PO_4)_3(OH)$ and chlorapatite $Ca_5(PO_4)_3Cl$ ores, the main constituents of phosphate rocks. In phosphate rocks, activity concentrations of uranium two isotopes (^{238}U and ^{235}U) and of their decay products are extremely variable and in some case quite high (Righi *et al.*, 2005). Fluorides and dust are emitted to the air from the fertilizer plant. All aspects of phosphate rock processing and finished product handling generate dust, from grinders and pulverizers, pneumatic conveyors, and screens. The mixer/reactors and dens produce fumes that contain silicon tetrafluoride and hydrogen fluoride. A sulfuric acid plant has two principal air emissions: sulfur dioxide and acid mist. If pyrites ore is roasted, there will also be particulates in air emissions that may contain heavy metals such as cadmium, mercury, and lead. Sulfuric acid plants do not normally discharge liquid effluents except where appropriate water management measures are absent (Mishra *et al.*, 2010). Solid wastes from a sulfuric acid plant will normally be limited to spent vanadium catalyst. Where pyrite ore is roasted, there will be pyrite residue, which will require disposal. The residue may contain a wide range of heavy metals such as zinc, copper, lead, cadmium, mercury, and arsenic. The phosphoric acid plant generates dust and fumes, both of which contain hydrofluoric acid, silicon tetrafluoride, or both (World Bank Group, 1998). Fugitive dust emission, transport and deposition from phosphate fertilizer industries may pose an environmental hazard to the surrounding environment (Kassir *et al.*, 2012). Sabiha showed that continuous application of fertilizers may result in buildup of heavy metals, thus, posing a threat to the environment, plants and human life (Sabiha, 2012). According to Mishra *et al.*, the air emissions contain fluorides and SO_2 along with heavy metals (Mishra *et al.*, 2010). Several diseases are known to be caused by the excessive presence of the toxic elements and among them gastrointestinal, pulmonary and kidney ailments are most noteworthy (Dissanayake and Chandrajith, 2009).

1.6.4 Air quality in the Greater Beirut Area

There are different monitoring programs inside the greater Beirut area. Saliba *et al.* (2006) and Afif *et al.* (2008-2009) measured different air pollutants concentrations and showed that beside local sources, long-range transport can account for an important source of SO₂ in Beirut and that the main emission source of the NO_x in Lebanon is traffic. AQRU studies also reported that annual average NO₂ concentrations in GBA exceed the WHO standard (Air Quality Research Unit, 2011). Moussa *et al.* showed that local anthropogenic emissions mainly vehicle emissions were the predominant source of carbonyl compounds measured in AUB and Hamra (Moussa *et al.*, 2006).

Table 1.15 shows PM levels in Beirut City listed chronologically by sampling period. Shaka *et al.* and Saliba *et al.* conducted extensive PM sampling and measurement in and around Beirut (Saliba *et al.*, 2010; Saliba *et al.*, 2006; Saliba *et al.*, 2007; Shaka' and Saliba, 2004). Shaka *et al.* measured PM concentrations (all particle sizes) over a four month period (2003), PM concentration during February and March were low (rainfall), whereas in April, the concentrations were higher (dust-storms). In summary, PM₁₀ and PM_{2.5} annual levels in all sampling sites in Beirut City exceeded WHO guidelines for PM₁₀ (20 µg/m³) and PM_{2.5} (10 µg/m³). In Lebanon, PM₁₀ variations have different root causes. Near the sea, high PM₁₀ levels were correlated with sea breezes which carry sea salt particles but in crowded suburbs, the high concentrations were related to local emissions (dust outbreaks, low precipitation, dust re-suspension, etc.). The main sources of PM_{2.5} include combustion processes and photo-chemical reactions combining precursors including NO₂ and SO₂ under increased humidity and high solar radiation.

In 2004, ionic composition was tested; Shaka and Saliba observed the presence of SO₄²⁻, NO₃⁻, SiO₄²⁻, CO₃⁻ and NH₄⁺ in higher concentrations in PM_{2.5} than in PM_{10-2.5} collected at the campus of the American University of Beirut (AUB). Higher concentrations of organic species were identified in the PM_{2.5} whereas high water concentrations were observed in the PM_{10-2.5} (Shaka' and Saliba, 2004). According to Kouyoumdjian and Saliba, the ions NO₃⁻, SO₄²⁻, CO₃²⁻, Cl⁻, Ca²⁺ and Na⁺ are the main ionic components of PM_{10-2.5}, while sulfates NH₄⁺ (as (NH₄)₂SO₄), Ca²⁺ and Na⁺ are mainly dominant in PM_{2.5} (Kouyoumdjian and Saliba, 2006). In this study, it was suggested that in summer, an increase of the nitrate ions and sulfate was noticed due to enhancement of photochemical reactions thus facilitating the conversion of NO₂ and SO₂ gases into NO₃⁻ and SO₄²⁻ and to ammonium sulfates and nitrates. High concentrations of chloride were also attributed to the incineration of waste near the site and the high calcium levels are a good indication of the abundance of crustal calcium in the region. In 2007, elemental composition was studied, crustal elements including Ca, Si, K, Ti, Mn and Fe were more abundant in PM_{10-2.5} (primary aerosols), increasing in stormy episodes, while enriched elements including S, Cu, Zn and Pb predominated in PM_{2.5} (secondary aerosols). Highly enriched elements like Cu and Zn were emitted from worn tires and brakes. Chlorine usually originating from sea salt aerosols and abundant in PM_{10-2.5}, was found in higher concentrations in PM_{2.5} due to the waste mass burning contribution according to the authors (Saliba *et al.*, 2007).

Table 1.15: PM levels in Beirut city listed chronologically by sampling period (MoE/UNDP/ECODIT, 2011)

Sampling site	Site description	Sampling period	Mean value during sampling period		Source
			PM ₁₀	PM _{2.5}	
AUB	Location exposed to different sources of PM (natural and anthropogenic)	2/2003-6/2003 (4 months)	118.9 µg/m ³	39.9 µg/m ³	Shaka <i>et al.</i> , 2003
Bliss Street	Urban area and coastal site	3/2003-6/2003 (3 months)	71.34 µg/m ³	40.95 µg/m ³	Saliba <i>et al.</i> , 2010
AUB Seagate	Urban area and coastal site	11/2003-3/2004 (4 months)	86.9 µg/m ³	-	Saliba <i>et al.</i> , 2010
Abdel Aziz (Hamra)	Urban area and coastal site	9/2004-12/2004 (3 month)	55.1 µg/m ³	-	Saliba <i>et al.</i> , 2010
Borj Hammoud (Beirut suburb, north)	Urban area, close to Beirut Harbor and awaste facility	1/2004-1/2005 (12 months)	103.78 µg/m ³	38.525 µg/m ³	Saliba <i>et al.</i> , 2007
Haret Hreik (Beirut suburb, south)	Urban area affected by post-war reconstruction	12/2006-8/2007 (8 months)	77.1 µg/m ³	28.14 µg/m ³	Saliba <i>et al.</i> , 2010
American University			54.69 µg/m ³	20.18 µg/m ³	Saliba and coresearchers, publication in progress
Lycee Abdel Kader	Urban areas	May 2009 -May 2010	60.77 µg/m ³	20.70 µg/m ³	
Grand Lycee Franco Libanais			74.69 µg/m ³	20.33 µg/m ³	
Sein El Fil	Urban area	18/05/2011-02/09/2011 (without July) (3months)	-	40 µg/m ³	Borgie, 2013
WHO Standards (WHO, 2005)	Annual average concentrations		20 µg/m³	10 µg/m³	

In 2012, Salameh *et al.* presents the first detailed characterization of the organic composition of Beirut atmosphere in summertime and wintertime. The analysis of the daily observations shows the traffic emissions footprint. Background levels are enhanced in air masses from Turkey and Southern Europe (Salameh *et al.*, 2012). In 2012, Saliba and Chamseddine showed that during the episodes rich in dust levels, PM₁₀ and PM_{2.5} concentrations increased by 80% and 75% respectively and an increase in nitrates in PM₁₀ (36%) and PM_{2.5} (54%) was attributed to the reaction of dust particles (CaCO₃) with HONO and HNO₃ which is improved in urban areas (Saliba and Chamseddine, 2012). In 2012, Waked A. *et al.* presented an atmospheric emission inventory of anthropogenic and biogenic sources in Lebanon. The annual emissions obtained from the inventory for the year 2010 for PM₁₀ and PM_{2.5} were 12 and 9 Gg. The spatial allocation of emissions in this study showed that the city of Beirut and its suburbs encounter a large fraction of the emissions from the on-road transport sector while urban areas such as Zouk Mikael, Jieh, Chekka and Selaata are mostly affected by emissions originating from the industrial and energy production sectors (Waked A. *et al.*, 2012). Another study done by Waked A. and Afif (2012) showed road transport emissions originating in normal and touristic periods in Beirut and compared these emissions to other developed and non-developed cities on the

Mediterranean basin. In 2013, the study done by Daher *et al.* showed that $PM_{2.5-0.25}$ concentrations were $22.6 \mu\text{g}/\text{m}^3$ in Riad Al Solh (AUB University, Beirut) and $50 \mu\text{g}/\text{m}^3$ in Jal El Dib (Beirut). This study showed that $PM_{10-2.5}$ fraction is largely composed of crustal elements (12-23% of its mass) (Daher *et al.*, 2013). While for $PM_{2.5-0.25}$ and $PM_{0.25}$, OC, EC and organic compounds (46-56%) dominate at the site near the highway as a contribution of diesel vehicles with PAHs concentrations (up to $11.5 \text{ ng}/\text{m}^3$), secondary ions (54-68%) were more abundant in the background site (Daher *et al.*, 2013). In 2013, Waked *et al.* showed that levoglucosan was the major most abundant single component of fine organic aerosols collected at a semi-urban site in Beirut (28 January-12 February 2012) with an average value of $306 \text{ ng}/\text{m}^3$, followed by saturated and unsaturated carboxylic acids and sugars with average values of 234 and $118 \text{ ng}/\text{m}^3$, respectively (Waked *et al.*, 2013a). Later on, a Polyphemus/Polair3D modeling system was used to investigate air pollution episodes in Beirut during 2 to 18 July 2011 (Waked *et al.*, 2013b). Waked *et al.* continued their studies in 2014 and they conducted a measurement campaign at a semi-urban site located in the suburbs of the city of Beirut during summertime (2-18 July 2011). Several classes of compounds represented by 18 individual organic tracers were determined. These tracers include levoglucosan, a tracer for biomass combustion, dicarboxylic acids, and several tracers for the photo-oxidation of isoprene, α -pinene and β -caryophyllene (Waked *et al.*, 2014). In 2015, Borgie *et al.* showed that ultrafine particles were found more enriched in trace elements, secondary inorganic ions, total carbon and organic compounds than the fine particles and that the average level of $PM_{2.5}$ obtained in urban areas ($40 \mu\text{g}/\text{m}^3$) is higher than that obtained in rural areas ($23 \mu\text{g}/\text{m}^3$), but these concentrations both exceed the annual standard set by the WHO ($10 \mu\text{g}/\text{m}^3$) (Borgie *et al.*, 2015). In January-December 2012, the annual average concentrations of $PM_{2.5}$ and PM_{10} in Beirut exceeded WHO's annual average limits by 150% and 200%, respectively (Nakhlé *et al.*, 2015). A comparative analysis done by Waked *et al.* (2015) suggested that on-road transportation and diesel generators used for electricity production are the major sources of atmospheric PM and should be targeted for emission reduction. This study suggested that cooking activities are a significant source of PM (Waked *et al.*, 2015). Salameh T. *et al.* measured 70 C2-C16 non-methane hydrocarbons (NMHCs) in suburban Beirut in summer 2011 and winter 2012 and the levels of NMHCs observed exceeded by a factor of two in total volume the levels found in northern mid-latitude megacities (Paris and Los Angeles), especially for the unburned fossil fuel fraction (Salameh T. *et al.*, 2015). In 2016, Salameh *et al.* identified NMHC sources and quantified their contribution to ambient levels; the major sources were traffic-related emissions (combustion and gasoline evaporation) regardless of the season (Salameh T. *et al.*, 2016).

1.6.5 Studies on air pollution in the North of Lebanon

Most air quality studies outside Beirut have focused on Chekka and Selaata; a region that is infamous for housing large industries including cement plants (Holcim and Cimenterie Nationale) and a phosphate fertilizer industry (Selaata Chemicals Company). The region is also affected by nearby

quarries, sea-spray, and long-range transport (secondary aerosols). North Lebanon used to be the second most active industrial Mohafaza after Mount Lebanon. The largest companies in the North (those having more than 250 workers) were related to the construction materials industry, mainly cement (MoI/ ALI/ UNIDO, 2010). Environment and Development Observatory (TEDO) and University of Balamand (UOB) are involved in air quality monitoring. TEDO started to measure and monitor TSP and PM in 2000 in the region of Al Fayhaa within Tripoli. UOB started in 2008 to monitor TSP in the industrial area of Chekaa and Selaata in North Lebanon. PM₁₀ and PM_{2.5} were measured at different sites in Tripoli in 2014 using the National Air Quality Monitoring Network (AQMN) with exceedances of the Lebanese standard for PM₁₀, and consequently WHO recommended values (MoE/UNDP/GEF, 2016). Table 1.16 presents an overview on air quality monitoring stations outside GBA. There are no known initiatives in air quality monitoring in the Bekaa Valley and South Lebanon.

Table 1.16: Air quality monitoring stations outside GBA

Period	Responsible unit	Measured pollutants	Sampling equipments	Location of equipments
2000 to present	TEDO	TSP, PM ₁₀ , PM _{2.5}	High volume sampler Low volume sampler	1) Urban station: down town of Tripoli; 2) Periurban station: TEDO building
		Benzene, Toluene, Xylene, HF, NO ₂ , SO ₂ , and O ₃	Passive sampling- Radiello Tubes	13 monitoring stations (according to Al Fayhaa community towns)
		Vehicle exhaust gas emissions: HC, O ₂ , CO, CO ₂	Exhaust gas analyzer	In the alleys of Tripoli City
2008 to present	UOB	TSP	High volume sampler	Chekka and Selaata

1.6.5.1 Chekka

In May 1996, Greenpeace proved that the cement, asbestos and chemical industries in Chekka and Selaata in Northern Lebanon are a major source of marine, soil and air pollution. Sample tests carried out by the Earth Research Center at Exeter University in England showed that ground water in Chekka is polluted by a wide range of toxic chemicals, while emissions from cement and asbestos factories pollute the air and the Mediterranean Sea. Greenpeace had showed the effect of industries on the environment by sampling groundwater, petro-coke used as cheap fuel in the cement plants, wastewater from industrial discharge pipes and nearby sediments, waste from cement factory filters, and surface sediment from the Asfur River in Chekka and dust from the factories (Greenpeace Mediterranean, 1998).

In 2001, the Lebanese Ministry of Environment has published the results of the air quality monitoring program in the Chekka region. SO₂ levels (1.28- 2 µg/m³), NO₂ (13.14- 20.76 µg/m³) and PM₁₀ (67- 315 µg/m³) found in all sampling sites, exceed the US EPA and Lebanese standards (MoE,

2001) (Table 1.17). Between September 2002 and February 2004, the Lebanese American University (LAU) monitored four criteria air pollutants (PM_{10} , CO, SO_2 and NO_2) in the framework of the USAID-funded project “Air Quality Management and Estimated Health Impact of Pollutants in Urban and Industrial Areas”. The program used monitoring stations in five locations in and around the cement industry zone in Chekka and the surrounding Koura villages (Kefraya, Kfarhazir, Fih, and Enfeh) (Table 1.16). Diffusion and dispersion of pollutants in these areas were also studied according to prevailing and recorded meteorological conditions. This study showed that the cement-related activities are the main source of NO_2 , SO_2 (Fiaa and Enfeh higher than Chekka) and PM_{10} concentrations, which exceeded the US-EPA standards in all monitoring locations especially in the sites located in the north of the cement plant, under the prevailing SW wind (Table 1.18). The main possible sources were identified as follows: the kiln smoke stacks of the two operating cement factories, the power plants of the two main factories with combined power in excess of 100 MW, the clinker mills inside the same factories, two limestone quarries (one for each factory), and the transport routes (conveyor belts and trucks). This study’ results confirmed that the industrial fumes do not represent more than a fraction (10-20%) of the observed pollution and that the local population is subjected to higher risks of mortality from cardiopulmonary disease and lung cancer than the overall Lebanese population (Karam and Tabbara, 2004).

In 2009, Kfoury *et al.* investigated the inorganic composition of aerosol samples in Chekka. This study focused on the chemical characterization of TSP (inorganic ions and major and trace elements). In terms of their ionic composition, NO_3^- (6182 ng/m^3) had the highest mean concentration linked to gas-particle conversion of NO_2 , followed by SO_4^{2-} (4048 ng/m^3), Ca^{2+} , NH_4^+ (1726 ng/m^3) and Cl^- (1278 ng/m^3). In terms of their elemental composition, potassium (4370 ng/m^3) (biomass combustion) was found in highest concentrations, followed by calcium (2134 ng/m^3) and sodium (1079 ng/m^3). Other crustal elements including Mg and Fe were detected in lower concentrations. The authors noted that the mass of the collected particles increases as the sites are approaching the industrial area (cement factories and quarries) (Kfoury *et al.*, 2009).

1.6.5.2 Selaata

In 2010, Yammine *et al.* studied the elemental composition of TSP in seven sampling points near the phosphate fertilizer industry (April-June 2008). Average levels of Na, Mg, Al and K were higher than all sampled sites in Lebanon. Phosphorous and Calcium concentrations exceeded $10,500 \text{ ng/m}^3$ which is attributed to rocks grinding and other processes. The concentrations of elemental phosphorus, calcium, nitrate and trace elements (Ti, V, Cr, Cu, Cd, Ni, and Mn) were higher in sites located north of the plant, under the prevailing wind than in those located in the South. This could highlight the contribution of the fertilizer plant to higher phosphate concentrations in the region, calcium (resuspension of dust loaded in calcium by the heavy machinery and/or the rock grinding), nitrate (emission of NO_2 by heavy vehicles and or engines used in the industrial site) and

trace elements (Ti, Cr, Ni and Mn) (possible source of the impurities in phosphate rock) (Yammine *et al.*, 2010).

Table 1.17: Summary of the air quality monitoring results in the Chekka region

Constituent	Concentration range in 1999 (Kobrossi <i>et al.</i> , 2000)	Concentration range in 2004 (Karam and Tabbara, 2004)	AAQS	Lebanese Standards in $\mu\text{g}/\text{m}^3$ MOE Decision 52/1 (1996)
SO ₂ (ppm)	0.45- 0.7	0-2	0.14 (24-hour average) 0.03 (annual average)	120 80
NO ₂ (ppm)	6.4- 10.11	6.4-10.11	0.053 (annual average)	100
CO (ppm)	0.33	0-2	9 (8-hour average)	10,000
PM ₁₀ ($\mu\text{g}/\text{m}^3$)	67-316	10-450	150 (24-hour average) 50 (annual average)	80 NA

Table 1.18: Air pollution field measurements results (Karam and Tabbara, 2004)

Location	Date	EPASTandards	150 $\mu\text{g}/\text{m}^3$	0.14 ppm	0.053 ppm	9 ppm
		AveragingTimes	24-hour	24-hour	Annual	8-hour
		PM ₁₀	SO ₂	NO ₂	CO	
Chekka	January 10 th to January 30 th	Peaks at 250	Below EPA Levels	Peaks up to 20 over more than one week	Below EPA Levels	
	May 10 th to May 20 th	Between 250 and 450	Below EPA Levels	Below EPA levels	Below EPA Levels	
	September 5 th to September 22 nd	Peaks at 170	0.3 to 0.4	Peaks up to 1	Below EPA Levels	
Enfeh	March 15 th to March 21 st	Between 200 and 300	Not Available	Not Available	Not Available	
	April 20 th to May 14 th	Peaks at 180	Below EPA Levels	Peaks up to 100 over one week	Below EPA Levels	
	September 12 th to October 2 nd	Below EPA levels	1.5 to 1.8	Peaks up to 10 over one week	Below EPA Levels	
Fiaa	June 15 th to July 3 rd	Below EPA levels	Below EPA Levels	Peaks up to 100 on a single day	Below EPA Levels	
	August 25 th to September 5 th	Peaks at 150	1.5 to 1.8	Peaks up to 10 over one week	Below EPA Levels	
KfarHazeer	March 4 th	Peaks at 250	Below EPA Levels	Peaks up to 2-3	Below EPA Levels	
	July 4 th to August 15 th	Varies between 130 to 170	Below EPA Levels	Peaks up to 10 over one week	Below EPA Levels	

1.6.5.3 Tripoli

Tripoli, 80 km from Beirut, is the capital of the North and the second largest city after Beirut. It forms with El Mina, and Beddawi the Urban community of Al Fayhaa. Since 2000, TEDO has been monitoring air pollutants in Al Fayhaa.

TEDO showed in its report that the problems of air pollution in Al Fayhaa are due to 16 sectors such as the transport sector, vessels (port), Deir Amar power plant, discharges, construction, dust resuspension from paved roads, transferring gasoline in trucks tanks, generators and domestic heating. It has been shown that these 16 sectors generate high levels of pollutants (TSP, PM₁₀, PM_{2.5}, NO₂, SO₂, O₃, benzene, toluene and xylene) that exceed the US EPA and Lebanese Standards (EU's Short and Medium Term Priority Environmental Action Plan, 2002). In 2008, TEDO installed fixed monitoring stations downtown Tripoli (urban) and on the roof of the TEDO building (peri-urban

station influenced by sea winds) to monitor TSP, PM₁₀ and PM_{2.5}. TSP and PM levels downtown Tripoli are showed in Table 1.19, covering a six-month period. They also operate 13 passive sampling monitoring stations, distributed across Al Fayhaa's 13 cadastral zones, to monitor benzene, toluene, xylene, HF, NO₂, SO₂, and O₃. In 24% of the sampling days, TSP concentrations values exceeded the Daily National Standard (120 µg/m³). Similarly, PM₁₀ values exceeded the Daily National Standard (80 µg/m³) in 45% of the sampling days; compared to the daily PM₁₀ WHO standard (50 µg/m³), the difference would be the number of sampling days. Mean PM_{2.5} in downtown Tripoli (34.6 µg/m³) was consistently higher than PM_{2.5} values at the seafront station (23.6 µg/m³), principally due to heavier traffic in downtown Tripoli. Generally, PM_{2.5} values recorded in downtown Tripoli and on the seafront were almost consistently higher than EPA's daily standard for PM_{2.5} (35 µg/m³) as well as WHO's annual standard for PM_{2.5} (10 µg/m³).

In 2013, Ghaleb studied the air quality in al Fayhaa in the thirteen sectors based on results inventory: the transportation sector is regarded as a major source of air pollution in al Fayhaa, specifically for the following pollutants: PM₁₀, PM_{2.5}, VOC, NMVOC, Benzene, Toluene, Xylene, CO, CO₂ and NO_x. According to this report the main pollutants located at UCF are particulate matter (PM₁₀ and PM_{2.5}), NO₂, SO₂ and O₃. The concentration of each pollutant changed from location to another according to the activity elaborated at each spot. In addition, the sources of different pollutant at al Fayhaa were determined and the main sources were traffic and transportation sector, Deir ammar power plant, port of Tripoli, fishing boat port and Tripoli controlled dump. A relationship was established between PM and meteorological parameters and it was found that the PM inversely correlates with wind speed and temperature (Ghaleb, 2013).

Therefore, these studies showed that the levels of air pollutants in Northern Lebanon are far exceeding the limits set by national and international standards. Furthermore, according to our knowledge, this region has been the subject of few studies on atmospheric aerosols and no study has been made of the effect of PM on public health.

Table 1.19: TSP, PM₁₀ and PM_{2.5} levels in Tripoli from Jan-Jun 2008 (MoE/UNDP/ECODIT, 2011)

Month	Mean monthly TSP (µg/m ³)	Mean monthly PM ₁₀ (µg/m ³)	Mean monthly PM _{2.5} (µg/m ³)	
			Downtown of Tripoli	TEDO building
Jan 2008	103	80	45	33
Feb 2008	93	79	49	26
Mar 2008	125	NA	23	27
Apr 2008	106	89	31	19
May 2008	83	76	31	19
Jun 2008	100	83	29	17
Mean	101	81.4	34.6	23.6
WHO std (daily)	150	50	25	25
WHO std (annual)	-	20	10	10
EPA std (daily)	75	150	35	35

1.6.6 Air pollution and Health studies in Lebanon

1.6.6.1 Epidemiological and toxicological studies

Ambient air pollution has been associated with a multitude of health effects, including mortality, respiratory and cardiovascular hospitalizations, changes in lung function and asthma attacks. The risk for childhood cancer is heightened by exposure to nitrogen dioxide, which is produced by car exhaust fumes (Ghaleb, 2013). This in fact reflects how serious the consequences of air pollution on public health in Lebanon are. Cancer is currently the most severe health condition Lebanese face due to increased air pollution, with higher figures being reported every other year (Abbas, 2010). To date, no direct link was established between air pollution and health effect in Lebanon. Dr. Coussa-Koniski from Rizk Hospital warned that the number of cases of asthma, rhino-sinusitis, and interstitial lung disease have been rising significantly over the past decade half (American University of Beirut, 2011). For instance, the prevalence of asthma in Lebanon is at least 50% higher than that in Europe or the United States. However, studies have shown that if air pollution is reduced to guideline levels, then the number of asthma cases would fall by 70% and bronchitis cases will be reduced.

Information about disease registries are not readily available, on the other hand, hospitals in Lebanon are not required to report detailed discharge summaries to the ministry of public health. A medical checkup conducted in some schools in the urban community of al Fayhaa provides data on allergies and epidemiological and respiratory diseases that are known to be caused by air pollution. Figure 1.32 shows the variation of airborne diseases from 2008 till 2012. The results of the medical checkup show that the diseases resulting from air pollution are increasing with years, which indirectly mean that air pollution in urban community al Fayhaa is increasing from year to year. This study also reveals that air pollution is harmful for those who are already vulnerable because of their age as children or existing health problems. Evidence suggests that adverse health effects are dependent on both exposure concentrations and length of exposure, and that long-term exposures have larger, more persistent cumulative effects than short-term exposure (Ghaleb, 2013). Health data for 11,567 individuals were collected over 12 months in a study done by Nakhlé *et al.* and variation of hospital admission causes was observed by age categories and gender (Nakhlé *et al.*, 2015).

A case study done by Kobrossi *et al.* in Chekka, Selaala, Koura, and Batroun (as exposed) and in Jbeil (as potentially non-industrialized) revealed the effect of atmospheric aerosols on the health of children. This study found a correlation between exposure and the risk of lung diseases (asthma, chronic bronchitis). Living in the more exposed areas of Koura and Batroun (0-4 km) was associated with a statistically significant increased adjusted risk for cough with colds, phlegm with colds, yearly episodes of cough and phlegm, yearly chest colds, and wheezing, as compared to living in the less exposed areas (4-7 km). Chest illnesses symptoms (chest illnesses keeping the child off his

activities during the past 3 years and severe chest illness before the age of 2 years), and bronchitis also increased but was not statistically significant (Kobrossi *et al.*, 2000).

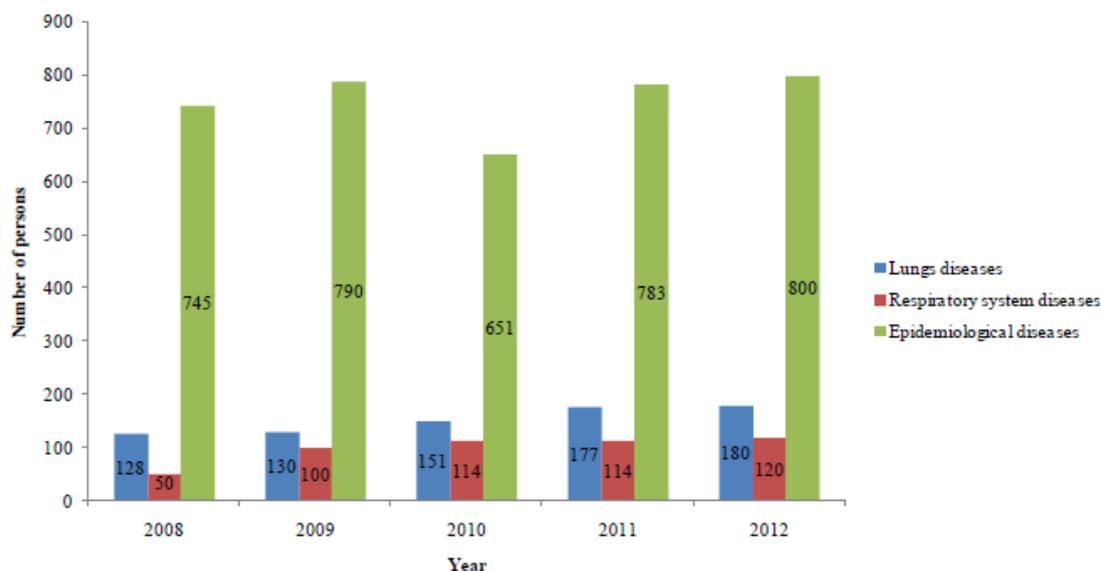


Figure 1.32: Variation of airborne diseases from year 2008 to 2012 (Ghaleb, 2013)

In Beirut, four epidemiological studies showed a relation between air pollution and health problems. A cross-sectional study conducted by Waked M. *et al.* between October 2009 and September 2010 showed that a substantial percentage of the non-smoking population may exhibit chronic bronchitis or chronic obstructive pulmonary disease (COPD) due to pollution (Waked M. *et al.*, 2012). A pilot case-control study, conducted by Salameh *et al.* in two tertiary care hospitals, proved that chronic bronchitis is associated with outdoor air pollution. This study showed that chronic bronchitis was moderately associated with passive smoking at home, older age, lower education, living close to a busy road and/or a local power plant, and electrical heating; the disease had an inverse association with heating home electrically (Salameh *et al.*, 2012). A hospital-based case-control study was conducted by Aoun *et al.*, where patients in a tertiary health care center were given a questionnaire to assess the possible risk factors for lung cancer. In this pilot study, it was found that in addition to smoking, outdoor and indoor pollution factors were potential risk factors of lung cancer (Aoun *et al.*, 2013). Outdoor air pollution is increasingly considered as a serious threat for cardiovascular diseases (CVD). A multicenter case-control study was conducted by Nasser *et al.* between October 2011 and October 2012. Cases were hospitalized patients diagnosed with CVD by a cardiologist while the control group subjects were free of any cardiac diseases. Information on sociodemographic characteristics, tobacco consumption, self-rated global health, pollution exposure, and other risk factors was collected using a questionnaire. The results of the logistic regression revealed that living near busy highway (OR= 5.04; 95% CI: 4.44-12.85), $P < 0.001$) and close to local diesel generator (OR= 4.76; 95% CI: 2.07-10.91), $P < 0.001$) was significantly associated with CVD.

The association between the CVD and exposure to outside pollutants differed by cigarette smoking status. A clear difference was noted between nonsmokers and current smokers OR= 4.6; 95% CI:1.10-19.25 and OR= 10.11; 95% CI:7.33-20.23, respectively (Nasser *et al.*, 2015). (CI is confidence interval and OR is odds ratio).

In Lebanon, very few studies were done on the toxicological effect of PM on health. Borgie *et al.* showed *in vitro* that pulmonary cells exposed to PM_{2.5-0.3} sample collected in Beirut caused DNA damages. This study showed an increase in the phosphorylation of H2AX, the telomerase activity and an up-regulation of the miR-21 in BEAS-2B cells in a dose- dependent manner. PM_{2.5-0.3} induced a significant increase in *CYP1A1*, *CYP1B1* and *AhRR* genes expression. The variable concentrations of transition metals and organic compounds detected in the collected samples might lead to accumulation of DNA damage which is critical for carcinogenesis (Borgie *et al.*, 2015). According to our knowledge, our study is the first study on the toxicological impact of particulate matter on health in Northern Lebanon.

1.6.6.2 Cancer trends in Lebanon

The first data on cancer incidence in Lebanon was performed in 1966 by Abou-Daoud and was based on pathology reports from eight institutions where histopathological diagnoses were made. The reported crude incidence rates were 102.8 per 100,000 in males and 104.1 per 100,000 in females (Abou-Daoud, 1966). Later on, several attempts were made to establish hospital-based registries in Lebanon and to examine the characteristics of major sites of cancer incidence; however, results were inconsistent (Adib *et al.*, 1998; Ghosn *et al.*, 1992). In 1998, the Lebanese Cancer Epidemiology Group (LCEG), a network of all hospitals with oncology specialties and pathology laboratories was established in an attempt to examine cancer incidence rates at the national level. The study results were based on a total of 4,388 cases diagnosed during the year 1998. The overall crude incidence rate for all cancers combined was 141.4 per 100,000 among males and 126.8 per 100,000 among females. This increase, in comparison to the data by Abou-Daoud, reflected long term changes in lifestyle and in the prevalence of risk factors coupled with an increase in life expectancy approaching to 69 years among males and 72 years among females as reported in the study. Among males, bladder (18.5%), prostate (14.2%), and lung cancer (14.1%) were the most frequently reported malignancies and among females, breast cancer alone accounted for over a third of all cancers, followed by colon cancer (5.8%), and cancer of the corpus uteri (4.8%). Sex differentials in incidence rates were highest for tobacco-related cancers (lung, larynx and bladder) (Shamseddine *et al.*, 2004). A “Cancer 2003” report was published in June 2003 (Ministry of Public Health, 2003). In 2004, breast cancer was the most commonly diagnosed cancer in Lebanon (19.7%), followed by lung cancer (10.8%) then bladder (9.3%), colorectal (8.2%), and prostate (7.7%) cancers (Harb, 2004). There has been a sharp increase of the adjusted age-standardized rates (ASRs) in both sexes between 1998 and 2004 with an

approximate increase of 60% in the reported cases. For males an ASR of 179.3 per 100,000 was observed with the highest ASR in lung cancer (28.5 per 100,000) followed by bladder (28 per 100,000) and prostate (27.6 per 100,000) cancers. For women the ASR was 190.3 per 100,000 with the highest ASR noted for breast (69.15 per 100,000) followed by lung (10.75 per 100,000) and Non-Hodgkin's lymphoma (10.6 per 100,000) (Shamseddine and Musallam, 2010). In 2014, a study done by Shamseddine *et al.* on cancer incidence showed that between 2003 and 2008, males and females presented an overall 4.5% and 5.4% annual increase, respectively. Significant increases were observed for cancers of the liver and prostate among males, and for cancers of the liver, thyroid, and corpus uteri among females. By 2018, incidence rates were projected to approach 296.0 and 339.5 cases per 100,000 for males and females, respectively. The most common five types of cancer are expected to be prostate, bladder, lung, non-Hodgkin, and colon among males; and breast, ovarian, non-Hodgkin, lung, and colon among females (Shamseddine *et al.*, 2014).

1.7 Hypothesis, objectives and methodology

Exposure to air pollution especially to air particles remains a major health risk (Loomis *et al.*, 2014). However, toxicity mechanisms of PM are not fully identified. PM toxicity occurs primarily by fine and ultrafine particles due to their physical and chemical characteristics. Northern Lebanon is affected by several sources of pollution: industries, transport section, and other human activities. Furthermore, knowledge about air pollutants and their impact on health remain very patchy in Lebanon and studies have focused on the emissions of gases. Up to our knowledge, still in Lebanon no study has examine the perception of air pollution and few epidemiological studies were done concerning respiratory diseases risk factors, although lung cancer is thought to be of primary importance in the region (Salim *et al.*, 2010) and has a relatively high incidence in Lebanon, particularly in males (Shamseddine *et al.*, 2014).

Hence, to contribute to fulfill this gap of knowledge and to better understand how air pollution is affecting the public health in Northern Lebanon, one perception and epidemiological study and a toxicological study were done. The perception and epidemiological study was carried out to determine the association between the most common risk factors and respiratory diseases in a sample of the Lebanese population. More specifically, this study aimed to investigate the association of these diseases with industrial activities, a common exposure in the Lebanese population. The toxicological study aimed to show genotoxicity mechanisms involved in the carcinogenicity PM_{2.5-0.3} collected in North Lebanon and to relate the PM composition to the observed mutagenic and genotoxic effects.

The principal objectives of this work aim to:

- Examine the levels of perceived air pollution annoyance and health risk and evaluate the relationship between the development of respiratory diseases and living close to industrial activities
- Assess the mutagenic and genotoxic effects of two fine particulate matter (PM_{2.5-0.3}) samples collected in background area or under industrial influence
- Relate the PM composition to the observed genotoxic effects

In order to accomplish these objectives, this study was divided into four successive actions performed following this sequence:

- Perception and epidemiological survey:

A survey was conducted in two areas in Northern Lebanon, the first one under industrial influence and the second one under rural influence (Figure 1.33). 310 questionnaires were filled in every area. In the area under industrial influence, questionnaires were filled in four villages: Zakroun, Barghoun, Bdeihoun, and Fiaa (Figure 1.34). And in the rural area, questionnaires were filled in three villages: Kaftoun, Btaaboura, and Kfar Hata (Figure 1.35).

The industrial area is affected by several sources of pollution: industries, transport section, and other human activities. The most important industries situated in this region are two cement factories and quarries in Chekka and a phosphate fertilizer industry in Selaata. The rural area was considered as a control site. Seasonal wind shows a dominant southwest in summer and northwest and southwest winds in winter. The chosen villages in the industrial area face the industries in Chekka and Selaata and in these wind conditions the industrial area appears affected by the industrial activities. The villages of the rural area were specifically chosen due to their location, and according to wind direction, they are not exposed to direct industrial emissions and they are quite protected by mountains chain.

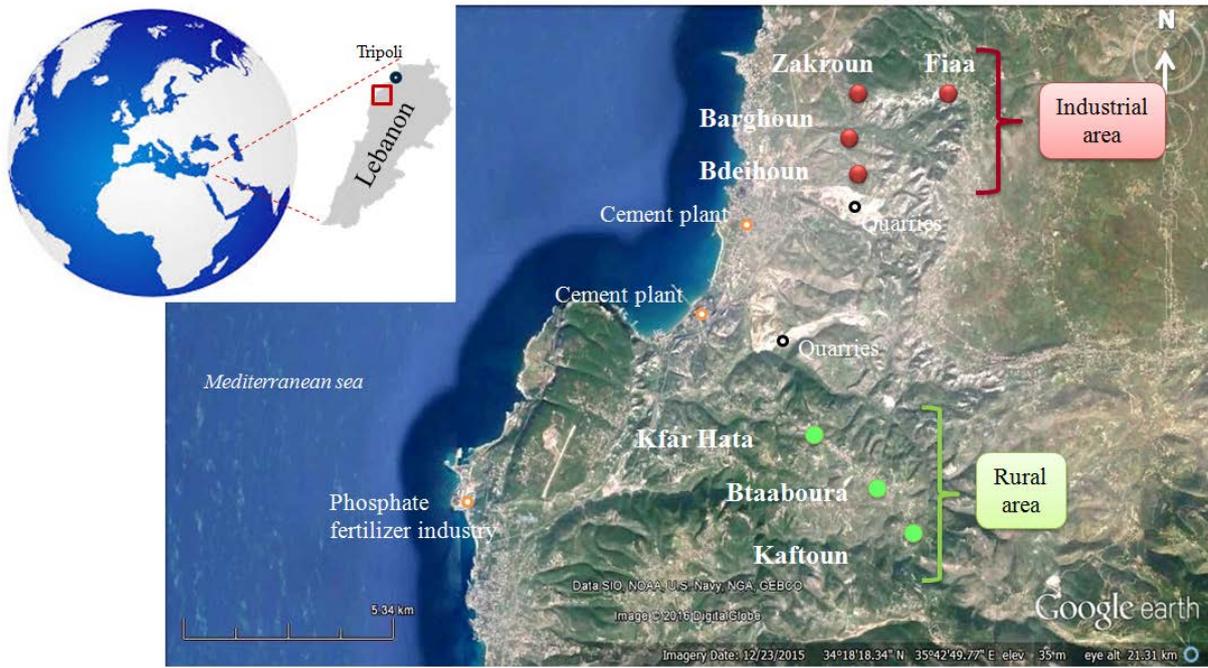


Figure 1.33: The study areas

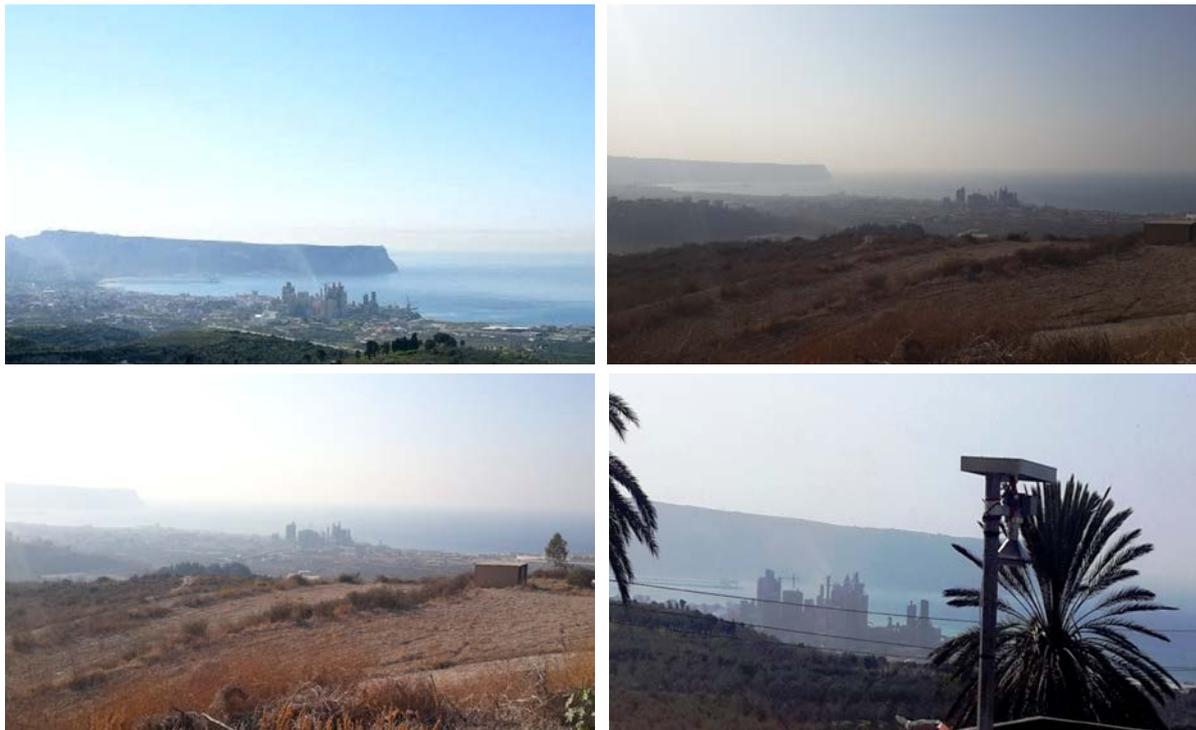


Figure 1.34: Villages of the industrial area facing the main source of pollution



Figure 1.35: Villages of the rural area

- Sampling of $PM_{2.5}$:

Sampling strategy consists of a simultaneous sampling of fine particles ($PM_{2.5-0.3}$) and quasi-ultrafine particles ($PM_{0.3}$) by cascade impaction using a TFIA-2 high volume air sampler at two sites in Northern Lebanon in Koura district: Zakroun, a site affected by industrial influences, and Kaftoun, a background rural site.

The site under industrial influence: Zakroun

The first sampling site, Zakroun, was chosen specifically in a way that allows us to sample under the effects of industrial emissions. Zakroun ($34^{\circ}21'4.95''N$; $35^{\circ}45'19.01''E$) is located close to the coastline of Northern Lebanon and the sampling site is on a building roof to prevent sampling of any punctual events localized at the street ground level. Considering the dominant SW wind direction, Zakroun, located at 78 m above sea level, appears affected by the industrial activities present in Chekka and Selaata mainly implanted 2 km southwest of the site and also to local human activities (traffic, domestic heating...). Zakroun is situated at 12 km and 57 km from the cities of Tripoli and Beirut, respectively. Zakroun is situated at 3 km from the Cimenterie Nationale (cement factory), 6 km from the Holcim (cement factory), 13 km from the phosphate fertilizer industry, 2 km from a quarry and 5 km from another quarry (Figure 1.30).

The site under predominantly rural influence: Kaftoun

The second sampling point is located in Kaftoun ($34^{\circ}15'56.98''N$; $35^{\circ}46'6.37''E$). Sampling was carried out on the roof of a building in a natural reserve. Kaftoun is situated at 300 m above sea level, at 20 km from Tripoli and 49 km from Beirut. It is situated at 8 km from the Cimenterie

Nationale (cement factory), 6 km from the Holcim (cement factory), 10 km from the phosphate fertilizer industry, 7 km from a quarry and 5 km from another quarry (Figure 1.31). Kaftoun, situated 10 km south of Zakroun, was specifically chosen due to its location, and according to wind direction and considering the protection by mountains chain, can be considered as a less exposed rural site.

- Physicochemical characterization of $PM_{2.5-0.3}$ and $PM_{0.3}$

After sampling, physicochemical analyses were performed: size distribution and morphology (Laser diffraction and SEM-EDX), identification of PM crystalline phases (XRD), inorganic composition, (ICP-MS, ICP-AES and Ion chromatography), total carbon composition (CHNS/O analyzer), and organic composition (GC-MS).

- Toxicological analysis of $PM_{2.5-0.3}$

- The bacterial mutagenicity and genotoxicity were studied, using the Ames fluctuation test (with *Salmonella typhimurium* strains TA 98, TA102, YG1041) and the SOS chromotest (with *Escherichia coli* PQ37).

- The global cytotoxicity of the $PM_{2.5-0.3}$ was determined in human bronchial epithelial cell line (BEAS-2B) by measuring the membrane integrity by testing the extracellular activity of lactate dehydrogenase (LDH), assessing cell viability by testing the activity of the mitochondrial dehydrogenase (MDH) and testing the extent of cell proliferation by measuring the incorporation of 5-bromo-2-deoxyuridine.

- Different genotoxic and epigenetic endpoints were then studied: gene expression of xenobiotic-metabolizing enzyme (CYP1A1), DNA adducts due to PAH metabolic activation (^{32}P post-labeling), DNA strands breaks (γ -H2AX quantification by flow cytometry analysis and in-cell western assay), and the repair process of bulky DNA adducts (XPA gene expression), the cellular oxidative stress (OGG1, NTH1, APE1, NUDT1 genes expression and OGG1 protein expression), and the DNA strand breaks (XRCC1 gene expression and PARP1 protein expression), and the methylation stability (DNMT1 and MGMT genes expression and DNMT1 protein expression).

CHAPTER 2

PERCEPTION AND EPIDEMIOLOGICAL STUDY

Perception and epidemiological studies are used to show the effect of air pollution on the environment and its impact on the general health of the population. Chapter 2 revolves around the perception and epidemiology study performed in two areas in Northern Lebanon, industrial and rural. This chapter begins with a detailed explanation of the study areas, design and procedure. The following part includes results of air pollution perception and the impact of respondents' gender, age and occupations according to the survey. The next section includes the results of air pollution epidemiology showing prevalence of the respiratory symptoms, the past illnesses, and the family history and children health. Later on, a principal component analysis (PCA) was performed showing the relation between perception and epidemiology. Followed by a section on methods and results, this chapter ends with a conclusion on the perception and epidemiology study done about air pollution in Northern Lebanon.

2.1 Introduction

2.1.1 Perception and epidemiological study

In the field of air pollution, the communication strategies traditionally developed by public agencies are not often based on a careful consideration of how individuals react to air pollution nor on how the public reacts to the information about pollution levels and effects (Blanken *et al.*, 2001). Research on the epidemiological effects of air pollution has extensively documented how air particles can affect the health of exposed individuals (WHO, 2014c). However, less attention has been paid to the individual and public responses to this environmental hazard. Perception is important in every society because responses from individuals and communities can significantly modify air pollution levels and air pollution effects through changes in the behavior of individuals and organizations. Second, because individuals may be forced to develop actions to modify their exposure to air pollution. Three types of health behavior are relevant in relation to air pollution: i) reduce air pollution; ii) protect oneself against the adverse effects of the pollution; iii) be aware and engaged in air pollution (being informed about air pollution levels and its health impact) (Oltra and Sala, 2014). Many factors related to the qualitative characteristics of the risk, the individual (from personality to lifestyle issues) and the context in which perception is developed (social amplification and attenuation of risk processes) might explain differences in perception among individuals in the same social group.

The research on air pollution perception started in the 1950-60 and most of the first studies were focused in North America and United Kingdom. Quantitative psychology theoretical approach dominates the research and it tried to measure social and psychological dimensions of air pollution

(people's awareness and effects on health). This approach used quantitative methods (questionnaires and opinion polls) focusing in measuring attitudes and cognitive processes related to air pollution. The main research topics in this first period were: public awareness and concern about air pollution, relation between experts and the public, correspondence between pollution measurement and public perception, and public perception among different groups. During the 90s, there was an increasing interest in how people perceived air quality and the field of research extended to other countries. At that time important aspects of the context had change: i) environmental degradation was increasingly recognized as a direct threat to human health and welfare; ii) some changes in the pollutants also occurred during this period: from carbon combustion to others pollutants from traffic, with less visual and olfactory impact; iii) urban and industrial air pollution climbed back to the policy agenda probably due to the newly discovered links between small particulate emissions and health (Bickerstaff and Walker, 2003; Catalán Vázquez, 2006; Omanga *et al.*, 2014). The second phase of studies developed a socio-cultural perspective which included contributions from various disciplines such as human geography, sociology and anthropology (Day, 2004). Social, cultural and political aspects were incorporated into the studies as important factors in determining environmental attitudes. It was assumed that public understanding on air pollution could not be explained only by individual factors, thus other social and cultural factors were included such as local knowledge and everyday experiences. During the last decades, there was an increasing acknowledgement and consolidation of a body of work and a more pronounced degree of convergence between the cultural and psychological approaches. The most important published research studies on the characterization of public perception of air pollution are related to: public awareness, physical experience of pollution, knowledge about the sources, pollutants, and pollution levels. Knowledge about the activities is to reduce pollution or to protect oneself, risk perception, experience of physiological or psychic effects, emotions, etc.

Different studies have pointed out that the main sources for air quality awareness among the public are: i) visual evidence: physical dirt or soot, smoke and fumes from "polluter" sources, color and contrast in landscape, observable effects on the environment (the color and growth of vegetation, tree or bird death) (Douglas and Waller, 1966; Galada *et al.*, 2009); ii) Olfactory evidence (Wakefield *et al.*, 2001); iii) experience of psychological and physical effects or impacts: fatigue, eye irritation, recurrent colds, etc. (Lercher *et al.*, 1995); iv) nuisance, annoyance and aesthetics impacts of pollution (dirtiness, dust and soot) (Claeson *et al.*, 2013); v) media coverage and publicity (weather forecast, media formats were more influenced than the governmental information services); vi) informal experience (interpersonal interaction and conversation, local knowledge); vii) social networks and lifestyle factors (for instance, time spent outdoors can influence perceptions).

Sensory visual perception of air pollution appears to increase with higher concentration of suspended particulates and dustfalls (Oltra and Sala, 2014). According to different studies, visible cues and bad smell were the most common indicators of air pollution after health impacts (Bickerstaff

and Walker, 2001; Cole, 1999). Claeson and colleagues also stressed the relevance of odor characteristics of air pollutants (Claeson *et al.*, 2013). Main perceived sources of pollution are road transport (Howel *et al.*, 2003; Jacobi, 1994; Smallbone, 2012) and industry (Bickerstaff and Walker, 1999; Howel *et al.*, 2003). Other sources such as agriculture (farms, fertilizers, burning of agricultural waste) or residential energy use (coal or wood for heating of individual households) were less perceived by the public (European Commission, 2013). Complaints about air pollution are usually related to visible or odorant pollutants (Amundsen *et al.*, 2008; Blanes-Vidal *et al.*, 2012) regardless of their chemical composition or toxic effects. Reactions to the presence of air pollution may differ among individuals depending on the intensity of odor/smell, dirt in the household and the presence of symptoms that cause annoyance, such as eye irritation, sore throat, cough and allergic reactions (Klæboe *et al.*, 2008). The concept of environmental annoyance is complex and subjective; it can be experienced as a perception, an emotion, an attitude or a mixture of these. Perceived annoyance is likely to be influenced by several factors, such as risk perception (Petrie *et al.*, 2001) and health symptoms, like pulmonary, eye, nose and throat irritation (Stenlund *et al.*, 2009), occurrence of health problems (Oglesby *et al.*, 2000), psychological effects (Llop *et al.*, 2008), location of residence (Rotko *et al.*, 2002), socio-demographic situation (Jacquemin *et al.*, 2007), access to information about air pollution levels (Klæboe *et al.*, 2008), and perception of dust levels and assessment of air quality (Blanes-Vidal *et al.*, 2012; Hyslop, 2009; Kim *et al.*, 2012).

Elements that influence risk perception could be divided into three broad categories:

- Risk-related factors which are specific characteristics of air pollution that influence how it is perceived (sensory characteristics, type of industries, level of air pollution...) (Brody *et al.*, 2004; Smith *et al.*, 1964).
- Psycho-social factors which are related to the subject and its social relations (gender, age, education, income levels, occupation or marriage status, presence of children, life style, personality characteristics, health status, social capital, attachment to place and commitment to the neighbourhood) (Elliott *et al.*, 1999; Howel *et al.*, 2003; Jacquemin *et al.*, 2007; Slovic, 1999).
- Institutional and contextual factors which are aspects of the context that have an influence in how the public perceive air pollution (neighborhood characteristics urban-industrial versus a rural setting, media coverage, distance to industry or source of pollution) (Brody *et al.*, 2004).

Air pollution is mainly related with health effects, especially respiratory diseases or allergic illnesses (recurrent colds, chronic bronchitis, hyper-reactive airways, acute bronchitis, hay fever, asthma, eczema). Some studies have shown that there is a lack of awareness amongst the general public regarding the links between air pollution and illness (Egondi *et al.*, 2013; Wakefield *et al.*, 2001). Precise health links were generally unclear, with symptoms such as chest pain, asthma, sore throats, headaches, fatigue and irritability. Awareness of the effect of air quality on those with heart conditions is generally low (Smallbone, 2012). There is little evidence of beliefs in strong causal links

between specific diseases and air pollution among members of the public; rather there is an understanding that chronic illnesses, such as allergies, asthma, and bronchitis, can be affected by air pollution. The “invisibility” aspect of air pollution discourages people from drawing strong links between air pollution and health (Saksena *et al.*, 2007). Several of the public opinion surveys revealed high-level identification of health impacts only when respondents were asked directly (Schusky, 1966). This has been explained in terms of psychological masking, in which a worrisome situation is hidden or put aside to avoid its overt recognition (Crowe, 1968). Those without a personal-risk from air pollution would not see it as relevant and people with profound physical conditions are more likely to perceive air pollution risks (Morton and Duck, 2001; van den Elshout *et al.*, 2008). For example the frequency of reporting annoyance reactions was higher among people with asthma (Forsberg *et al.*, 1997).

Finally, regarding beliefs about ways to reduce air pollution, reducing car use and upgrading to more energy efficient appliances are the most common individual actions to reduce emissions. Stricter pollution controls on industrial activity and energy production are seen as the most effective way to tackle air-related problems. Providing higher financial incentives for low emitting products is perceived as a good measure to tackle air pollution. More public information on the health and environmental effects of air pollution is also seen as a measure to reduce pollution, probably by increasing awareness and motivation (European Commission, 2013).

Most evidence linking the various human health effects to ambient levels of air pollution and air particulate matter (PM) comes from the fields of epidemiology by short-term (time series studies, panel studies, meta-analysis, case-crossover analysis) and long-term studies (cohort studies) (Lavy *et al.*, 2014; Moolgavkar *et al.*, 2013; Shah *et al.*, 2015; Tsai *et al.*, 2014; Ward and Ayres, 2004; WHO/Europe, 2014; Zheng *et al.*, 2015). Time series studies estimate the influence of temporal (usually daily) variations in air pollutant concentrations on mortality or morbidity, using statistical models linking the daily counts of health events in a geographically defined population with daily measures of air pollution and other time-dependent variables. In panel studies, a group of individuals, ideally homogeneous, is followed up prospectively for a short period of time. Multiple measurements are obtained from each subject at different times and analyzed using time series methods. A meta-analysis consists of a statistical synthesis of data from a number of independent but comparable studies of a problem, leading to a quantitative summary of the pooled results. Case-crossover analysis focuses on individual deaths rather than death counts, it is possible to control for factors that may modify or influence the effects of air pollution on mortality at the individual level. Cohort studies estimate chronic health effects associated with air pollution by examining the health risk (e.g. death) in relation to long-term average pollution exposure, usually by comparing people living in different geographical locations (WHO, 2005). These studies use different methods for estimating health risks associated with variation in exposure across spatial and temporal gradients. Study types are often distinguished by their use of individual- or aggregate-level data on exposure, health, and modifying

and confounding factors. Most air pollution studies use exposure indicators aggregated over a specific geographic area, and some studies use a mixture of individual- and group-level exposures (Samet and Jaakkola, 1999). Most contemporary epidemiological research of air pollution's health effects uses either cohort or time-series approaches. Some studies are cross-sectional in design, and associations between environmental pollutants and health outcomes are assessed at one point in time. Time-series studies assess the effects of short-term changes in air pollution on acute health effects by estimating associations between day-to-day variations in air pollution and in mortality and morbidity counts (Bell *et al.*, 2004).

The epidemiology of the health effects of fine particulate air pollution, provides an early, somewhat speculative, interpretation of the contribution of epidemiology to evaluating biologic mechanisms, and evaluates who is at risk or is susceptible to adverse health effects (Bell *et al.*, 2004, 2007; Pelucchi *et al.*, 2009; Pope, 2000). Many studies showed that short-term exposure to particle components, including secondary aerosols, carbon, particle number concentrations and metals, and especially to fine particle mass (PM_{2.5}) has been associated with adverse health effects (Atkinson *et al.*, 2015). The outcomes of the short-term PM exposure were mortality, hospital admissions for cardiovascular and respiratory diseases, and physician visits for respiratory diseases. Based on preliminary epidemiologic evidence, it is speculated that a systemic response to fine particle-induced pulmonary inflammation, including cytokine release and altered cardiac autonomic function, may be part of the pathophysiologic mechanisms or pathways linking PM pollution with cardiopulmonary disease. According to these studies, the elderly, infants, and people with chronic cardiopulmonary disease, influenza, or asthma are the most susceptible to mortality and serious morbidity effects from short-term acute elevated exposures. Others are susceptible to less serious health effects such as a transient increase in respiratory symptoms, decreased lung function, or other physiologic changes. Chronic exposure studies suggest relatively broad susceptibility to cumulative effects of long-term repeated exposure to fine particulate pollution, resulting in substantive estimates of population average loss of life expectancy in highly polluted environments. Many epidemiological studies have been used to assess the health consequences of living in the vicinity of industries (García-Pérez *et al.*, 2015; Ginns and Gatrell, 1996; Simkhovich *et al.*, 2008). Major health themes were cancers, morbidity, mortality, birth outcome and mental health. While studies were available from many different countries, a majority of papers came from the United Kingdom, Italy, and Spain. Geographical ecological designs were largely used for studying cancer and mortality, including statistical designs to quantify a relationship between health indicators and exposure. Morbidity was frequently investigated through cross-sectional surveys on the respiratory health of children. Few multicenter studies were performed. In a majority of papers, exposed areas were defined based on the distance to the industry and were located from < 2 km to > 20 km from the plants (Pascal *et al.*, 2013).

2.1.2 Population data in Lebanon

Lebanon's last population census was conducted in 1932. All population estimates have since been based on surveys and extrapolations. The most reliable source of population data in Lebanon therefore remains the Central Administration of Statistics (CAS). CAS conducted in 1996 a national survey of population data and living conditions and revised their data in 2008 (Table 2.1). According to the 2008 update, Lebanon's resident population in 2007 was 3.7 million, excluding an estimated 425,000 Palestinian refugees (CAS, 2008). The total population in 2008 including refugees was therefore about 4.2 million. The Ministry of Energy and Water (MOEW) assumed a population growth rate of 1.75% between 2007 and 2009 (MoEW, 2010b). The country witnessed a sharp transition: 25% were aged under 15 and 10% were older than 65 years which indicated that almost half of the population was active with a dependence rate of 52%. Furthermore, the fertility rate (1.9) was relatively low. Average life expectancy was 74 years, ranging from 71 years for men and 77 years for women (USJ/OMS/MOPH, 2012). The United Nations estimated that the average life expectancy in Lebanon would rise to 78.7 years between 2009 and 2050, leading to an increase in the median population age from 28.8 to 41.7 years as well as the percentage of older population aged 60 years and above from 10.3% to 25.8% (Shamseddine *et al.*, 2014).

Table 2.1: Administrative regions and Lebanon's resident population in 2007 (CAS, 2008)

Mohafaza	Percent of Total	2007
Beirut	9.61%	61,366
Mount Lebanon	39.49%	484,474
North Lebanon	20.32%	763,712
South Lebanon	17.55%	659,718
Beqaa	13.03%	489,865
Lebanon	100.00%	3,759,135

2.1.3 Objectives of the perception and epidemiological study in Northern Lebanon

This study was conducted in the North of Lebanon, specifically in two areas in: rural and industrial (310 treatable questionnaires/ area) as mentioned in *paragraph 1.7*. The best of our knowledge, this was the first survey that studied the perceptions of air pollution in Lebanon. Few epidemiological studies were done concerning respiratory diseases risk factors, although lung cancer is thought to be of primary importance in the North region (Salim *et al.*, 2010) and has a relatively high incidence in Lebanon, particularly in males (Shamseddine *et al.*, 2014b). This study was performed to determine the association between the most common risk factors and respiratory diseases in a sample of the Lebanese population; more specifically, the present study aimed to investigate the association of these diseases with industrial activities, a common exposure in the Lebanese population.

Five factors motivated the conduction of this survey and the selection of the towns for this study: i) it was found that in addition to smoking, outdoor and indoor pollution factors were potential

risk factors of different respiratory diseases and lung cancer, and additional studies were necessary to confirm these findings (Aoun *et al.*, 2013a; Nyberg *et al.*, 2000); ii) exposure to fine particle mass has been associated with adverse health effects (Atkinson *et al.*, 2015); iii) the presence of the industries in North Lebanon and its being the largest employer in the area and therefore a backbone of the region's economy; iv) the increase of cancer incidence in the region as mentioned in *paragraph 1.6.6.2*; v) no community level studies on pollution have been carried out before in the area.

The survey instrument was a questionnaire developed by reviewing and adapting questionnaires from literature (*detailed in paragraph 2.2*). The prepared questionnaire used two scales:

- Perception study: to examine the opinions people express when they are asked to characterize and evaluate air quality and pollution
- Epidemiology study: to study the patterns, causes, and effects of health and disease conditions in defined populations

Therefore, this survey' objectives were:

- Examine the levels of perceived air pollution annoyance and health risk in both an industrialized area and a rural area to establish associations between the perceived annoyance and the specific factors associated to air quality and demographic characteristics
- Show the relationship between respiratory diseases and the industrial activities and provide quantitative data to demonstrate the need to improve air quality
- Confirm the need to conduct a toxicological study in order to identify potential biomarkers of exposure to air pollution (especially to atmospheric particulate matter) and effects associated with early events in carcinogenesis (cytotoxic, genotoxic and epigenetic effects)
- Contribute to the awareness of governments on the harmful effects of air pollution, help articulate policy objectives in risk minimization, and contribute to the design of programs for the evaluation of risk management and organizational structures to identify, monitor and control risks

2.2 Study design and Procedures

The questionnaire (Appendix 1) contains five sections: personal information, general environmental concerns, health state, past illnesses, family history and children health. Data for this study was collected face to face between the pollster and the subjects. Some questions in the second part (general environmental concern) of the survey are based on the survey conducted in Dunkerque (France) in 2008 (Zwarterook, 2010). Health state and past illnesses questions were based on the standardized questionnaire ATS-DLD-78 prepared by the British Medical Research Council (Fletcher *et al.*, 1959).

The interviewers were trained to be articulate, able to read the questions out loud easily and able to follow the instructions. They became familiar with the flow of questions. All the scientific

terms used in the questionnaire were defined with a precise written definition so that the respondent would not doubt the meaning of the question, and for the interviewer to know the exact meaning of each term. The investigators tried to have a homogenous sample of subjects. The filter characteristics adopted that the subjects should be older than 18 years old and that the subjects should be in the particular address for more than 2 years in order to ensure a representative picture of people's views living in a permanent way in the villages. For people that did not accept to participate in the survey, the interviewer noted on an extra sheet the place of residence, age, sex, and occupation of these persons. In a same family, the pollster interviewed each member alone and wrote down any comments that could be made to complete a closed question.

In order to minimize the bias: i) the investigators tried to have two paired samples, rural and industrial (without differences in the socio-demographic variables); ii) the whole questioning period was not more than 2 months (July-August 2015); iii) the survey was done in parallel in the two sites during the weekdays, from Monday till Friday; iv) every pollster interviewed the same number of people in the two sites; v) the data entering in the statistical software was done regularly and by the same person. A total of 310 questionnaires were filled in each area. All quantitative analyses were done using SPSS (Statistical Package for the Social Sciences, IBM France). Bivariate analysis was performed to compare variables and to identify characteristics related to perceptions. Chi-square (χ^2) test was used to test for the correlation, as all variables were categorical. Statistical levels of significance were set at 0.05. Multivariate analysis revealed factors associated with risk perception of the study population. The odds ratio and the relative risk were calculated for the epidemiology variables with their 95% confidence interval.

2.3 Results and discussion

2.3.1 Air pollution perception

2.3.1.1 Statistical analysis

The first section of the interview is based on obtaining the personal information of the respondents. The information gathered comprised: staying duration in the city of residence, if ever lived in an urban area, socio-demographic characteristics (age, sex, marital status); present occupation; occupational history (exposure to dust, gas or chemical fumes); current and past smoking habits (cigarette and shisha smoking, age of starting, smoking number of cigarettes or bowls of shisha, age of quitting); and indoor pollution (heating). In the early part of the interview respondents were asked how long they have lived in the particular address (< 2 years, 2-5 years and > 5years), if they were present in this particular address in a permanent or temporary way (and if in a temporary way, what was the second city of residence), and if they ever lived in Beirut or Tripoli (and if yes, how

long). These socio-demographic variables are important because they affect the perception on the environmental problems and the bias is minimized by having two paired samples, rural and industrial.

All the interview respondents (100%) on the industrial area and 98.7% of the interview respondents in the rural area lived in their particular address for more than 5 years. The difference between the two sites was not significant ($p= 0.134$). Most of the respondents were present in a permanent way and there was no difference between the two areas ($p= 0.157$). The number of respondents who lived in Beirut or Tripoli was low and similar in the two areas ($p= 0.101$).

The occupations were classified in five categories: primary for the outdoor professions, secondary for professions in the industries, tertiary for the indoor professions, the fourth category for those who do not work or are retired, and the fifth category for students. No significant difference was shown between the two samples (industrial and rural) regarding gender distribution ($p= 0.260$), age range distribution ($p= 0.889$), marital status ($p= 0.410$), respondents that have or do not have children ($p= 0.872$) and occupation categories ($p= 0.074$); therefore the two samples are homogenous (Table 2.2).

Concerning occupational history, 16.8% and 12.9% were exposed to a dusty job for a year or more in the industrial and rural area respectively (Table 2.3). Among the exposed respondents, 9.0% and 6.8% of the respondents answered that the exposure was mild, 6.5% and 5.2% answered that the exposure was moderate, 1.3% and 1% answered that the exposure was severe, in the industrial and rural area respectively. The difference between the two populations was not significant regarding the percentage of exposed respondents to dust ($p= 0.175$) and the level of exposure ($p= 0.989$). Only 1.6% and 1% of the respondents were exposed to gas or chemicals fumes, in the industrial and rural area respectively. Among the exposed respondents, 1.3 % and 0.6% of the respondents claimed that the exposure was mild, 0.3% and 0.6% answered that the exposure was moderate, and 1.6% and 1.0% answered that the exposure was severe in the industrial and rural area respectively. The difference between the two populations was not significant regarding the percentage of exposed respondents to gas or chemicals fumes ($p= 0.477$) and the level of exposure ($p= 0.760$) (Table 2.3).

When asking the inhabitants if they ever smoked, 28.7% and 19.4% of the respondents in the industrial and rural area respectively were cigarette smokers, and 28.4% and 19% out of the smokers are still smoking, in the industrial and rural area respectively. There is a significant difference between the industrial and the rural sample in the percentage of cigarette smokers ($p= 0.006$) with a higher percentage in the industrial area, and even in the percentage of the respondents that are still smoking ($p= 0.006$). Most of these are heavy smokers with no significant difference between the industrial and rural site. 14.6% and 13.3% smoke from less than 5 years and 85.4% and 86.7% smoke for 5 years or more in the industrial site and rural site respectively ($p= 0.513$). 41.6% and 39% smoke less than 140 cigarettes per week, 49.4% and 52.5% smoke between 140 and 300 cigarettes per week and 9.0% and 8.5% smoke more than 300 cigarettes per week in the industrial and rural site respectively ($p= 0.934$). Since shisha is also a cultural practice of tobacco consumption in Lebanon,

respondents were asked about it. 20.3% and 15.2% of the respondents in the industrial and rural area respectively were shisha smokers and still smoking. On the other side, there is no significant difference in the percentage of shisha smoking respondents between rural and industrial ($p= 0.093$) and in the percentage of the persons that are still smoking ($p= 0.114$). 36.7% and 28.3% smoke for less than 5 years and 61.3% and 71.7% smoke for 5 years or more, in the industrial site and rural site respectively ($p= 0.177$). 61.3% and 43.5% smoke less than 4 bowls per week, 35.5% and 52.2% smoke 4 to 8 bowls per week and 3.2% and 4.3% smoke more than 8 bowls per week, in the industrial and rural site respectively ($p= 0.185$) (Table 2.3). For respiratory diseases including lung cancer, multiple causal factors are relevant and people exposed to more than one risk factor may be subject to risks beyond those anticipated from the individual agents acting alone. Smoking has adverse health effects on the entire lung, affecting every aspect of lung structure and function, including impairing lung defenses against infection and causing the sustained lung injury that leads to chronic obstructive pulmonary disease (COPD) (Rückerl *et al.*, 2011). Tobacco smoking is associated with mortality from lung cancer and this association has been demonstrated by both case history and cohort studies in a variety of countries, and has been thoroughly summarized in innumerable review articles (Anderson, 1964; Cornfield *et al.*, 2009). Tobacco smoking using shisha, also known as arguileh, hookah and waterpipe, is an older form of tobacco consumption traditional of the eastern Mediterranean region. Although shisha tobacco contains the same chemicals found in cigarettes, few studies have assessed the association between shisha smoking and lung cancer. A review of the available evidence showed that waterpipe smoking may be associated with lung cancer (Akl *et al.*, 2010). A recent Indian study found a 6-times elevated risk for lung cancer in shisha smokers, in a region where shisha smoking is popular (Koul *et al.*, 2011). Meo *et al.* showed that different lung function parameters were significantly decreased in shisha smokers relative to their control group (Meo *et al.*, 2014). A review by El-Zaatari *et al.* (2015) showed that the shisha acutely leads to increased heart rate, blood pressure, impaired pulmonary function and carbon monoxide intoxication. Chronic bronchitis, emphysema and coronary artery disease are serious complications of long-term use. Lung, gastric and oesophageal cancer are associated with shisha as well as periodontal disease, obstetrical complications, osteoporosis and mental health problems (El-Zaatari *et al.*, 2015).

Among the 310 respondents in the industrial area, 38.1%, 24.8%, 14.8%, 14.5%, 6.1% and 1.6% used electrical, diesel, wood, gas, hot air and central home heating respectively. Similarly in the rural area, 30.6%, 29%, 21%, 12.6%, 6.1% and 0.6% of respondents used electrical, diesel, wood, gas, hot air and central home heating respectively (Table 2.3). Therefore, the main type of home heating is electrical or diesel in the two areas with no significant difference between the two populations ($p= 0.133$). It was found in multiple studies that the use of fuel and especially solid fuel for heating and cooking is associated with elevated risk of lung cancer (Aoun *et al.*, 2013; Hosgood *et al.*, 2010; Kurmi *et al.*, 2012; Lissowska *et al.*, 2005). Indoor pollutions such as non-electrical heating; wood-smoke, biomass fuel are associated with COPD (Salameh *et al.*, 2012).

Table 2.2: Socio-demographic characteristics of the study participants

Variable	Industrial n=310 (%)	Rural n=310 (%)	p-value
Staying duration in the city of residence			
<2 years	0	2 (0.6)	0.134
2-5 years	0	2 (0.6)	
>5 years	310 (100)	306 (98.7)	
Presence in the city of residence			
Temporary way	0	2 (0.6)	0.157
Permanent way	310 (100)	308 (99.7)	
Ever lived in Beirut or Tripoli			
Yes	42 (13.5)	29 (9.4)	0.101
No	268 (86.5)	281 (90.6)	
Gender			
Male	157 (50.6)	171 (55.2)	0.260
Female	153 (49.4)	139 (44.8)	
Age (yrs)			
18-25	75 (24.2)	65 (21)	0.889
26-35	54 (17.4)	54 (17.4)	
36-45	61 (19.7)	61 (19.7)	
46-55	59 (19)	65 (21)	
above 55	61 (19.7)	65 (21)	
Marital status			
Single	132 (42.6)	121 (39)	0.410
Married	152 (49)	170 (54.8)	
Widowed	21 (6.8)	14 (4.5)	
Separated/Divorced	5 (1.6)	5 (1.6)	
Have Children			
Yes	157 (50.6)	155 (50)	0.872
No	153 (49.4)	155 (50)	
Present occupation			
Primary (outdoor)	70 (22.6)	78 (25.2)	0.074
Secondary (industries)	11 (3.5)	3 (1)	
Tertiary (indoor)	120 (38.7)	100 (32.3)	
Don't work	62 (20)	72 (23.2)	
Student	47 (15.2)	57 (18.4)	

Table 2.3: Subject's exposure to active smoking, occupational toxins and indoor pollution

Variable	Industrial n=310 (%)	Rural n=310 (%)	p-value indus/rural
Exposed to dusty job for a year or more			
Yes	52 (16.8)	40 (12.9)	0.175
No	258 (83.2)	270 (87.1)	
Level of the exposure			
Mild	28 (9)	21 (6.8)	0.989
Moderate	20 (6.5)	16 (5.2)	
Severe	4 (1.3)	3 (1)	
Exposed to gas or chemicals fumes			
Yes	5 (1.6)	3 (1)	0.477
No	305 (98.4)	307 (99)	
Level of the exposure			
Mild	4 (1.3)	2 (0.6)	0.760
Moderate	1 (0.3)	1 (0.3)	
Severe	5 (1.6)	3 (1)	
Ever smoked cigarettes			
Yes	89 (28.7)	60 (19.4)	0.006
No	221 (71.3)	250 (80.6)	
How long (yrs)			
< 5	13 (4.6)	8 (3.3)	0.513
= or > 5	76 (85.4)	52 (86.7)	
Number of cigarettes per week			
< 140	37 (41.6)	23 (39)	0.934
140-300	44 (49.4)	31 (52.5)	
> 300	8 (9)	5 (8.5)	
Still smoking			
Yes	88 (28.4)	59 (19)	0.006
No	222 (71.6)	251 (81)	
Number of cigarettes per week			
< 140	36 (41.4)	21 (37.5)	0.884
140-300	43 (49.4)	30 (53.6)	
> 300	8 (9.2)	5 (8.9)	
Ever smoked shisha			
Yes	63 (20.3)	47 (15.2)	0.093
No	247 (79.7)	263 (84.8)	
How long (yrs)			
< 5	24 (36.7)	13 (28.3)	0.177
= or > 5	38 (61.3)	33 (71.7)	
Number of bowls per week			
< 4	38 (61.3)	20 (43.5)	0.185
4-8	22 (35.5)	24 (52.2)	
= or > 8	2 (3.2)	2 (4.3)	
Still smoking			
Yes	62 (20)	47 (15.2)	0.114
No	248 (80)	263 (84.8)	
Number of bowls per week			
< 4	39 (62.9)	17 (37.8)	0.037
4-8	20 (32.3)	24 (53.3)	
= or > 8	3 (4.8)	4 (8.9)	
Heating home			
by gas	45 (14.5)	39 (12.6)	0.133
by wood	46 (14.8)	65 (21)	
by diesel	77 (24.8)	90 (29)	
electrically	118 (38.1)	95 (30.6)	
by hot air	19 (6.1)	19 (6.1)	
centrally	5 (1.6)	2 (0.6)	

The second section of the questionnaire was about the perception on general environmental concerns. When asking the inhabitants about the three major environmental problems in Lebanon, significant differences were found between the two sites ($p < 0.05$) (Figure 2.1). 82.0% of the industrial site respondents chose air pollution, 16.5% chose water pollution and 3.5% chose soil pollution as the first major environmental problem. Whereas, 58.3% of the rural site respondents selected air pollution, 33.0% water pollution and 8.7% soil pollution as the first major environmental problem. Therefore air pollution was considered as a major problem according to the two populations with a higher importance given at the industrial site. Environmental water pollution was the second major environmental problem in Lebanon according to the industrial site respondents (56.2%) and the rural site respondents (36.0%). Soil pollution was chosen as the third major environmental problem in Lebanon at both sites (Figure 2.1).

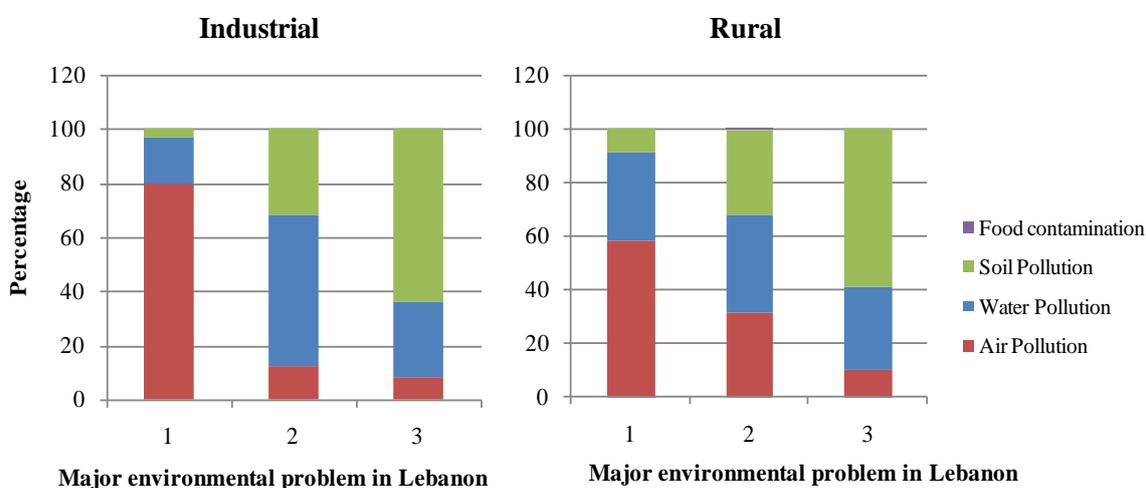


Figure 2.1: Major environmental problems in Lebanon

First major problem= 1, second= 2, third= 3

When asking the inhabitants about the air quality in Northern Lebanon, there was also a significant difference in the responses of the two sites ($p < 0.05$) (Figure 2.2). 44.5% of the respondents in the industrial site considered the air quality to be poor whereas 68.4% of the respondents at the rural site claimed it was good and 10.6% that it was very good quality. These answers may be influenced by the fact that industrial respondents could see industrial waste and releases. This influence was verified in the next set of questions.

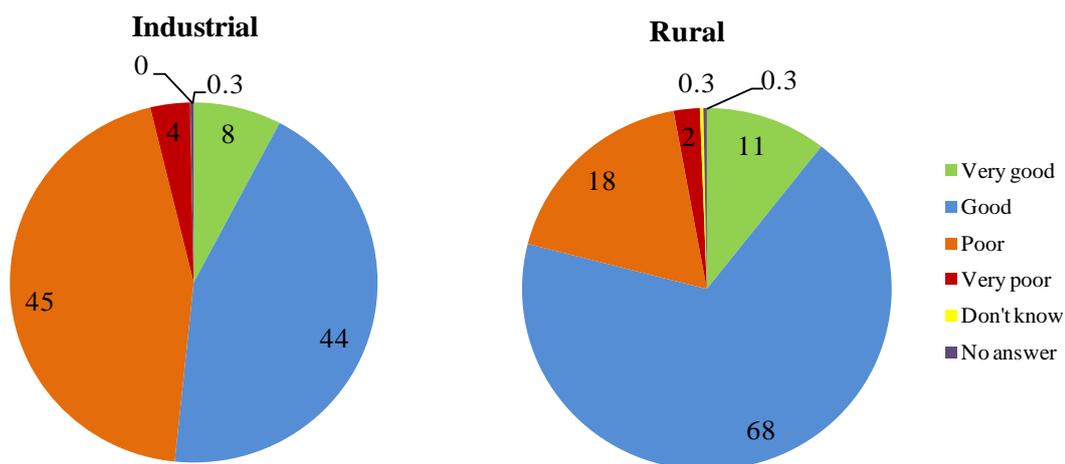


Figure 2.2: Air quality in Northern Lebanon

In the two sites, most of the respondents perceived air pollution as dust, followed by smoke then odor (Figure 2.3). There was a significant difference in the responses between the two sites ($p < 0.05$). 16.5% of the respondents in the rural site had “no answer” since they perceived their site free of air pollution. These respondents could not see the industries nor the quarries since their village is separated from industries by mountains. Similar results were obtained in a study done in Dunkerque (France) and Vitoria (Spain), two industrialized sites, where respondents also perceived dust as the main form of air pollution (Machado De Melo, 2015). According to Hyslop (2009), the annoyance caused by particulate matter is related to the perception of the amount of dust in residential and urban areas (Hyslop, 2009).

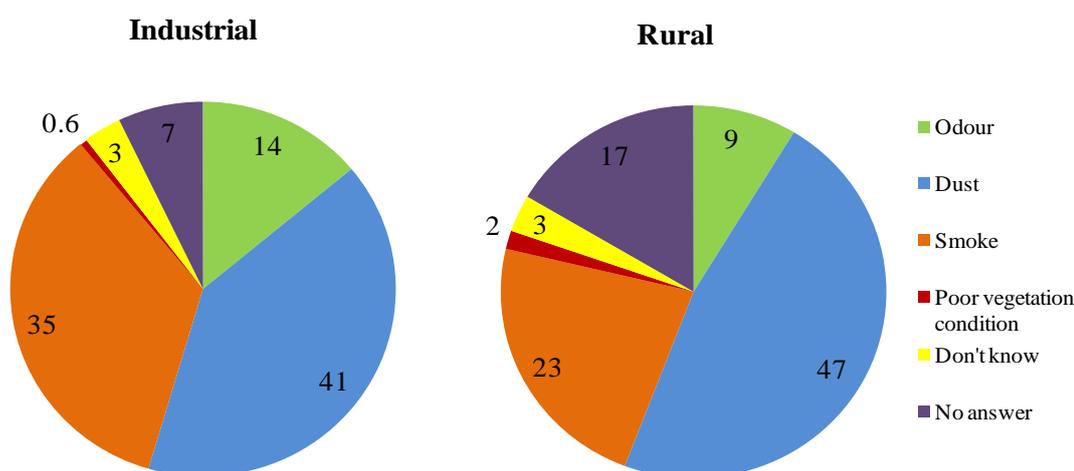


Figure 2.3: Perception of air pollution

When asked how often the inhabitants notice the presence of air pollution in their region, the difference between the answers on the two sites was significant ($p < 0.05$) (Figure 2.4). In the industrial site, 48.7% of the respondents answered “often”, 22.9% “seldom” and 20% “always”.

38.1% of the rural area respondents answered “often”, 37.7% “seldom” and 11.9% “never”. These answers were affected by the distances separating both the industrial area and the rural area from the industries, and the fact that the rural villages are protected by a series of mountains. If these answers are linked to the first question in this section, 79.0% of the rural respondents find that the air quality in Northern Lebanon is good or very good, thus only 5.5% answered that they “always” noticed the air pollution.

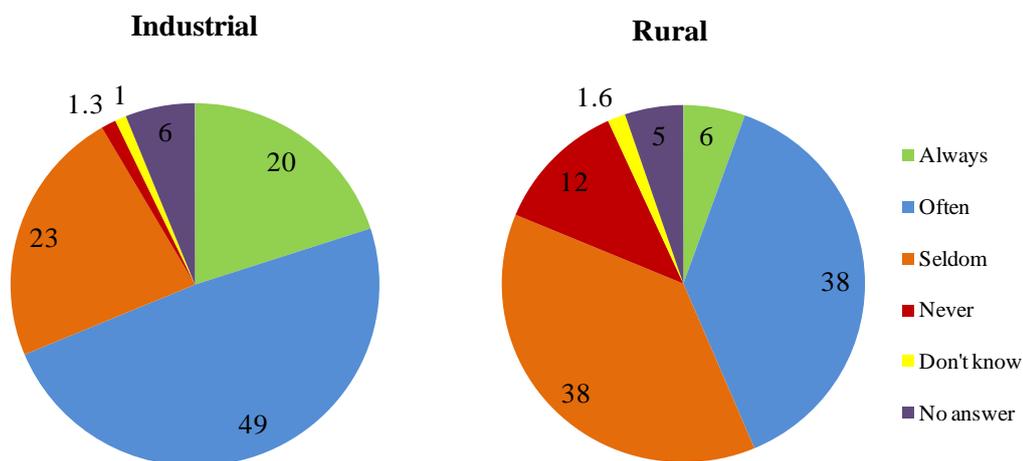


Figure 2.4: Air pollution frequency in each area

According to most of the inhabitants of the industrial area (45.5%), the air quality in their village is worse than other villages in the North; 25.5% perceive it better and 18.7% perceive it as similar to other villages in the North. According to most of the inhabitants of the rural area (50.0%), the air quality in their village is better than other villages in the North; 21.0% perceive it much better and 13.5% perceive it as similar to other villages in the North. The difference between the answers on the two sites was significant ($p < 0.05$) (Figure 2.5). The industrial discharges may have influenced the respondents' answers in the industrial site.

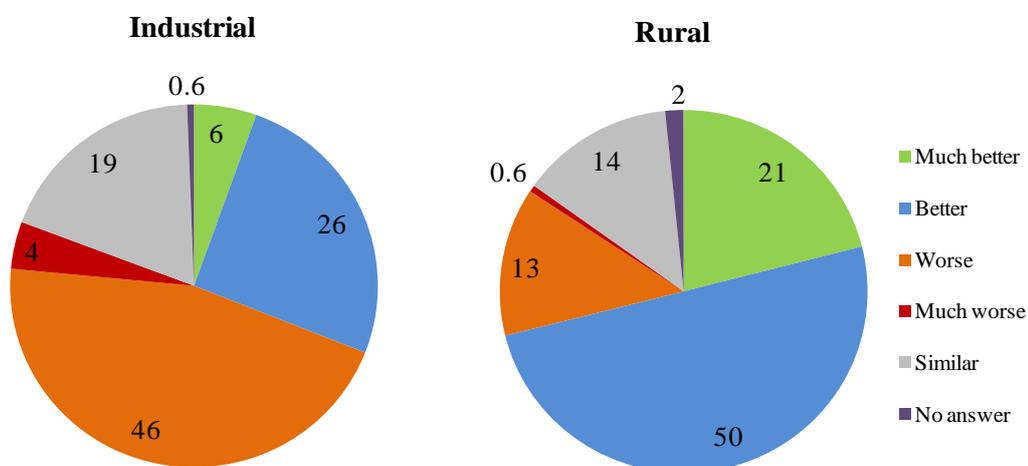


Figure 2.5: Air quality in their areas compared to other villages in the North

In the industrial site, 39.7% of the inhabitants feel the pollution during the day and at night and 36.5% feel it mostly at night. On the other hand, in the rural site, 36.8% feel the pollution at day and night, 25.2% had “no answer” and 8.4% do not know, since they believe that their region is not polluted. The difference between the two areas is significant ($p < 0.05$) (Figure 2.6). Even at night, the industries present in Chekka and Selaata are active and release black smoke; the inhabitants at the industrial site can directly see these discharges.

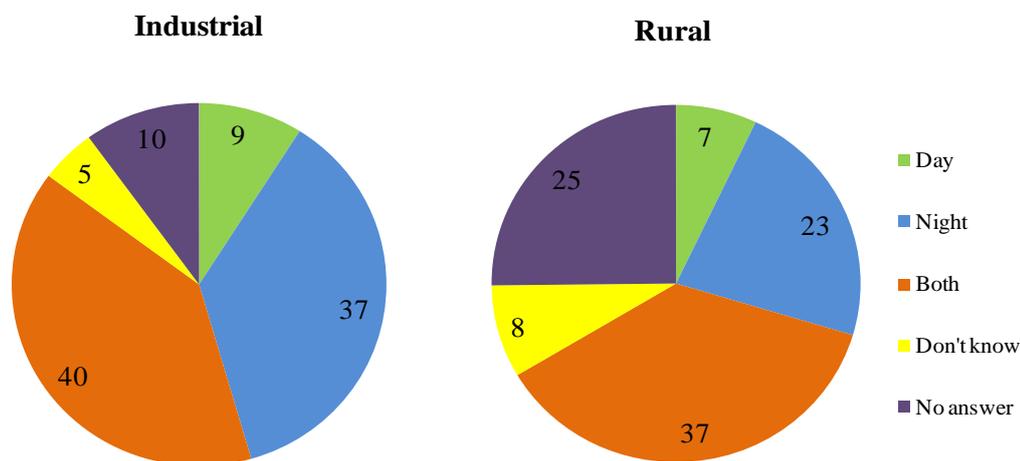


Figure 2.6: Period of air pollution highest level

When asked about the effect of weather conditions on air pollution, respondents at both sites perceived that there was more pollution in summer than in winter (Figure 2.7). The percentage of “no answer” was high at the rural site (18.1%). The difference between the two areas is significant ($p = 0.017$). Same results were obtained in Dunkerque where the majority of respondents reported that perceived air pollution can be influenced by weather conditions (season), 45.0% of respondents reported that air pollution is worse during the summer (and only 8.0% in winter) (Machado De Melo, 2015).

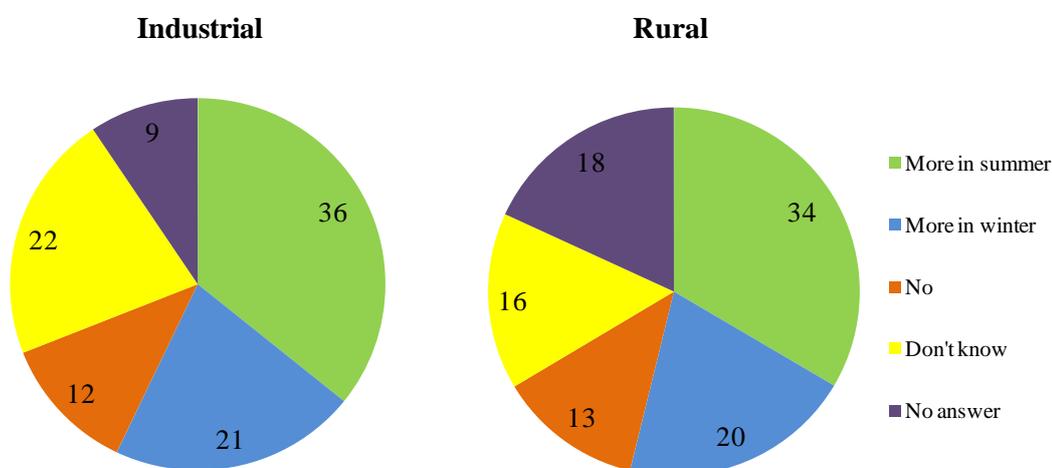


Figure 2.7: Effect of weather conditions on air pollution

According to most of the inhabitants at the industrial site, the impact of air pollution on their health is important (54.8%) or very important (28.1%), only 11.3% answered that the effect is low (Figure 2.8). 49.4% claimed that air pollution affected the health of someone of their family or friends (Figure 2.9). 41.3% of the inhabitants of the rural site answered that the impact is important, 30.0% that it is very important and 18.1% answered by “no impact”. 65.8% of them added that air pollution did not affect the health of any of their family or friends. The difference between the two populations is significant ($p < 0.05$). This question was general and the effect of air pollution is well known to be important, even if the respondents were not exposed directly to air pollution or did not show any health problems. 49.4% of the industrial respondents and 23.2% of the rural respondents claimed that air pollution affected the health of any of their family and friends, therefore industrial respondents presented a double percentage than the rural ones showing that the industrial influence is higher in the industrial area.

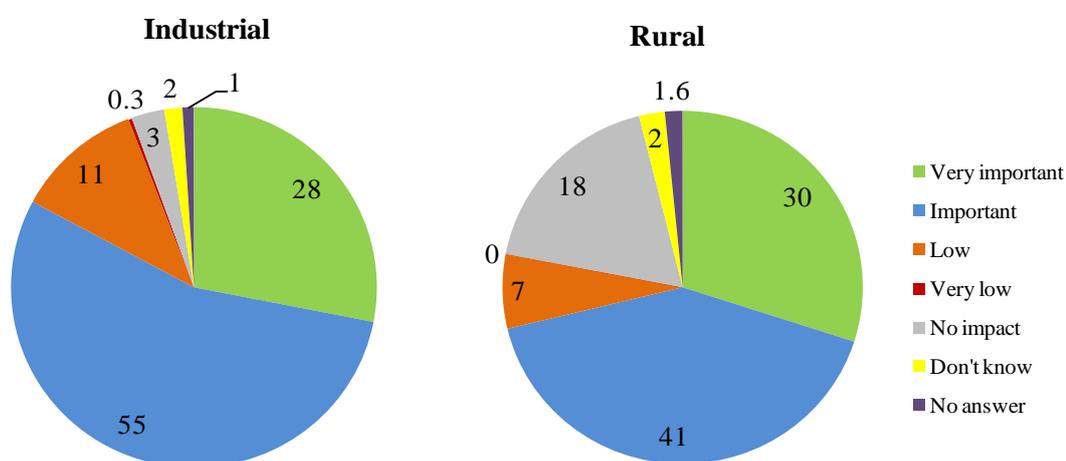


Figure 2.8: Impact of air pollution on the inhabitant's health

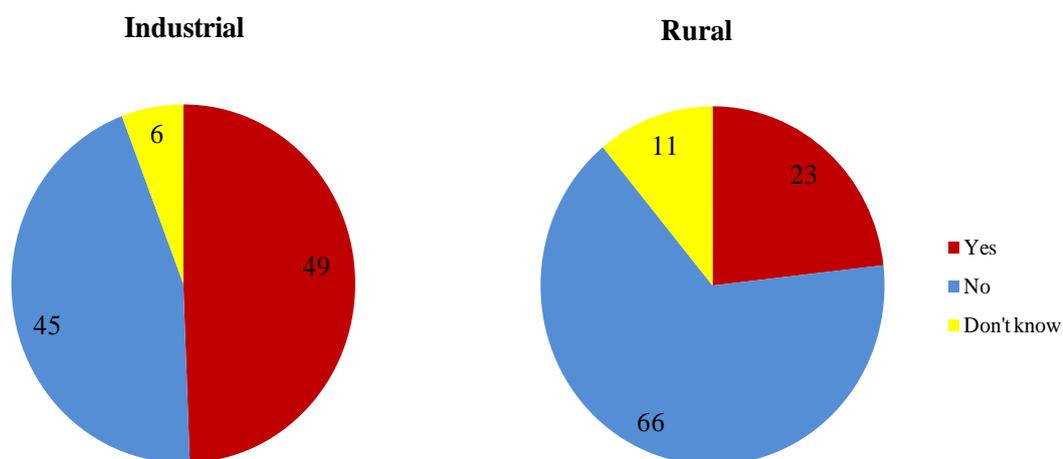


Figure 2.9: Impact of air pollution on the health of any of the inhabitants' family or friends

When the respondents were asked what were the three main sources of air pollution in Northern Lebanon (1 to 3 from most to least important), both populations considered industries as the first main source, transport as the second main source and agriculture as the third main source (Figure 2.10). 29.7% and 25.5% of respondents considered transport, 40.3 % and 50.3% considered industries, 30.0% and 24.2% considered agriculture as the first main source of pollution in Northern Lebanon at the industrial and rural site respectively ($p= 0.042$). 68.0% and 65.8% of respondents considered transport, 21.8% and 23.9% considered industries, 10.2% and 10.3% considered agriculture as the second main source of pollution in Northern Lebanon at the industrial and rural site respectively ($p= 0.818$). 3.8% and 8.9% of respondents considered transport, 38.4% and 27.3% considered industries, 57.8% and 63.8% considered agriculture as the second main source of pollution in Northern Lebanon at the industrial and rural site respectively ($p= 0.002$). A similar result was found by Stenlund *et al.* (2009) who suggested that air pollution perception was related to the proximity to the industry. Therefore, the closer the respondents were living to the industries the more they will perceive this source as the major source of pollution. However, rural inhabitants are informed about the effects of the cement industries and the phosphate fertilizer industries present in the Northern Lebanon on its environment.

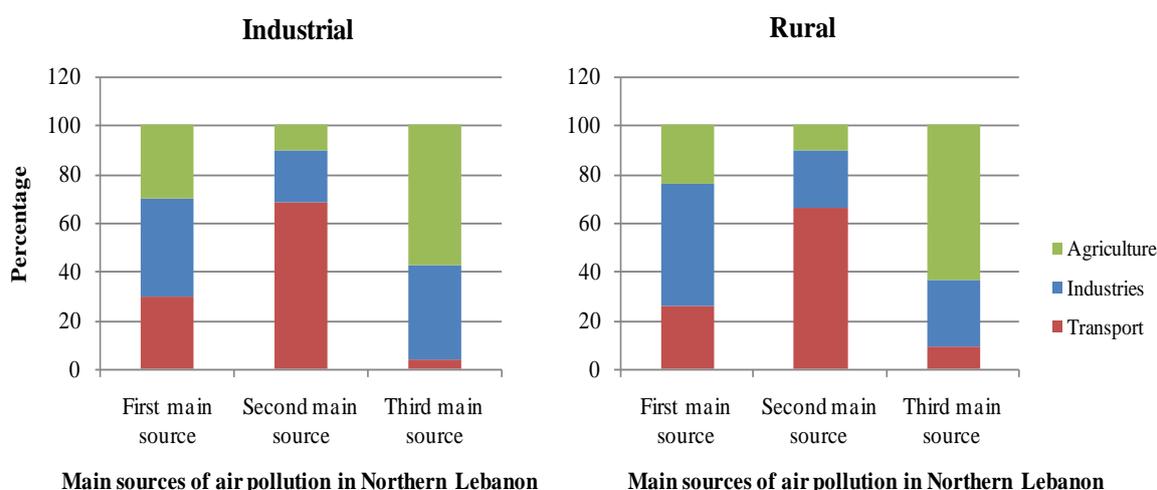


Figure 2.10: Main sources of air pollution in Northern Lebanon

According to the respondents at the two sites, the major impact of the industries was the harmful effect to health and then to environment, and there was no significant difference between the answers in the two areas ($p= 0.844$) (Figure 2.11). This question verifies that according to all respondents industries are the major source of pollution in the Northern Lebanon and that their major impact was that they are harmful to health and to the environment. Moreover, most of the employers of the industries located in the North are from the villages surrounding it, where this survey was done; therefore answers on questions related to industries and the effects of their releases may not be objective.

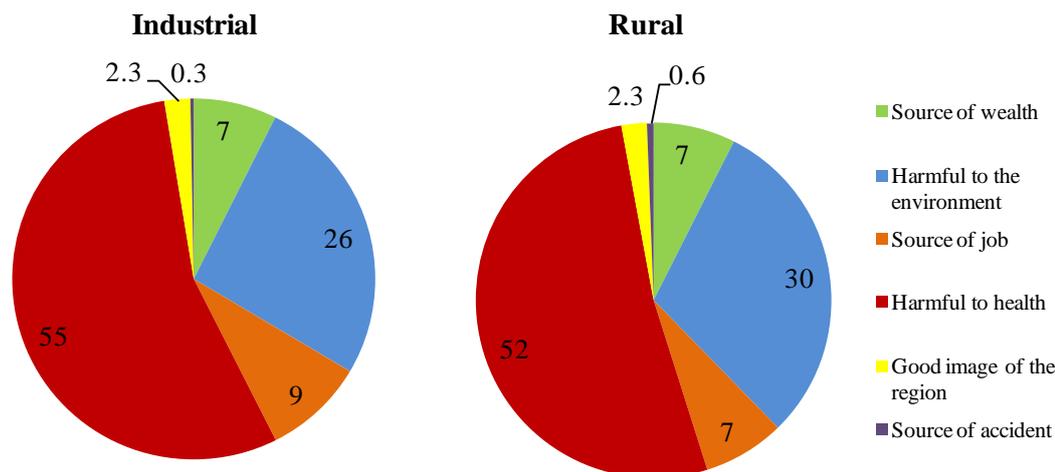


Figure 2.11: The impact of the industries

From the survey, it could be concluded that: people know what air pollution is; they know that air pollution is a major environmental concern in Lebanon; they know that the longer they are exposed to air pollution; the greater the risk they will suffer from air pollution related diseases; they know that clear air is better for human health; and they understand the impact of industrial emissions on health and on the environment.

After further analysis, it is clear that there were significant differences between the inhabitants' perceptions in the two areas, and therefore an indicator of the real situation in these areas. The first finding discusses that most of the industrial inhabitants perceive that air quality in Northern Lebanon is poor and that the air quality in their village is worse than other villages in the North, whereas most of the rural inhabitants perceive that the air quality in Northern Lebanon is good and it is better in their village than other villages in the North. The public's perception of air quality is not always a reliable indicator of the actual levels of air pollution in their area (Williams and Bird, 2003). In our study, there was a real difference in fine air particles concentrations between the two studied sites. The mean concentration of $PM_{2.5}$ was $36.5 \mu\text{g}/\text{m}^3$ in Zakroun (industrial influence) and $22.8 \mu\text{g}/\text{m}^3$ in Kaftoun (background site).

In the section about people's feelings and perceptions on air pollution, at the two sites, most of the respondents perceived air pollution mostly as dust, then as smoke, then odor. In both areas, people mostly selected summer season as the most polluted. The inhabitants of the industrial site felt the pollution during both day and night and mostly the last time. In the rural area, a high percentage of the respondents did not answer, since they did not perceive their area as air-polluted.

Similar to other studies, most of the industrial and rural site inhabitants rated the impact of air pollution on their health as important or very important. However, most of the industrial site respondents added that air pollution affected the health of someone of their family or friends. Whereas, most of the inhabitants of the rural site added that air pollution did not affect the health of any of their family or friends. According to the respondents at the two sites, the major impact of the

industries was the harmful effect to health and then to the environment and these results are similar to those of the study done by Ngo *et al.* (2015), a pilot study that assessed health risk perceptions of air pollution for civic-minded residents in Mathare, Kenya.

Our survey reveals how seriously the industrial public regards air pollution from industrial sources and its effects on their quality of life and health. It also identifies the attitudes of the public towards nuisance from industrial emissions. The responses were affected by many factors: i) some of the inhabitants of the industrial area work or worked in these industries; ii) the industrial area faces directly the factories and the rural area is far away from the industries and surrounded by mountains; iii) the respondents in the rural site perceived their region free from air pollution whereas, the respondents in the industrial site can see and feel the industrial emissions. This survey showed that the majority of the participants from all the occupational groups and ages considered improving air quality essential should be the responsibility of both the government and individual citizen. It is important to note that people who are accustomed to relatively poor air quality may be more sensitive to questions on air quality perceptions.

2.3.1.2 Impact of respondents' gender on air pollution perception

According to the calculated percentages and χ^2 , no significant difference was shown among female and male respondents in the whole population and in the industrial and rural samples (Appendix 2). Only at the industrial site, when asking what are the three major environmental problems in Lebanon (1 to 3 from most to least important), there was a significant difference between female and male answers about the first major environmental problem ($p= 0.022$) and the second major environmental problem ($p= 0.011$). Even if both women and men perceived in the same order (air, then water and soil pollutions), more females perceived air pollution as a first major environmental problem than males. Some studies showed that men tend to have lower knowledge of air pollution as compared to women (Hema and Jamal, 2004; Liao *et al.*, 2015) and that women reported feeling more annoyed than men (Machado De Melo, 2015). Other studies showed that male respondents were more familiar with air pollution and environmental conditions because more males than females work outside the house (Elosta *et al.*, 2013).

2.3.1.3 Impact of respondents' age on air pollution perception

Respondents' age appears to have a significant importance on air pollution perception in both areas. People with different age did not share the same perceptions about air pollution (Appendix 3). There was no significant difference, due to age, in ranking the major environmental problems in Lebanon and the main source of air pollution in Northern Lebanon. However when the respondents, especially from the industrial site were asked, about the air quality in Northern Lebanon, most of the respondents who answered very good or good, were above 36 years old, and those that answered poor

or very poor were younger. Most of respondents that answered “Don’t know” or didn’t answer were respondents above 46 years old. When asked how the respondents perceived air pollution, only young respondents (18-25 years old) answered “poor vegetation condition”. When asked if the air quality in their village was better or worse than other villages in the North, respondents of different age replied in a similar way but at the rural area, there was a significant difference between different ranges of age ($p= 0.021$). Respondents at different ages perceived pollution time (day/night) differently in the industrial ($p= 0.013$) and the rural ($p= 0.031$) sites. There was a significant difference, at the rural site, among respondents with different ranges of age ($p= 0.011$) with respect to the effect of weather conditions. Mostly respondents above 55 years did not answer this question. When asked if air pollution ever affected the health of any of the respondents’ family or friends, the respondents above 46 mostly answered “No” and younger respondents answered “Yes”. At the rural area only, there was a significant difference between the different ranges of age when asked about the industries in the Northern Lebanon ($p= 0.007$), and most of who considered the industries as a source of wealth were respondents above 55 years old. Most of those who did not answer throughout the perception section of the survey were respondents above 55 years old. Furthermore, the younger population and students reported relatively high levels of annoyance, appear to be more aware about environmental issues, and are more conscious of air pollution. Fischer *et al.*, (1991) conducted a study in USA and found that young people were more concerned about environmental issues, while older respondents were more likely to emphasize health and safety issues.

2.3.1.4 Occupational Impact on Perception of Air Pollution

For the treatment of the results, different occupations were classified into five groups: i) primary including people who work outside; ii) secondary including people working the industries; iii) tertiary including people working inside; iv) people who don’t work; v) students. In general and for the 620 respondents on the sites, people from different occupations did not have the same perceptions about air pollution except when asked about their opinion about the impact of air pollution on their health. All the respondents answered that the impact on health is important. Similarly for the industrial sample, significant differences were found between different occupational categories. However in the rural area, people from different occupations had the same perceptions about air pollution except when asked about how they perceived air pollution (Appendix 4). The comparison was harder due to the low number of respondents per occupational category. It was evident that the secondary class of occupations, corresponding to people who work in industries had a different perception on air pollution, especially in the industrial area. For example when asking about the air quality in Northern Lebanon, only the secondary class respondents answered good as a first choice. Answers on the last perception question also showed that 63.6% of the industrial respondents corresponding to the secondary occupational group stated that the industries are a source of wealth, whereas the other groups reported that it was harmful to health and the environment. People

corresponding to the tertiary class (working inside) perceived air pollution differently, 55% of the industrial respondents whereas 20% of the rural respondents perceived air quality in Northern Lebanon as poor.

2.3.2 Air pollution epidemiology

The second part of the survey revealed an association between air pollution and health status. This part studies the prevalence of symptoms in populations at the selected sites, the relationship of these symptoms to one another, and to the effect of industries. The odds ratio (ORs) and the relative risks (RRs) were calculated for the variables with their 95% confidence interval. The ORs and RRs were calculated to show the relationship between health status and rural and industrial sites, duly adjusting for matching factor such as the sex and other potential confounding variables such as smoking.

2.3.2.1 Prevalence of respiratory symptoms

Health and more particularly pulmonary problems were investigated by asking a number of questions about cough, expectoration, and other symptoms.

Cough and phlegm - In recording replies to questions 1-3 on cough and phlegm the interviewers were instructed to answer in order to determine the grade of the symptoms and the severity of the case. When comparing rural and industrial respondents (Appendix 5), little increase of recorded cough and phlegm production at the industrial area was noticed but this increase was not statistically significant. Only two industrial respondents out of 310 had periods of increased cough and phlegm lasting for 3 weeks or more each year whereas, none on the rural site had such symptom.

Breathlessness on Exertion or **shortness of breath** - Subjects were classified into five grades of breathlessness on their replies to question 6 in this section similar to those described by Fletcher (1952). Grade 1 is considered in case of shortness of breath when hurrying or walking up a slight hill. Grade 2 is considered if the person has to walk slower than people of his age because of breathlessness. Grade 3 is when the person has to stop for breath when walking at his own pace. If the person ever has to stop for breathing after walking about 100 meters (or after a few minutes), this subject is classified as Grade 4. Moreover, Grade 5 is considered when the person is too breathless to leave the house or breathless while dressing or undressing. There was a significantly greater prevalence of breathlessness of grades 1 to 4 in the industrial inhabitants than in the rural ones. A slight but not significant excess of grade 5 in the industrial respondents compared with the rural ones (Appendix 5).

Other Symptoms - A number of respondents admitted to occasional wheezing apart with colds. Wheezing was higher with colds in the industrial site compared to the rural site. More industrial respondents admitted that they had a wheezing attack that had made them feel short of breath, had two

or more such episodes and some even required medicine or treatment for these attacks. When the inhabitants were asked if when they get a cold it usually goes to their chest, or if during the past 3 years they had any chest illnesses that have kept them off work, or if they produce phlegm with any chest illnesses, higher positive answers were obtained at the industrial site, but this difference is not statistically significant.

In summary, the main difference between the two sites was the shortness of breath, and only this symptom was significantly more prevalent in the industrial site. These results are similar to studies performed in industrial cities such as in the polluted industrial estate of Vapi in India which suggested significant respiratory morbidity among residents that was associated with age and distance from the development corporation (Patel *et al.*, 2008). A study done by Zhang *et al.* showed a significant increase in the prevalence of respiratory symptoms such as coughing, wheezing, and decreased pulmonary function. Experimental exposure research and epidemiological studies have indicated that exposure to particulate matter, ozone, nitrogen dioxide, and environmental tobacco smoke have a harmful influence on development of respiratory diseases and are significantly associated with cough, wheeze and shortness of breath (Zhang *et al.*, 2015).

In particular, PM_{2.5} and PM_{0.1} interact with alveolar epithelial cells and pulmonary alveolar macrophages and can directly reach the small airways and pulmonary alveoli. Additionally, nano-sized, ultrafine particles can directly infiltrate pulmonary alveoli and enter the bloodstream, which may produce a harmful effect on different body organs (Nemmar *et al.*, 2002). High concentrations of PM_{2.5} are a major factor in the development of haze and also of respiratory or other diseases (Zhang *et al.*, 2015). Nightingale *et al.* showed that controlled indoor exposure of normal subjects to 200 µg/m³ diesel exhaust particles led to neutrophilic inflammation and neutrophil release (Nightingale *et al.*, 2000). In a study on asthma patients walking along a congested London street for 2 h, a decline in FEV₁ (forced expiratory volume in 1 second) and pulmonary neutrophilic inflammation indicated that these effects were associated with exposure to organic carbon and ultrafine particles (McCreanor *et al.*, 2007). Results of the cross-sectional study done by Ho *et al.* which investigated the prevalence of irritative symptoms and chronic respiratory symptoms among 109 workers exposed to PM as a result of incense burning in a temple in Taiwan, and 118 unexposed workers in a control group, indicated that chronic cough was common in the exposure group and that working in a temple increased the risk of acute irritative symptoms of the nose and throat (Ho *et al.*, 2005). A study on 36 children with asthma and exposed to air pollution showed a longitudinal association between PM_{2.5} from indoor and outdoor sources with cough and wheeze symptoms (Habre *et al.*, 2014). This study using a hybrid model linked daily signs of cough and wheeze with PM_{2.5} exposure; the odds ratios with an standard deviation increase in PM_{2.5} from indoor sources were 1.24 (cough) and 1.63 (wheeze), respectively. Ozone (O₃) levels correlated with wheezing, and cough correlated with indoor PM_{2.5} from ambient sources. In Switzerland, a study was performed by Schindler *et al.* on 7019 subjects tested at baseline in 1991, followed up in 2002, and exposed to PM₁₀ during the 12 months before each health

evaluation. The mean exposure to PM₁₀ in 2002 was not higher than that in 1991 and the results of that study indicated that symptoms caused by exposure to PM₁₀ were as follows: fewer than 259 subjects had regular cough, fewer than 179 subjects had chronic cough or sputum, and fewer than 137 subjects had wheeze and shortness of breath (Schindler *et al.*, 2009). However, in an experiment on children aged 24 months in New York City from 1998 to 2006, increased ambient nickel and vanadium levels were significantly related to wheeze, and increased elemental carbon levels were significantly related to cough during cold and flu season; however, total PM_{2.5} did not show an association with wheeze or cough (Patel *et al.*, 2009).

Furthermore, air pollutant gases have a significant association with respiratory symptoms, including cough and wheeze. Hoffmeyer *et al.* showed that there were lower cough thresholds with enhanced exposure to O₃ (Hoffmeyer *et al.*, 2013). Belanger *et al.* (2013) indicated that each 5 µg/L increment (based on a threshold of 6 µg/L) in NO₂ exposure was associated with a dose-dependent increment in asthma severity including night symptoms, rescue medication use, and wheezing. Children exposed to indoor NO₂ at levels below the US EPA outdoor standard (53 µg/L) are at risk for enhanced asthma morbidity (Belanger *et al.*, 2013). A cross-sectional study on 10-year-old children showed that elevated PM₁₀ and carbon dioxide (CO₂) levels present during normal daily activities were associated with a dry cough at night, wheezing, and rhinitis in 654 children and acoustic rhinitis in 193 children (Simoni *et al.*, 2010). Another study demonstrated that exposure to SO₂ activated the transient receptor potential vanilloid 1 (TRPV1) receptor in the nodose ganglia which increased sensitivity and cough as a response to capsaicin (McLeod *et al.*, 2007). Exposure to indoor NO₂ at levels well below the Environmental Protection Agency outdoor standard (53 µg/L) is associated with respiratory symptoms among children with asthma in multifamily housing; This study demonstrated increased wheezing, polypnea, and chest distress in study participants (Belanger *et al.*, 2006). Different studies reveal that respiratory symptoms was far greater among children who lived in locations near a busy road, close to a factory or industrial chimney, and also in children who had a coal-burning device or recent renovations in their home, or who had family members who smoked at home (Zhang *et al.*, 2015). A survey across 18 regions in Liaoning Province showed TSP levels ranging from 188 to 689 mg/m³, SO₂ from 14 to 140 mg/m³, and NO₂ levels from 29 to 94 mg/m³. This survey showed an increase in constant cough (21-28%), constant sputum (21-30%), and asthma (39-56%) associated with increments in levels of those air pollutants (172 mg/m³ for TSP, 69 mg/m³ for SO₂, and 30 mg/m³ for NO₂) (Zhao *et al.*, 2011).

Therefore, chest symptoms and shortness of breath were more prevalent in the industrial site than the rural site and industrial pollution could be the reason.

2.3.2.1.a Stratification over gender

The comparison between men and women showed that in general men were more susceptible to air pollution or showed more respiratory symptoms. Relative risk values (shown in Appendix 6) confirmed that the prevalence of each grade of symptom was higher in men such as cough, phlegm, shortness of breath. Significantly more men than women admitted to bring up phlegm of their chest 5.04 times more than women (RR= 5.044; 95% CI: 1.53-18.17). For nearly every symptom studied, the prevalence was higher in men than in women.

Same results were found in a study based on a similar questionnaire that showed that cough and phlegm were significantly more prevalent in men than in women (Fletcher *et al.*, 1959). Dong *et al.* showed that ambient air pollution effects were more evident in males without an allergic predisposition; however more associations were detected in females with allergic predisposition (Dong *et al.*, 2011). Effects of criteria air contaminants on the respiratory system are well documented, and several studies have reported differing outcomes for males and females (Granados-Canal *et al.*, 2005; Luginaah *et al.*, 2005).

When comparing prevalence of symptoms for men and women in the rural and industrial site separately, some differences were shown. More individuals showed respiratory symptoms in the industrial site and this was more expressed in men respondents. According to Bravo *et al.*, health impacts of air pollution may differ depending on sex, education, socioeconomic status, location at time of death, and other factors (Bravo *et al.*, 2016). There is growing epidemiologic evidence of differing associations between air pollution and respiratory health for females and males. Meta-analyses of respiratory effect modification of sex and gender are difficult to complete due to varying exposure mixes, outcomes and analytic techniques, however, more studies report stronger effects among women and girls than among men and boys, but the literature is far from consistent. Importantly, it is unknown whether observed modification is attributable primarily to biological differences between men and women, to exposure differences (e.g., work-related coexposures), or to some interplay thereof. Gender analysis, which aims to disaggregate social and biological differences between men and women (e.g., hormonal status), may help to elucidate this difference, identify key mechanisms, and design more effective interventions (Clougherty, 2010).

2.3.2.1.b Symptoms and smoking habits

The bivariate analysis results (Appendix 7) showed that former cigarette smokers had higher risk of lung problems compared with non-smokers. All symptoms, cough, phlegm, chest whistling and shortness of breath were significantly more common in both the rural and industrial site in smokers than in non-smokers respondents. Smokers showed higher prevalence when asked about the presence of cough (OR= 1.81; 95% CI: 1.02-3.2), if as much as 4 to 6 times a day, 4 or more days of the week (OR= 2.72; 95% CI: 1.35-5.5), if during the rest of the day or at night (OR= 2.58; 95% CI: 1.11-6.01) and if they usually cough like this on most days for 3 consecutive months or more during the year

(OR= 3.02; 95% CI: 1.2-7.58). Significantly higher symptoms were prevalent in smokers than in non-smokers when asked if they bring up phlegm of their chest (OR= 1.38; 95% CI: 1.38-8.29), if as much as twice a day, 4 or more days out of the week (OR= 8.29; 95% CI: 1.59-43.19), if during the rest of the day or at night (OR= 6.59; 95% CI: 1.19-36.35) and if on most days for 3 consecutive months or more during the year (OR= 9.83; 95% CI: 1.01-95.23). There was a significantly greater prevalence of breathlessness of grades 1 to 5 in smokers than in non-smokers and chest whistling (OR= 2.66; 95% CI: 1.29-5.49). The prevalence of symptoms increased significantly with increasing tobacco consumption in both sites. In literature, a consistent relationship was found between cough, phlegm, wheeze, shortness of breath, chronic obstructive pulmonary disease (COPD) and smoking or environmental tobacco smoke (ETS) (Bentayeb *et al.*, 2013; Willemse *et al.*, 2004; Zhang *et al.*, 2015). The effect of smoking on respiratory symptoms prevalence is well known and not surprising; our results confirmed the effect of tobacco consumption on the prevalence of cough, phlegm, chest whistling and breathlessness.

Waked M. *et al.* also showed that a significant percentage of the population of non-smokers could have chronic bronchitis or COPD (Waked M. *et al.*, 2012). Therefore, when considering only the non-smoker population, only the prevalence of cough increased significantly at the industrial site compared to the rural site revealing the presence of another factor than smoking.

2.3.2.2 Past illnesses

The second part is related to past illnesses; therefore the inhabitants were asked if they had any lung problems before the age of 16 years old, attacks of bronchitis, pneumonia, hay fever, chronic bronchitis, emphysema, asthma or any chest illness, chest operations or injuries, heart problems or high blood pressure.

There were some differences between the industrial site and the rural site when the inhabitants were asked about past illnesses (Appendix 8). The industrial inhabitants had a significantly 29-fold risk of having lung troubles before the age of 16 than the rural inhabitants (OR= 29.48; 95% CI: 3.98-218.37) (Section 4 Question 1). There was no significant difference between the two sites when asked about past illnesses such as bronchitis, pneumonia, hay fever, chronic bronchitis, and emphysema. Higher prevalence of respondents in the industrial site had asthma but this increase was not statistically significant (OR= 1.42; 95% CI: 0.62-3.25) and had other chest illnesses (OR= 3.06; 95% CI: 0.82-11.41) such as allergy and chest operations.

When asked about heart problems and high blood pressure, more industrial inhabitants than rural ones had heart problems (OR= 1.29; 95% CI: 0.69-2.41), high blood pressure (OR= 1.51; 95% CI: 0.99-2.31), had treatment for heart problems (OR= 1.23; 95% CI: 0.66-2.31) and treatment for high blood pressure (OR= 1.46; 95% CI: 0.95-2.24) in the past 10 years, but this increase was not statistically significant.

Therefore our results suggest that air pollution could increase asthma and allergy prevalence in the industrial site compared to the rural site but that was not statistically significant, and these results were consistent with many studies. The detrimental effects of air pollution on health have been recognized for most of the last century but the mechanisms of how these pollutants exert their effects are likely to be different. A substantial body of research on the effects of air pollution on asthma has been published in the past 10 years, adding to the body of knowledge that has accumulated over several decades. Complex mixtures like fine particulate matter and tobacco smoke have been associated with respiratory symptoms and hospital admissions for asthma (Goldsmith and Kobzik, 1999; Leikauf, 2002). Kim *et al.* showed that exposure to environmental (both outdoor and indoor) pollutants may partially account for the prevalence of asthma and allergies (Kim *et al.*, 2013). Presently, short-term exposures to ozone, nitrogen dioxide, sulphur dioxide, PM_{2.5}, and traffic-related air pollution (TRAP) is thought to increase the risk of exacerbations of asthma symptoms (Guarnieri and Balmes, 2014). Increasing amounts of evidence also suggest that long-term exposures to air pollution can contribute to new-onset asthma in both children and adults (Gowers *et al.*, 2012). From a mechanistic perspective, air pollutants probably cause oxidative injury to the airways, leading to inflammation, remodeling, and increased risk of sensitization. Although several pollutants have been linked to new-onset asthma, the strength of the evidence is variable (Guarnieri and Balmes, 2014). A study done in proximity of a major industrial park in Oman confirmed that living within 10 km from an industrial zone showed a greater association with acute respiratory diseases (RR= 2.5; 95% CI: 2.3-2.7), asthma (RR= 3.7; 95% CI: 3.1-4.5), conjunctivitis (RR= 3.1; 95% CI: 2.9-3.5) and dermatitis (RR= 2.7; 95% CI: 2.5-3.0) when compared with the control zone (Alwahaibi and Zeka, 2015).

Furthermore, our results showed an increase in heart problems in the industrial site but this increase was not statistically significant. A strong epidemiologic association is observed between acute and chronic exposures to PM and the occurrence of pulmonary diseases, cardiovascular events, coronary artery disease, cerebrovascular disease and venous thromboembolism, especially among older people and people with diabetes and previous cardiovascular conditions. The most serious effects of air pollutants are related to PM, because that contain a broad range of toxic substances and are considered reliable indicators of other pollutants, such as nitric oxides, and hence of the global adverse effect of air pollution (Brook, 2008; Schwarze *et al.*, 2006). PM has been associated with increased plasma viscosity (Peters *et al.*, 1997); changes in the characteristics of the blood (Franchini and Mannucci, 2011); and anomaly indicators of autonomic function of the heart including increased heart rate, decreased heart rate variability, and increased cardiac arrhythmias (Sun *et al.*, 2010). These findings provide possible pathways by which PM affects the cardiopulmonary system. Studies confirmed that the association between particulate matter and mortality risks of cardiopulmonary diseases was stronger than all-cause mortality risk (Bateson and Schwartz, 2004; Chen *et al.*, 2012). From an array of experimental, epidemiologic, and clinical studies it emerged that air pollution is an important acquired cardiovascular risk factor that adds to the traditional ones. Alterations

induced by exposure to particulate air pollution contribute, in the long term, to the development and progression of atherosclerosis and in the frame of short-term exposures, to triggering thrombosis and acute cardiovascular events. The most frequent cardiovascular diseases (coronary artery disease and cerebrovascular disease) are definitely and consistently increased by PM exposure (Franchini and Mannucci, 2011).

Preliminary evidence suggests that air pollution and PM exposure may be a risk factor for the increase in asthma, allergies and heart problems at the industrial site.

2.3.2.2.a Stratification over gender

The differences between the responses of men and women about their past illnesses were not statistically significant. However, men had a lower prevalence of lung trouble before the age of 16 (RR= 0.42; 95% CI: 0.18-0.92), attacks of bronchitis and the presence of other chest illness such as allergy. However for the rest of the questions related to past illnesses, men showed higher prevalence especially for heart problems and high blood pressure (Appendix 9).

More studies report stronger effects among women and causal mechanisms for this remain not totally elucidated, though experimental and clinical studies have corroborated this assertion and offer at least a partial explanation. Environmental health studies pay particular attention to risk disparities among males and females because some autoimmune disorders show strong sex differences, and furthermore environmental exposures are known risk factors for some of these disorders. For example, lung PM deposition characteristics differ among men and women and the relative amount of deposition of is greater in women due to higher flow rates (Kim and Hu, 1998). Stress can potentiate or attenuate impacts of air pollution, and both hormonal and sociocultural characteristics of sex and gender, respectively, can moderate stress levels (Clougherty and Kubzansky, 2009). Furthermore, as measured by fatigue and pulmonary function, women are slightly more sensitive than men to effects of 2-propanol and *m*-xylene vapors, which are among VOCs released by petrochemical processing and combustion engine exhaust (Ernstgård *et al.*, 2002). During the reproductive period of life, cyclical fluctuations in sex hormones increase the prevalence rates of atopic disease in women, though rates are lower compared to males during childhood and after menopause (Becklake and Kauffmann, 1999). Women have a lower risk of developing asthma in their childhood, equal risk during adolescence, and higher risk during early adulthood, which is attributed to smaller airway caliber and hormonal factors, while air pollution is known to exacerbate asthma (Burra *et al.*, 2009; de Marco *et al.*, 2000; Johnston and Sears, 2006). Interestingly, immunoglobulin E (IgE) serum levels are higher in males throughout life, but sex specific differences in allergen sensitivity are inconsistent. Air pollutants can trigger IgE responses, and a population-based study in London, observed increased primary care consultations for allergic rhinitis in association with outdoor air pollution (Hajat *et al.*, 2001). Sex and gender differences in susceptibility to air pollution are expressed beyond the respiratory system. There is also evidence in support of hormonal status attenuating the effect of

particulate matter on heart disease in women less than 60 years of age (Zeka *et al.*, 2006). Males are at a higher risk of developing lung cancer compared with females (Jemal *et al.*, 2011). Environmental health studies demonstrate significant associations between air pollution exposure profiles and sociocultural, socioeconomic and demographic factors, all of which can be influenced by gender (Jerrett *et al.*, 2005; Oiamo *et al.*, 2011).

2.3.2.2.b Difference in past illnesses prevalence between smokers and non-smokers

When comparing past illnesses prevalence between smokers and non-smokers in the total population (620 respondents), significant differences were shown only when we asked if they had chest illnesses, if they had chest operations, and if they had heart problems and got any treatment in the past 10 years (Appendix 10). It is evident that smokers had a 3-times higher risk of chest illnesses compared with non-smokers (OR= 3.31; 95% CI: 1.05-10.42), 13-times more chest operations (OR= 13.2; 95% CI: 1.46-119.05), 2-times more higher risk of heart problems (OR= 2.02; 95% CI: 1.06-3.86) and 2-times more treatment for heart problems in the past 10 years (OR= 2.01; 95% CI: 1.09-4.03).

When comparing past illnesses prevalence between smokers in the industrial and the rural site, higher prevalence was shown in the industrial site but this increase was not statistically significant. When comparing past illnesses prevalence between non-smokers in the two sites, higher prevalence was shown in the industrial site but this increase was only statistically significant when inhabitants were asked if they had lung trouble before the age of 16 years old (OR= 20.73; 95% CI: 2.74-157.09).

Pollution is the second major cause for many respiratory diseases after smoking (Mihălțan *et al.*, 2015). Our results correspond to that of literature where smoking has been established as the main risk factor for developing respiratory diseases and lung cancer in multiple studies in different countries (Cornfield *et al.*, 2009). Emphysema, respiratory bronchiolitis, desquamative interstitial pneumonia, pulmonary Langerhans' cell histiocytosis and other histologic findings are recognized in cigarette smokers (Franks and Galvin, 2015). Several studies have documented the uptake of several toxicants and carcinogens during waterpipe smoking that is strongly associated with harmful health effects (Bou Fakhreddine *et al.*, 2014). These studies have demonstrated its adverse health effects on many organs but primarily the cardiovascular and respiratory systems where there is documentation of CAD, obstructive pulmonary disease and increased risk to develop lung cancer. In addition, perinatal effects in smoking mothers, periodontal disease and other health effects have been described in this group of smokers (El-Zaatari *et al.*, 2015).

2.3.2.3 Family history and children health

In this section of the questionnaire, inhabitants were asked about their family history: if any of their parents or family members is currently working or worked in an industry, if either of their

natural parents had a chronic lung condition such as: chronic bronchitis, asthma, emphysema, lung cancer or other chest conditions, and if anyone of their family died of lung cancer (Appendix 11). Parents were asked four questions about their children's health: if their child had any chest illness or chest cold, if he/she ever been hospitalized for a severe chest illness or chest cold before the age of 2 years, if a physician stated that their child had an allergic reaction to pollen or dust, and to what extent air quality affected their children's respiratory health (Appendix 11). Significantly higher prevalence of parents or family members at the industrial site worked or are working in the industries (OR= 4.82; 95% CI: 2.8-8.31). Similarly, more prevalence was found for industrial respondents that had their natural parents diagnosed for chronic lung condition such as chronic bronchitis, asthma, emphysema, lung cancer or other chest conditions. Industrial inhabitants had 2-times higher prevalence of members of their family that died from lung cancer (OR= 2.34; 95% CI: 1.36-4.03). The prevalence of children having chest illnesses or chest cold in the industrial site was 13-times higher than in the rural site (OR= 12.67; 95% CI: 1.63-98.69). The prevalence of children having an allergic reaction to pollen or dust in the industrial site was 3-times higher than in the rural site (OR= 3.07; 95% CI: 1.69-5.58). Higher prevalence of parents in the industrial site admitted that air quality seriously affected their children's respiratory health.

Our results revealed that air pollution could have an effect on the prevalence of lung cancer in the industrial site which is consistent with literature. However, a perception study done by Mazières *et al.*, (2015) showed that major proportion considered lung cancer to be a tobacco-induced, life-threatening disease that involved major treatment, and a minor proportion considered it to be an environmentally induced disease. The prevalence of children having chest illnesses or chest cold, and an allergic reaction to pollen or dust in the industrial site was much higher than in the rural site. Air pollution has many effects on the health of both adults and children, but children's vulnerability is unique. Children spend a lot of time outdoors and engage in physical activities that increase their breath rate, which leads to larger deposits of environmental pollutants in the respiratory tract. Higher ventilation rates and mouth-breathing may pull air pollutants deeper into children's lungs, thereby making clearance slower and more difficult. A cohort study showed a higher correlation between exacerbations of asthma and environmental pollution (Gauderman *et al.*, 2007). A study of 352 infants showed that in the first year of life, the morbidity of persistent cough, wheezing, and lower airway infection was 6.3%, 26.1% and 30.4% respectively. With a $10 \mu\text{g}/\text{m}^3$ increment in ambient NO_2 concentration, the adjusted odds ratio of persistent cough was 1.40. These results indicate that when infants are exposed to outdoor air pollution in the first year, the threat of persistent cough is enhanced (Esplugues *et al.*, 2011). As a child's lungs are still growing, early exposure to environmental pollutants can more easily alter lung development and lung function (Esposito *et al.*, 2014). Studies have demonstrated an association between reduced lung growth and PM concentrations, and have shown that air pollution alters lung development (Heinrich and Slama, 2007; Pinkerton and Joad, 2006; Valent *et al.*, 2004). It was found that children with asthma living in an

area with high PM concentrations showed reduced lung growth, and that long-term exposure to NO₂ and PM₁₀ was associated with a small but significant reduction in lung volume and lung function impairment (Gao *et al.*, 2013; Gauderman *et al.*, 2004; Mölter *et al.*, 2013).

Therefore, children are very susceptible to adverse effects from air pollution due to their developing lungs, immature metabolic pathways, high ventilation rates per bodyweight, increased time exercising outdoors, and greater exposure than adults. Even exposure *in utero* might affect postnatal risk of respiratory diseases such as asthma and asthma exacerbations. The largest reported effects are associated with prenatal exposure to PM, nitrogen dioxide, and tobacco smoke (Vieira, 2015).

There was a no significant difference between men and women when asked about their family history and children health (Appendix 12), however women had more “yes” answers to questions related to family history and children health. When asked about family members working in the factories, women had more answers in the industrial site than in the rural site. Women also had a higher percentage of positive answers than men: when asked if their child had any chest illness or chest cold, or had an allergic reaction to pollen or dust. Both men and women answered that air quality seriously affected their children’s respiratory health.

In summary, the second part of the survey indicates:

- higher prevalence of chest symptoms (shortness of breath), respiratory diseases, chest operations, heart problems, high blood pressure cases, chronic lung conditions and mortality by lung cancer were found in the industrial site
- more men than women showed cardio-respiratory symptoms and diseases, but women had more lung trouble before the age of 16, attacks of bronchitis and allergy
- prevalence of respiratory symptoms and illnesses and heart problems increased significantly with increasing tobacco consumption in both sites
- in the non-smoker population, prevalence of cough increased significantly at the industrial site compared to the rural site showing the presence of another factor than smoking
- the prevalence of children having chest illnesses and/or allergy in the industrial site was much higher than in the rural site

2.3.3 Comparison of perception and epidemiological data

Principal Component Analysis (PCA) was performed in order to cross-compare the data obtained in the perception study versus the epidemiological study ones. PCA is an unsupervised multivariate method that allows reduction of the data matrix and evaluation of samples' clustering tendency. All the data analyses were performed using SPSS software (SPSS 20.0, IBM France). This analysis was useful to identify specific profiles between the two datasets. Barycentre of the

distribution were used to represent the centre of the distribution. All data of the study were treated as binary data or assimilate as binary data for PCA.

When correlating the perception data of the population about air quality in Northern Lebanon (Section 2) and their health state (Section 3), the PCA identified two major patterns that explained 74.71% of variation. Principal Component 1 (PC1) was strongly correlated with questions about health pollution impact and PC2 was correlated with question about air quality in Northern Lebanon. Graphical representation of the respondents, based on their answers about health state, allowed us to discriminate two distinct populations. People with lung troubles like chronic bronchitis, asthma, shortness of breath, cough or phlegm generally claimed that air quality is poor or very poor and that it could have a health effect (Total variance explained= 74.1%) (Figure 2.12).

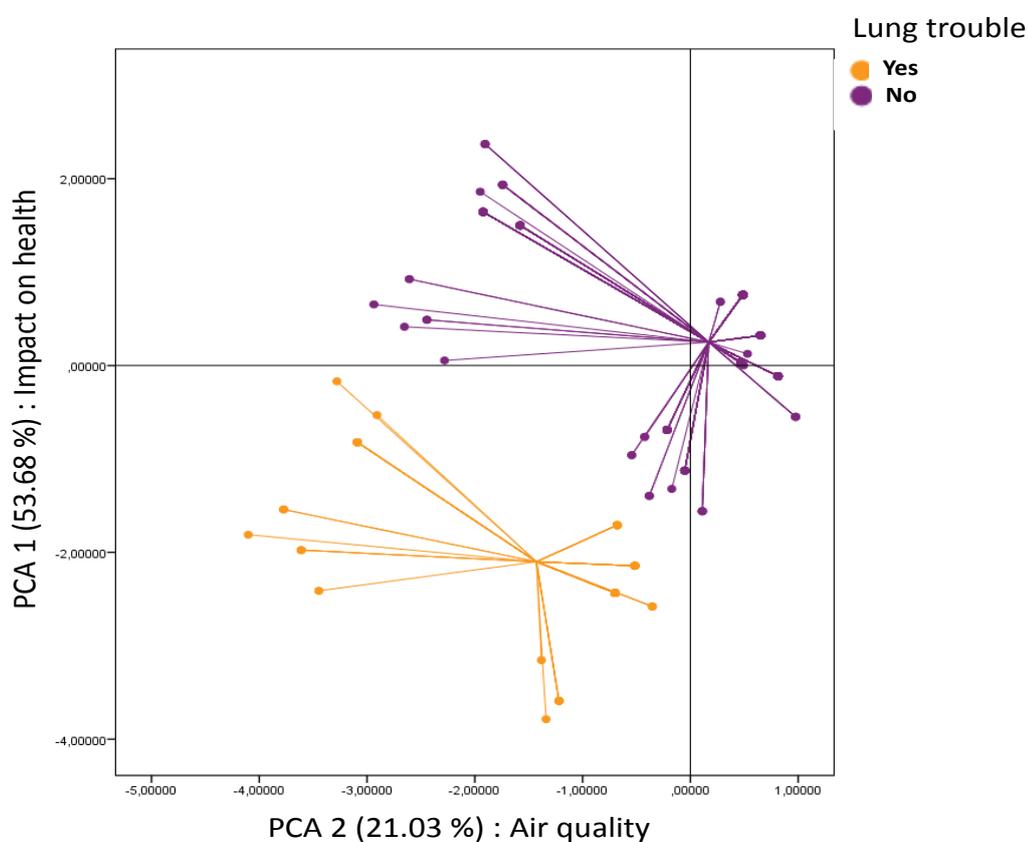


Figure 2.12: PCA analysis of air quality in Northern Lebanon and the health state of the population

Furthermore, when correlating health concerns (Sections 3 and 4) with air quality (Section 2), we observed two different populations in this PCA (Figure 2.13). Component diagram allows us to identify two distinct components and may explain 40.91% of the variance of the dataset: the first component (PC1) was correlated with health state and the second one (PC2) was correlated with questions about past illnesses. Graphical representation of respondents, based on their responses about global air quality in Northern Lebanon, showed two distinct populations. People with health trouble

(health state, section 3) such as cough or phlegm, and/or pathologies like chronic bronchitis or asthma (past illnesses, section 4) perceived air quality as poor or very poor while people with little or no health problems are more confident about air quality.

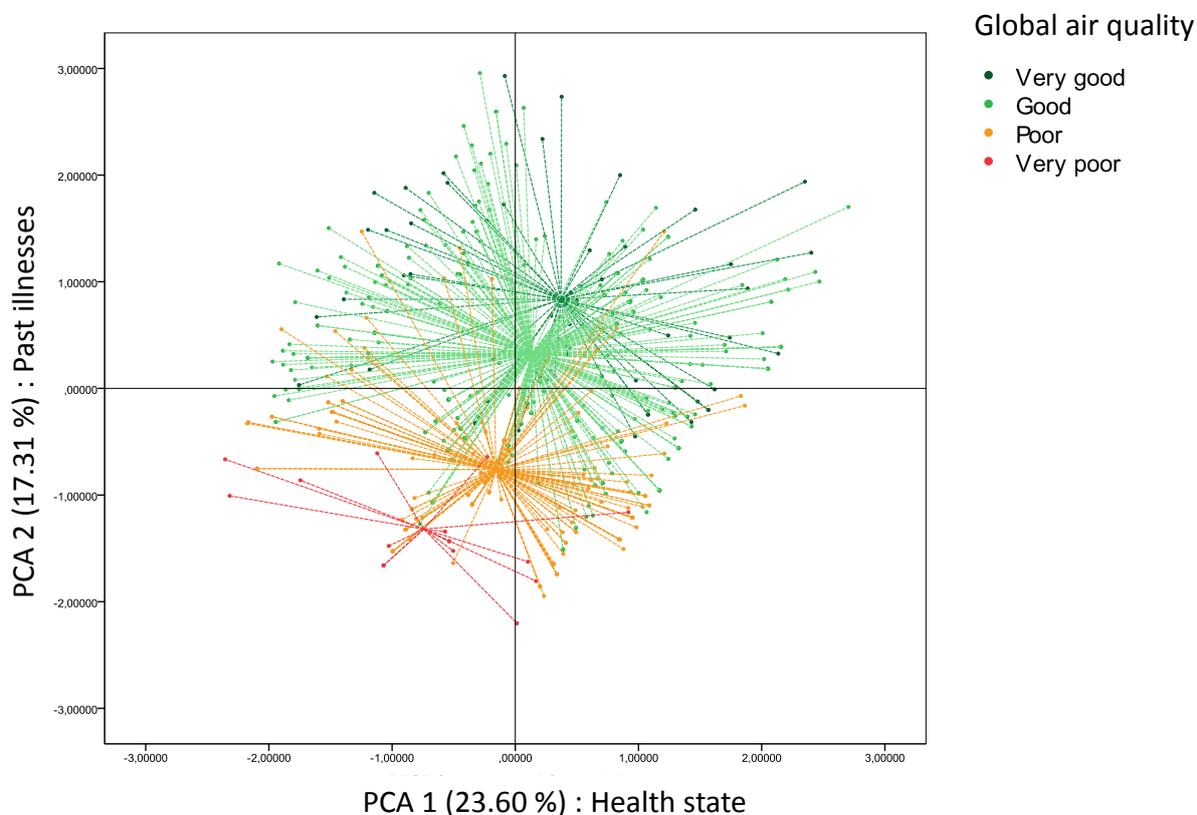


Figure 2.13: Correlation between health concerns and global air quality

Deep green: very good quality of air, Green: good quality of air,
Orange: poor quality of air, Red: very poor quality of air

Multivariate analysis and PCAs showed that people perception about air quality is conditioned not only by the environmental parameters of the place where they live but also by their health state and their past illnesses.

2.4 Conclusion

Air pollution is a major environmental health risk in both developing and developed countries. It has many negative effects on health and it is recognized as a serious health hazard. In general, people living in less polluted cities have better respiratory and cardiovascular conditions. Related diseases burden can be reduced by improving the ambient air quality.

In order to obtain perception data about air quality in North Lebanon and about the health state of people, this survey was conducted in two Lebanese areas in the North Lebanon: rural and industrial (310 treatable questionnaires/ area). The questionnaire used two scales, perception and

epidemiology, in order to examine the opinions people express when they are asked to characterize and evaluate air quality and pollution. This questionnaire also studied the patterns, causes, and effects of health and diseases in the two populations.

According to the analysis, there were significant differences between the inhabitants' perceptions in the two areas, and this may be an indicator of the real situation in these areas. The survey results confirmed how seriously the industrial public regards air pollution from industrial activities in terms of their quality of life and health and identifies the attitudes of the public towards nuisance from industrial emissions. The answers were affected by many factors such as working in the industries, the distance from the industries, and seeing and feeling the industrial releases. People who are accustomed to relatively poor air quality may be more sensitive to questions on air quality perceptions.

This study confirmed that living in an industrial area has a significant association with respiratory symptoms and diseases. Preliminary evidence suggests that industrial air pollution and PM exposure may be a risk factor for the increase in breathlessness, asthma, allergies and heart problems at the industrial site. Air pollution is the second major cause for many respiratory diseases, after smoking. Our findings were similar to those of other researchers for several etiologic factors. Besides air pollution, smoking, and advanced age have also been reported to increase the risk of respiratory diseases. Children were vulnerable to ambient air pollution as they spend more time outdoors, are more physically active, and have higher ventilation rates than adults.

Multivariate analysis and PCAs confirmed that people perception about air quality is conditioned by the surrounding environmental parameters and by their health state and their past illnesses.

The results of this study compared the levels of perceived air pollution annoyance and health risk in the industrialized area with respect to the rural area and the relationship between respiratory diseases and industrial activities. It confirmed the need to conduct a toxicological study in order to identify potential mechanisms that might contribute to the pathogenesis of air pollution and especially PM-associated respiratory diseases.

CHAPTER 3

PHYSICOCHEMICAL CHARACTERISTICS OF FINE PARTICLES

This chapter is divided in two parts.

The first part includes a detailed explanation of the chosen sites, materials and methodology of the sampling, sampling periods, meteorological data, concentrations in collected particulate matter and preparation of composite samples for the toxicological study. The next section in this part includes the analysis materials and procedures used for the chemical characterization of the collected PM_{2.5} samples: size distribution and morphology (Laser diffraction and SEM-EDX), ICP-AES and Ion chromatography, total carbon composition (CHNS-O analyzer), and organic composition (GC-MS). In total, seven different methods were used for the physicochemical characterization of fine (FP) and quasi-ultrafine particles (UFP) collected under rural and industrial influences. The second part focuses on presenting and discussing the physicochemical results.

3.1 Materials and methods for PM sampling and characterization

Our project scope is to study the physicochemical characteristics of PM_{2.5} in Northern Lebanon, to assess their toxicological effects and to study different genotoxic and epigenetic endpoints. Our sampling strategy consists of a simultaneous sampling of fine particles (PM_{2.5-0.3}) and quasi-ultrafine particles (PM_{0.3}) at two sites in Northern Lebanon in Koura district: Zakroun (a site affected by industrial influences) and Kaftoun (a background rural site) (as mentioned in *paragraph 1.7*).

3.1.1 PM Sampling

3.1.1.1 Methodology

Sampling of fine particles (FP) and quasi-ultrafine particles (UFP) was carried out using high volume cascade impactor. This technique was used to collect sufficient masses of native PM_{2.5} (without using any filter) to be able to study both physicochemical characteristics and toxicological endpoints.

Cascade impactors consist of a series of plates (stage), with specific slots arrangement (slots are offset from one plate to the other to enable the impaction), and collection surface. Cascade impactors operate on a physical principle of fluid mechanics, the separation is based on differences in particles' inertia in a stream of air. Particles laden air is drawn into the impactor, flowing sequentially through the stages; slot size and total slot area decrease with stage number. As particles pass through

the slots, they either remain entrained in the air stream, which is directed through a right angle at the exit of the slot, or break through the lines of flow, impacting on the collection surface. Particles with sufficient inertia are collected, the rest pass onto the next stage. Each stage of the impactor is therefore associated with a cut-off diameter, defining the size of particles that are retained on the collection surface of that stage. Ideal collection efficiency is that all of the particles above a certain size would be captured and those below it would pass through. In reality there is a curve from which D_{p50} , the stage cut-off diameter, is determined (Figure 3.1). As slot size decreases, velocity increases, allowing the collection of increasingly small particles, any non impacted particles being captured in a final filter (Back up).

The sampling of particles was done continuously, from 12 February till 28 April 2014 and simultaneously at the two sites, by cascade impaction using a TFIA-2 (Staplex[®], USA) high volume air sampler at a constant aspiration flow rate (68 m³/h) equipped with a Model 235 (Staplex[®], USA) cascade impactor (Figure 3.2) and a flow controller that delivers constant air flow. The Model 235 is composed of five stages (plates provided with calibrated slots) allowing the separation and collection of aerosols based on their equivalent aerodynamic diameter (D_{ae}). Particles are thereby separated into a series of size fractions. For Staplex[®] Model 235, impactor cut-points are nominally: 5.08, 2.10, 1.04, 0.64, 0.33 μ m (Figure 3.3).

A slotted quartz fiber filter (5,63"x5,38", TFAQS810, Staplex[®], USA), used as impaction substrate, was placed on the first stage in order to retain particles with aerodynamic diameters higher than 5.08 μ m, and no impaction substrate was used on the following impaction stages. A "back-up" filter (8"x10", Staplex fiberglass filters) was used in the last level to recover the quasi-ultrafine particles (PM_{0.3}). The choice of filters was linked to the objectives of the project. It is related to the analytical procedure and the chemical species to be studied in this work. Glass fiber filters have a low vacuum loss allowing maintaining a constant aspiration flow rate over a relatively long period and are suitable for high flow rate impactors. The collected particles on the back-up filter have a $D_{ae} < 0.33 \mu$ m, which is the D_{p50} of the 5th impactor stage and corresponds to quasi-ultrafine particles (it contains the ultrafine fraction, PM_{0.1}).

The impactor was assembled with the filters in the laboratory under the hood, packed in a plastic bag and then transported to the site. The impaction system was dismantled every 12 days. Impaction plates were dried under laminar flow hood during 48h, and then PM_{2.5-0.3} were recovered from the collection plates using a brush and pooled regardless of their aerodynamic diameter. The collected PM were placed into a Teflon-PFA vial with two PTFE balls and homogenization was done using magnetic agitation during two hours and then stored at -20°C. The mass of particles was determined using a precision balance (Cubis[®] 225S, Sartorius, Germany). Back-up filters, retaining UFP, were dried under the hood, wrapped in aluminum foil, sealed in a plastic bag, and stored at -20°C.

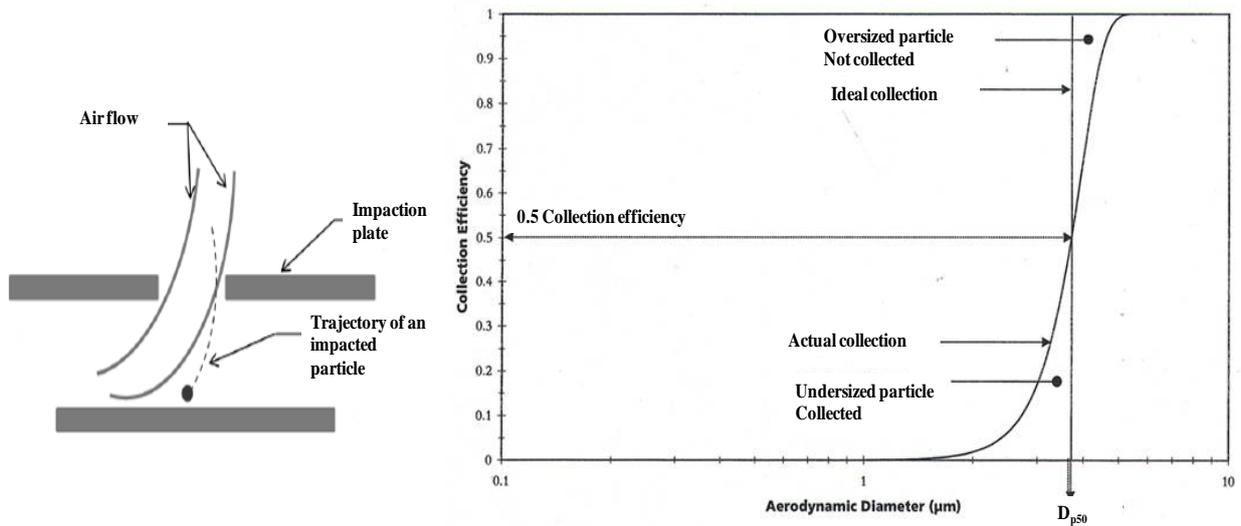


Figure 3.1: Cascade impaction principle and collection efficiency curve



Figure 3.2: High volume air sampler and cascade impactor

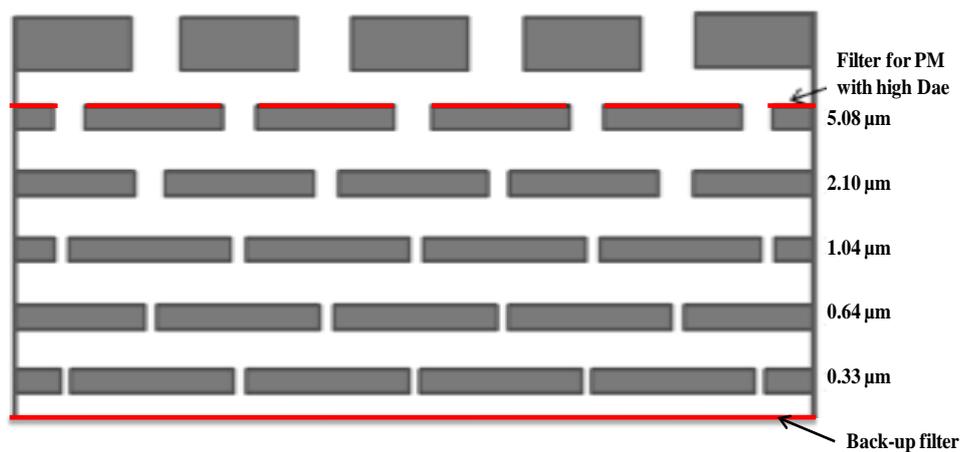


Figure 3.3: The Model 235 impactor five stages schematic

3.1.1.2 Sampling periods and meteorological data

Sampling of FP and UFP was carried in Zakroun and Kaftoun simultaneously from 12 February till 28 April 2014 during five periods (P1 to P5) (Table 3.1).

Table 3.1: Sampling periods of fine and quasi-ultrafine particles

		Period	FP samples	UFP Samples (on filters)
Industrial: Zakroun	P1	12/02/2014 – 24/02/2014	FP-I1	UFP-I1
	P2	28/02/2014 – 07/03/2014	FP-I2	UFP-I2
	P3	20/03/2014 – 24/03/2014	FP-I3	UFP-I3
	P4	01/04/2014 – 15/04/2014	FP-I4	UFP-I4
	P5	16/04/2014 – 28/04/2014	FP-I5	UFP-I5
Rural: Kaftoun	P1	12/02/2014 – 24/02/2014	FP-R1	UFP-R1
	P2	28/02/2014 – 07/03/2014	FP-R2	UFP-R2
	P3	20/03/2014 – 28/03/2014	FP-R3	UFP-R3
	P4-5	01/04/2014 – 28/04/2014	FP-R4-5	UFP-R4-5

Meteorological data (i.e., wind speed, wind direction, temperature and precipitation) were obtained from the port of Tripoli and retrieved from www.infoclimat.fr database. These data were used to draw wind roses, precipitation trends and temperature trends for the sampling periods. Wind velocities were divided into four major classes illustrated in Table 3.2.

Table 3.2: Classification of the encountered wind speeds during the sampling campaigns

Wind speed (m/s)	Speed type	Description
> 8	High	Fresh breeze to fresh gale
4 to 8	Moderate	Gentle to moderate breeze
2 to 4	Low	Light to gentle breeze
< 2	Very low	Calm to light air

A closer look at wind roses (Figure 3.4) shows that most frequent wind directions were southeast (SE) with very low speed winds (< 2 m/s). When the wind speed is inferior to 3 m/s, even if a wind direction is recorded, it corresponds rather to turbulent and stagnant atmosphere without any well-defined origin. Predominant wind direction associated with higher speed winds (> 2 m/s) came from southwest and west during the sampling period. These observations are in agreement with the historical seasonal trends (from 2005 to 2014) recorded at Beirut Rafic Hariri International Airport showing a prevailing SW wind direction.

The total precipitation in the region (Tripoli weather station) during the sampling period was 17.7 mm. The rainfall was mostly in the first sampling period (16.6 mm in P1, 0.2 mm in P4, 0.9 mm in P5). This corresponds to normal weather conditions in Lebanon (Appendix 13A), since most of the precipitation occurs between November and March, due to wet winds coming from the West or Southwest bringing large masses of humid air over the littoral.

The temperature in the region (Tripoli weather station) ranged between 10 and 20°C and reached 25°C during P5 (Figure 3.5). This corresponds to normal temperature trends in Lebanon (Appendix 13B).

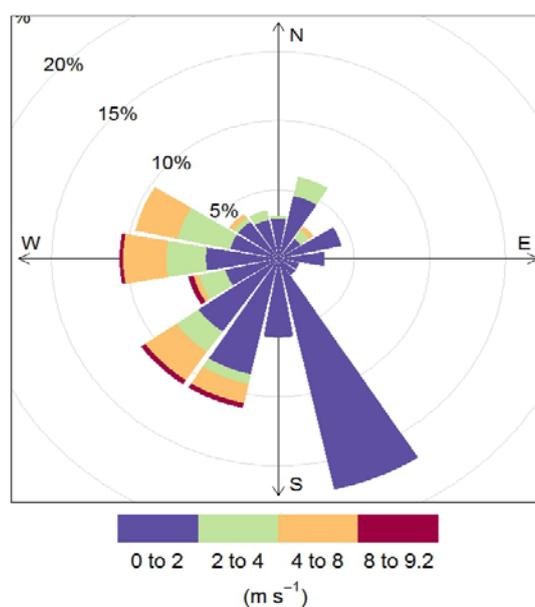


Figure 3.4: Wind roses for the sampling period from 12/02/2014 to 28/04/2014 recorded at Beirut (from data retrieved from infoclimat.fr website)

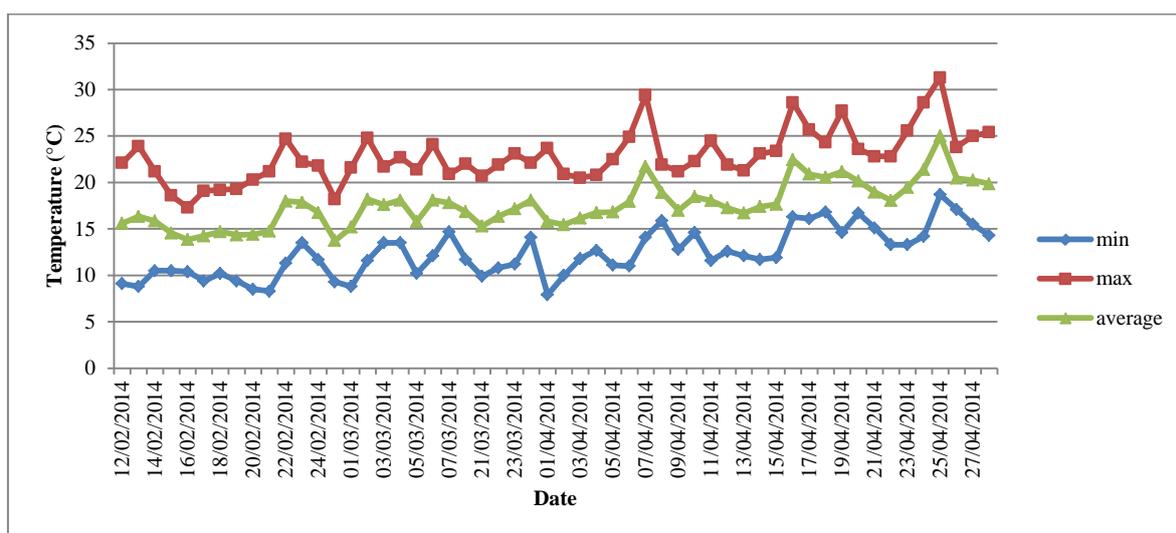


Figure 3.5: Evolution of temperatures in Tripoli during the period of study

In order to determine the origin of air masses during the sampling period, backward trajectories were drawn using Hybrid Single-Particles Lagrangian Integrated Trajectory (HYSPLIT) with the following parameters (Draxler and Rolph, 2013):

- Vertical Motion: Model vertical velocity
- Total run time (hours): 72
- Start a new trajectory every: 12 hours
- Level 1 height: 100 m (above ground level)
- Label Interval: 12 hours

For the whole sampling period, we drew backward trajectory every 12 hours (Figure 3.6). In the first sampling period (P1: 12/02/2014 – 24/02/2014), Northern Lebanon received continental air masses from Europe and Asia. In the second sampling episode (P2: 28/02/2014 – 07/03/2014), air

masses came from Africa through Egypt and others from Europe after their passage over the sea. This period was characterized by sandy wind that could have originated from the dry desert air blown from North Africa. In the third sampling period (P3: 20/03/2014 – 24/03/2014), air masses came from the littoral passing by Syria before reaching our sampling site from the North. In the fourth sampling period (P4: 01/04/2014 – 15/04/2014), air masses came from the South of Europe passing by the littoral. This type of air masses is usually warmer and slightly humid after its passage over the sea but is not sufficiently saturated to bring rain. In the fifth sampling period (P5: 16/04/2014 – 28/04/2014), continental air masses came from Italy, Greece and Turkey, and after its passage over the sea, it reached the sampling sites mainly from the North.

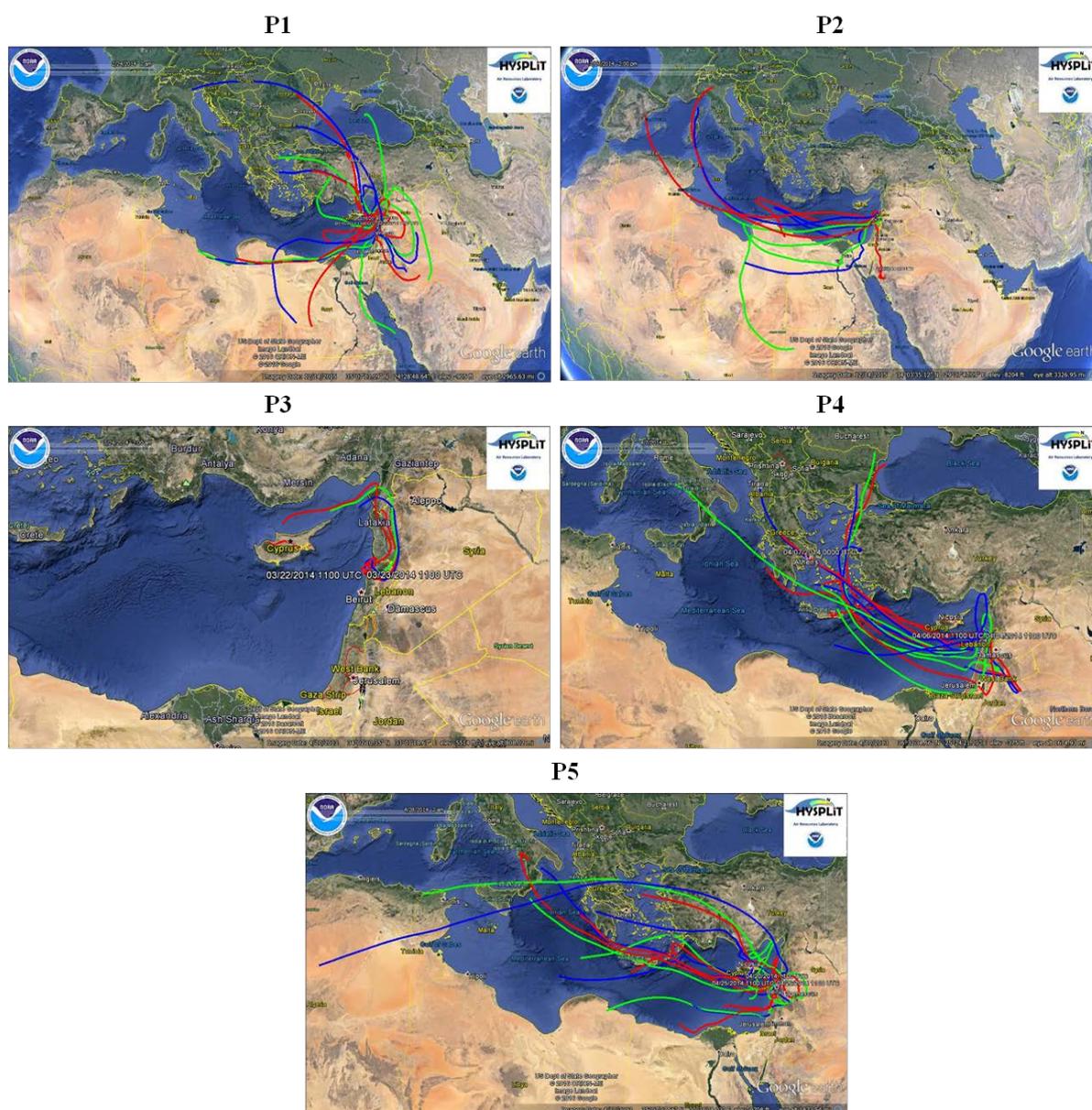


Figure 3.6: Air mass back trajectories for the sampling periods from HYSPLIT model (Draxler and Rolph, 2013)

3.1.1.3 Concentrations of collected particulate matter and preparation of composite samples

The concentrations of PM_{2.5} in the air were evaluated by considering the mass of particles collected on the plates and the volumes of air having passed through the impactor (Table 3.3). It should be noted that these concentrations could be slightly underestimated because losses may occur, during the collection of the particles from the plates.

Table 3.3: Concentrations of industrial and rural particles

	Period	FP samples	PM _{2.5-0.3} concentrations (µg/m ³)	UFP Samples	PM _{0.3} concentrations (µg/m ³)	PM _{2.5} concentrations (µg/m ³)
Industrial: Zakroun	12/02/2014 – 24/02/2014	FP-I1	17	UFP-I1	11	28
	28/02/2014 – 07/03/2014	FP-I2	34	UFP-I2	13	47
	20/03/2014 – 24/03/2014	FP-I3	22	UFP-I3	23	45
	01/04/2014 – 15/04/2014	FP-I4	21	UFP-I4	20	41
	16/04/2014 – 28/04/2014	FP-I5	14	UFP-I5	23	37
Rural: Kaftoun	12/02/2014 – 24/02/2014	FP-R1	9	UFP-R1	10	19
	28/02/2014 – 07/03/2014	FP-R2	82	UFP-R2	14	96
	20/03/2014 – 28/03/2014	FP-R3	8	UFP-R3	13	21
	01/04/2014 – 28/04/2014	FP-R4-5	15	UFP-R4-5	10	25

Before commenting the data, in both sites and especially in the rural one, we have to mention that high PM_{2.5-0.3} mass was obtained during the second sampling period (28/02/2014 – 07/03/2014), which could be explained by a local sandy wind. The concentrations of FP (PM_{2.5-0.3}) in the atmosphere varied between 14 and 22 µg/m³ for the samples collected in Zakroun, except for the FP-I2, for which the concentration was 34 µg/m³. For the rural site, the concentration varied between 8 µg/m³ and 15 µg/m³ except for the FP-R2, for which the concentration was 82 µg/m³.

Regarding the UFP, the masses retained on filters were determined by summing the concentrations for all analyzed elements in the collected particles (major and trace elements, inorganic ions and total carbon) (Gherzi *et al.*, 2012). The calculated UFP concentrations ranged from 11 to 23 µg/m³ for the industrial site and from 10 to 14 µg/m³ for the rural site. From these values, it is possible to estimate the PM_{2.5} concentrations, which are the sum of the concentrations of FP (PM_{2.5-0.3}) and UFP (PM_{0.3}). The mean PM_{2.5} concentrations in Zakroun (industrial site) and Kaftoun (rural site) were 36.5 and 22.8 µg/m³ respectively; the PM_{0.3} fraction represents 50% of PM_{2.5} in both cases.

FP and UFP collected during each of the sampling period, called "individual" samples, in the industrial influenced and rural sites were used for physicochemical analyses. In order to study the toxicological effects of fine particles, two mean PM_{2.5-0.3} samples, called "composite" were prepared from individual samples collected in the two sites. The second sampling period was dismissed from the prepared composites due to the occurrence of a local sandy wind, as said before. Therefore, an industrial influenced composite, FP-I, was prepared by pooling and carefully homogenizing representative masses from the industrial individual samples (FP-I1, FP-I3, FP-I4 and FP-I5). A similar procedure was done using the rural individual samples (FP-R1, FP-R3 and FP-R4-5) in order to prepare a rural composite sample, FP-R. The prepared composites were stored at -20°C until their use for chemical characterization and toxicology assessment. Before each analysis, FP (powder) and

UFP (on filters) were placed at room temperature for one hour. As for the back-up filters, prior to analysis, the contours, part of the filter in contact with the seal that does not retain particles, have been cut and removed and the filters portions loaded with particles were then weighed.

All individual samples of FP and UFP listed in Table 3.3 and composite samples were analyzed to determine their composition and identify their properties.

3.1.1.4 Size distribution

In order to validate our sampling technique, size distributions of the particles in the composite samples, FP-I and PF-R, were determined by laser diffraction (LS13320 analyzer, Beckman Coulter, USA) using an aqueous suspension of composite FP and carrying on sonication prior to measurements. The principle of this technique is summarized in Appendix 14A and results are shown in Figure 3.7.

The cumulative frequencies of the $PM_{2.5}$ particle size correspond to 98.7% and 97% respectively for composite samples, FP-I and PF-R. A significant proportion of PM_1 can be noted for FP-I (97.2%) and FP-R (80%). The latter are able to penetrate to the alveolar region of the lung and exert their toxicity (Harrison and Yin, 2000). According to epidemiological evidence, the greatest health risks correlate with smaller PM (Levy *et al.*, 2000). Our results agree with those obtained in other studies conducted in our laboratory (Borgie, 2014; Dergham, 2012; Dieme *et al.*, 2012), where the sampling technique by cascade impaction was used to collect $PM_{2.5-0.3}$ and this allows to validate this sampling technique.

3.1.2 Physicochemical characterization of PM

3.1.2.1 Morphology and single particle analysis

Morphology and single particle analysis were performed using the scanning electron microscope with energy dispersive X-ray analysis (SEM-EDX) using Leo 438 VP microscope and IXRF analysis system (LEO Electron Microscopy Ltd, Carl Zeiss, United Kingdom). The principle of this technique is showed in Appendix 14B. The analyses were performed at “Maison de Recherche en Environnement Naturel” in ULCO. For SEM-EDX analysis, 1 mg of composite fine particles were suspended in an-hexane solution using ultrasonic treatment for 5 minutes, and then filtered through a 0.45 μm porosity polycarbonate Nuclepore® (Whatman, United Kingdom) membrane to obtain well-distributed and dispersed PM. A piece of the polycarbonate membrane was placed on a stub using an adhesive conductive carbon tape and coated with carbon prior to the analysis.

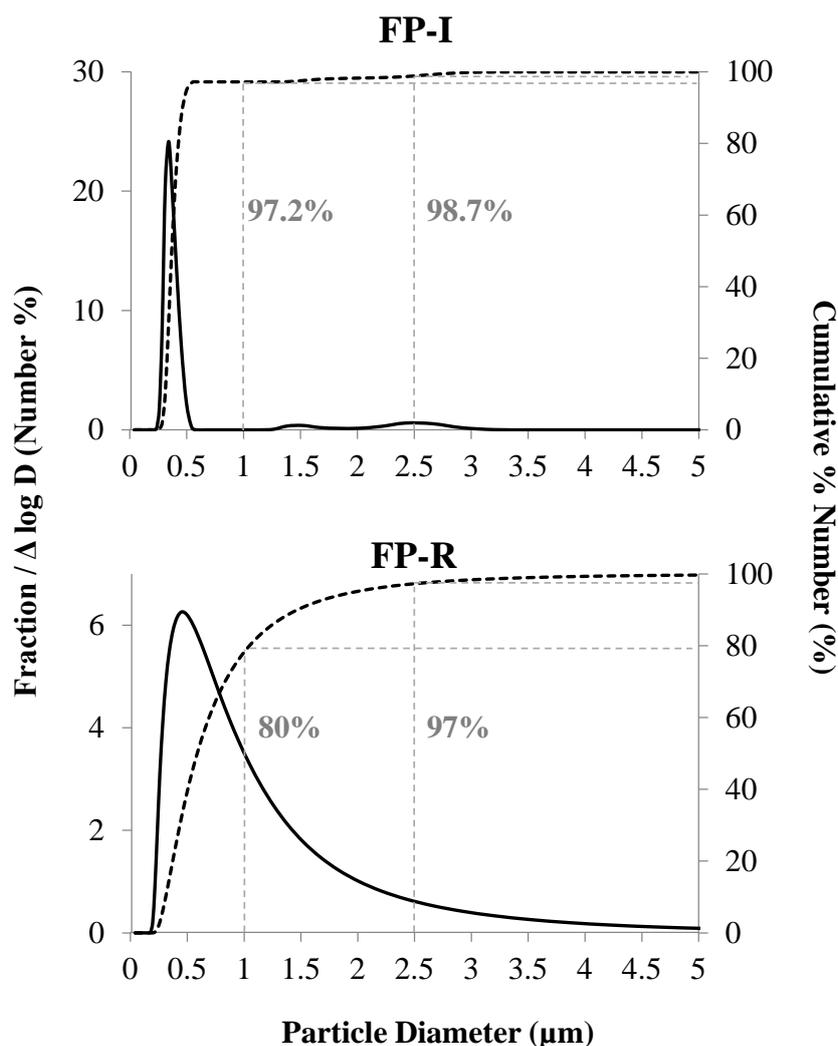


Figure 3.7: PM size distribution of FP-I and FP-R

3.1.2.2 Identification of crystalline phases

The identification of crystalline phases of the FP composites was done using X-ray diffraction (XRD) at room temperature by the X-Ray Diffractometer AXS D8 Advance, Brüker, Germany. The diffractometer is equipped with a copper anticathode that emits $K\alpha$ radiation ($\lambda=1.5405\text{\AA}$), a LynxEye detector, a $\theta/2\theta$ goniometer and a rotating sample holder. Scattering intensities were measured at an angular range of $10^\circ < 2\theta < 70^\circ$ with a step-size of $2\theta= 0.02^\circ$ and a step-time of 10 seconds. The principle and more details are in Appendix 14C. Peak and phase identification were conducted by comparing the obtained diffraction patterns with standard XRD reference patterns from the JCPDS (Joint Committee on Powder Diffraction Standards), established by the ICDD (International Center for Diffraction Data).

3.1.2.3 Determination of inorganic elements

To determine the composition of the particles in inorganic elements, ICP-AES (Inductively Coupled Plasma- Atomic Emission Spectroscopy) and ICP-MS (Inductively Coupled Plasma- Mass

Spectrometry) were used for major elements, and minor and trace elements respectively. These two techniques are most adequate to measure components within the particles; they allow a rapid and simultaneous analysis of a large number of elements. The ICP-AES, with a high linearity range (ppb to hundred ppm), has detection limits of a few $\mu\text{g/L}$ and therefore is very effective for quantifying the major elements. The ICP-MS detection limits are lower in $\mu\text{g/L}$ and this technique is used to the detection of trace elements and ultra-trace elements (Salomon *et al.*, 2002).

Fine composite and individual samples (FP) and quasi-ultrafine individual samples collected on filter (UFP) were analyzed.

3.1.2.3.a Acid digestion of the particles

Approximately 1/16 of the back-up filter retaining the quasi-ultrafine particles or about 3 mg of the fine particles are weighed and placed in a 15 mL SAVILLEX® PTFE flask (polytetrafluoroethylene or Teflon). These samples are mineralized by acid digestion, at 120°C for 4 hours, with a mixture of three concentrated acids Suprapur® Merck: nitric acid (HNO_3), hydrofluoric acid (HF) and perchloric acid (HClO_4), in respective proportions of 4: 1: 0.5 by volume. After an almost complete evaporation of the acid at 170°C for about four hours, the residual drop is taken in 2 to 3 mL of ultrapure water and then raised to 90°C for one hour to solubilize all components concentrated in the remaining drop. The cooled solution was diluted with MilliQ® water up to 15 mL added with 0.2% of nitric acid and filtered on PTFE membrane (0.45 μm , 25 mm) prior to analysis. The filtrate, containing the elements to be analyzed is collected in a polyethylene bottle and stored at -20°C until the day of analysis. The same procedure was applied to a blank filter to determine the contribution of the filter fiber and the used acid mixture to take into account the possible contamination during the phases of preparation and analysis (Ledoux *et al.*, 2006).

A wide range of elements were analyzed to best characterize the particles collected in industrial and rural influenced areas. In this work, we looked at the following items of which the majority is known to have toxic effects on humans (Baccarelli and Bollati, 2009). Thus the solutions obtained were used for the analysis of Al, Ba, Cr, Cu, Fe, Mn, P, Pb, Sr, V and Zn by ICP-AES and Ag, As, Cd, Ce, Co, La, Nb, Ni, Rb, Sb, Se, Sn, Te and Ti by ICP-MS.

3.1.2.3.b Inductively Coupled Plasma- Atomic Emission Spectroscopy

The principle of the ICP-AES is summarized in Appendix 14D. The analyses were carried out using the iCAP 6000 Series from Thermo SCIENTIFIC®, UK. The instrument used for the analysis can also detect emitted rays using two modes: radial detection and direct axial detection. The latter is the most sensitive between the two methods, and hence it is selected for our analysis. For the calibration, standard solutions (blank, 5, 10, 25, 50, 100, 200, 500, 2000 ppb) were prepared from a multi-element solution (10 ppm). The dilutions were achieved using MilliQ® ultra pure water.

Calibration solutions were also acidified with 0.2% HNO₃ Suprapur[®]. The list of elements quantified using this technique, along with the respective analytical conditions are illustrated in Table 3.4.

Table 3.4: List of elements analyzed by ICP-AES, their calibration ranges and detection limits

Element	Wavelength(s) (nm)	Calibration range (ppb)	Detection limit (ppb)
Al	2367	50- 20000	4
Ba	4554	0.5- 200	0.4
Cr	2677	5- 2000	3
Cu	3247	5- 2000	2
Fe	2382; 2599	50- 20000	21
Mn	2576; 2605	5- 2000	1
P	1782	5- 2000	3
Pb	2203	5- 2000	3
Sr	4077	0.5- 200	0.2
V	2908	5- 2000	2
Zn	2138	5- 2000	2

3.1.2.3.c Inductively Coupled Plasma- Mass Spectrometry

The principle of the ICP-MS (coupled with a collision reaction cell to minimize polyatomic interferences) is summarized in Appendix 14E. Analyses were performed using a Varian[®] 820-MS, USA with a Collision Reaction Interface (CRI) in which we used helium as collision gas in addition to air used to quantify elements with minimum polyatomic interferences. For the calibration, standard solutions were prepared from a multi-element solution and acidified (0.2%) with HNO₃. For most analyzed isotopes, there are one or more polyatomic interferences. This required a precise choice of the mass for each element and collision gas that minimized the interference and maintain good detection sensitivity. Table 3.5 illustrates the main characteristics of the ICP-MS method.

Table 3.5: List of elements analyzed by ICP-MS, their calibration ranges and detection limits

Element	Atomic mass	CRI gas	Calibration range (ppb)	Detection limit (ppb)
Ag	107		0.2- 1	0.077
As	75		0.5- 5	0.162
Cd	110		0.2- 10	0.038
Ce	140		0.5- 20	0.245
Co	59		1- 50	0.059
La	139		0.5- 10	0.065
Nb	93		0.2- 5	0.087
Ni	60	Helium	1- 50	0.032
Rb	85		1- 100	0.059
Sb	121		0.5- 20	0.112
Sc	45		0.2- 50	0.010
Se	77		0.2- 50	0.105
Sn	118		0.5- 25	0.105
Te	123		0.2- 50	0.084
Ti	47		0.2- 50	0.039

3.1.2.4 Determination of inorganic ions

The identification and quantification of the major water-soluble ions such as fluorine F⁻, chlorides Cl⁻, nitrates NO₃⁻, sulfates SO₄²⁻, phosphates PO₄³⁻, ammonium NH₄⁺, sodium Na⁺,

magnesium Mg^{2+} , potassium K^+ and calcium Ca^{2+} were done by ion chromatography after water extraction. Fine composite and individual samples and quasi-ultrafine individual samples were tested.

3.1.2.4.a Sample preparation

Approximately 1/16 of the back-up filter retaining the ultrafine particles or about 4 mg of the fine particles are weighed, placed in a beaker and covered with 5 mL of Milli-Q[®] ultrapure water. The beaker is placed in an ultrasonic bath for 30 min, and the leachate containing the ions is collected using a syringe and filtered through a Sartorius[®] cellulose acetate membrane (0.2 μm , 25 mm) previously rinsed with ultrapure water. The leachate is then placed in a polyethylene flask, whereas the filters were submerged again in few milliliters of ultrapure water Milli-Q[®] before undergoing another ultrasonic bath and filtration. The same procedure was repeated three times successively. The filtrates obtained are gathered and stored at 4°C until analysis. A Blank filter was treated with the same protocol applied to the samples to take into account the contribution of the quartz fiber filter.

3.1.2.4.b Ion Chromatography

The principle of this technique is summarized in Appendix 14F. Two initial stock solutions of 500 mg/L were prepared using EMSURE[®] MERCK NaF, NaCl, NaNO₃, Na₃PO₄ and Na₂SO₄ salts for anions and NaCl, KCl, NH₄Cl, MgCl₂ (6H₂O) and CaCl₂ for cations. The calibration covered a concentration range between 0.5 and 100 ppm. Simultaneous analysis of anions (F^- , Cl^- , NO_3^- , PO_4^{3-} and SO_4^{2-}) and cations (Na^+ , NH_4^+ , K^+ , Mg^{2+} and Ca^{2+}) was carried out using a Dionex DX 100[®] coupled to a Dionex ICS 900[®] ion chromatography column, equipped with an electrochemical suppressor CSRS 300[®] and a conductivity meter. For anion analysis, a mixture of sodium carbonate solution (Na₂CO₃, 35 mM) and sodium bicarbonate (NaHCO₃, 1mM) was used as mobile phase with a flow rate of 1.2 mL/min. On the other hand, a solution of methane sulfonic acid (CH₃SO₃H, 20 mM) is used as mobile phase in the soluble anions analysis, in a 1.2 mL/min eluent flux.

3.1.2.5 Elemental microanalysis by the CHNS-O Analyzer

The elemental microanalysis is used to determine the C, H, N, S and O elements in a sample. Its principle is based on oxidation-reduction reactions of the tested elements, which will lead to the formation of gases that will be separated and analyzed by gas chromatography (Appendix 14G). The analyses were performed using an organic elemental analyzer Thermo Fisher[®] Flash 2000.

Fine composite and individual samples, and quasi-ultrafine individual samples collected on filter were tested. Accurately weighed samples of approximately 2 mg of FP or 1 cm² of UFP are wrapped in a light weight tin capsule or two tin foils. The sample is then introduced in the apparatus leading to oxidation of C to CO₂ that is detected and quantified using a thermal conductivity detector. For the total carbon measurement, in order to take into account the possible contamination during preparation phases and analyzing samples of FP, and to determine the contribution of filters to the

total carbon content in the samples of UFP, the same analyses were performed for empty tin capsules and a blank filter. Methionine (9.35% N, 40.26% C, 7.37% H, 21.46% S and 21.55% O) was used as standard for calibration. The detection limit (DL) for total carbon (TC) analysis was calculated using the standard deviation (SD) equation applied on 10 measurements of TC content in Tin paper blanks: $DL = 3 \times SD$. The DL of the analyzer was 0.00367 mg of carbon, which is verified to be lower than the contents of all analyzed samples in this study.

3.1.2.6 Determination of Organic Compounds

The identification and quantification of the organic compounds in the collected particles were carried out by Gas Chromatography-Mass Spectrometry (GC-MS): polycyclic aromatic hydrocarbons compounds (PAHs), paraffins, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs). The analyses of PAHs and paraffins were performed by the “Centre Commun de Mesures” in ULCO while the analyses of PCDD, PCDF and PCB were performed by “Micropolluants Technologie SA” (Saint-Julien les Metz, France). Composite fine particles and individual quasi-ultrafine particles were tested for PAHs and paraffins. Only composite fine particles were tested for PCDD, PCDF and PCB.

3.1.2.7.a Sample preparation for PAHs and paraffins analyses

75 mg of FP or 1/8 of the filters containing the UFP particles were submitted to a soxhlet extraction with dichloromethane (100 mL, 24 hr). The extracts are then concentrated under nitrogen flow until having a 200 μ L solution.

3.1.2.7.b Sample preparation for PCDD, PCDF and PCB analyses

10 mg of the FP composites were homogenized by stirring for 2 hours in 4 mL of HCl 0.1 M and filtered on a fiberglass filter. After drying, extraction markers (mixtures of dioxins, furans and PCB-C¹³) were added and the retaining filter is extracted with toluene. The extract was then concentrated under a nitrogen stream, until having a 20 mL solution. This solution was purified on a silica column eluting with hexane. The eluate was concentrated to about 20 mL before being purified and separated on an alumina column: PCBs are eluted with toluene and concentrated to 100 μ L, while PCDDs and PCDFs are eluted with a dichloromethane/ hexane mixture and concentrated to 10 μ L.

3.1.2.7.c GC-MS

For the analysis of PAHs and paraffins, organic extracts were analyzed using a Varian 3400 chromatograph equipped with a FactorFour™ Varian VF-5ms capillary column (30 m length, 0.25 mm internal diameter, a film with 25 μ m thickness) and coupled to a mass spectrometer 1200 Triple Quad, Varian. Concentrations of the 16 priority PAHs listed by the United States Environmental

Protection Agency and even and odd paraffins were investigated. The principle of this technique is explained in Appendix 14H.

For the analysis of PCDD, PCDF and PCBs, Agilent Technologies 7890A gas chromatograph equipped with a DB 5MS Agilent Technologies capillary column (40 m length, 0.18 mm internal diameter, a film with 0.18 μm thickness) and coupled to a high resolution Agilent Technologies P800 mass spectrometer were used (HRGC/ HRMS).

For the analysis, 1 μL of the sample was injected and the quantification of the analytes is based on an internal calibration principle.

Table 3.6 is a summary of the different analyses that were done on the different samples to determine their composition and physico-chemical characteristics.

Table 3.6: Summary of the performed analyses

	Analysis	Technique	Individual samples				Composite samples	
			Fine particles		Ultrafine particles		FP-I	FP-R
			FP-I1 till FP-I5	FP-R1 till FP-R4-5	UFP-I1 till UFP-I5	UFP-R1 till UFP-R4-5		
Physicochemical analysis	Size distribution morphology, and individual composition	Laser diffraction and SEM-EDX					X	X
	PM crystalline phases	XRD					X	X
	Inorganic composition	ICP-MS, ICP-AES and Ion chromatography	X	X	X	X	X	X
	Organic composition HAP and PAR	GC-MS			X	X	X	X
	PCDD, PCDF and PCB	GC-MS					X	X
	Total carbon composition	CHNS/O analyzer	X	X	X	X	X	X
Toxicological study						X	X	

3.2 Results and discussions

3.2.1 Comparison of obtained $PM_{2.5}$ concentrations with data collected in the Mediterranean sites

As mentioned before in *paragraph 3.1.1.3*, mean concentrations of $PM_{2.5}$ in Zakroun (industrial influenced site) and Kaftoun (background rural site) are 36.5 and $22.8 \mu\text{g}/\text{m}^3$ respectively. $PM_{2.5}$ concentrations were higher in the industrial site than in the rural site, and the concentrations in both sites are higher than the annual standard set by the WHO ($10 \mu\text{g}/\text{m}^3$).

Moreover, the $PM_{2.5}$ average concentration in Zakroun is similar to those measured in Mediterranean Basin sites, such as Amman (Jordan, residential-commercial site, $34.9 \mu\text{g}/\text{m}^3$), Istanbul (Turkey, urban site, $40 \mu\text{g}/\text{m}^3$), Athens (Greece, urban site, $41 \mu\text{g}/\text{m}^3$), Escuelas Aguirre (Madrid) (Spain, urban, $34 \mu\text{g}/\text{m}^3$) and Thessaloniki (Greece, industrial-urban, $37 \mu\text{g}/\text{m}^3$) (Table 3.7).

However, the industrial influenced $PM_{2.5}$ concentration in this study was higher than in Kocaeli, (Turkey, industrial area, $23.5 \mu\text{g}/\text{m}^3$ in summer and $21.8 \mu\text{g}/\text{m}^3$ in winter), Iturrama and Plaza Cruz (Spain, urban sites, 15 and $17 \mu\text{g}/\text{m}^3$ respectively), Lecce and Corso Firenze (Italy, urban sites, 19 and $13 \mu\text{g}/\text{m}^3$ respectively), and in Helwan (Egypt, residential site, $29.4 \mu\text{g}/\text{m}^3$). For an extended list of Mediterranean sites and its $PM_{2.5}$ concentrations, please refer to Table 3.6. The higher $PM_{2.5}$ concentrations were measured in Egypt: $62 \mu\text{g}/\text{m}^3$ in Al Zamalek (residential site), $84.6 \mu\text{g}/\text{m}^3$ in El Qualaly (urban site), and $49.7 \mu\text{g}/\text{m}^3$ in Kaha (background site). The rural $PM_{2.5}$ concentration in this study ($22 \mu\text{g}/\text{m}^3$) was close to the concentration found in Bejje (Lebanon, rural site, $23 \mu\text{g}/\text{m}^3$) but higher than other Mediterranean rural sites such as Erdemli (Turkey, rural site, $9.7 \mu\text{g}/\text{m}^3$).

Compared to other locations in Lebanon, the concentration in Zakroun was higher than that found in HaretHreik (Beirut suburb, south, $28.1 \mu\text{g}/\text{m}^3$, 2006-2007) and in three other sites in Beirut ($20.2 \mu\text{g}/\text{m}^3$, $20.7 \mu\text{g}/\text{m}^3$, $20.3 \mu\text{g}/\text{m}^3$, 2009-2010). Higher concentrations were found in Sin El Fil (Beirut, $41 \mu\text{g}/\text{m}^3$, 2011). The study done by Daher *et al.* (2013) showed that $PM_{2.5-0.25}$ concentrations were $22.6 \mu\text{g}/\text{m}^3$ in Riad Al Solh (AUB University, Beirut) and $50 \mu\text{g}/\text{m}^3$ in Jal El Dib (Beirut). In Tripoli, TEDO has been monitoring air pollutants since 2000. The mean $PM_{2.5}$ concentration was $23.6 \mu\text{g}/\text{m}^3$ at the seafront station, and consistently lower than values in downtown Tripoli ($34.6 \mu\text{g}/\text{m}^3$), principally due to heavier traffic in downtown Tripoli.

Table 3.7: PM_{2.5} mass concentrations in Lebanon and different Mediterranean sites

Country	City	Characteristics of the site	PM _{2.5} concentrations (µg/m ³)	Studies
Lebanon	Zakroun	Industrial	36.5	This study
	Kaftoun	Background	22.8	
	Sin El-Fil	Urban	41	(Borgie, 2014; Borgie <i>et al.</i> , 2016)
	Beje	Rural	23	
	HaretHreik	Urban	28.1	(Saliba, <i>et al.</i> , 2010)
	Three sites in Beirut	Urban	20.18, 20.7 and 20.3	
	Riad Al Solh (AUB University)	Urban	22.6	(Daher <i>et al.</i> , 2013)
	Jal El Dib (Beirut)	Urban	50	
	Downtown Tripoli	Traffic	34.6	(TEDO, 2009)
Tripoli (seafont station)	Urban	23.6		
Egypt	Al Zamalek	Residential	62	(Abu-Allaban <i>et al.</i> , 2002)
	El Qualaly	Traffic	84.6	
	Helwan	Residential	29.4	
Jordan	Kaha	Background	49.7	(Sarnat <i>et al.</i> , 2010)
	Amman	Residential-commercial	34.9	
Palestine	Jerusalem-East	Urban	23.8	(Sarnat <i>et al.</i> , 2010)
	Hebron	Industrial-urban	21.1	
	Nablus	Residential-commercial	27.6	
Occupied Palestinian Territory	Gedera	Rural	25	(Mamane <i>et al.</i> , 2008)
	Ashdod	Industrial-urban	23.9	
Turkey	Istanbul	Urban	40	(Szigeti <i>et al.</i> , 2013)
	Kocaeli	Industrial	23.5 in summer 21.8 in winter	(Pekey <i>et al.</i> , 2010)
	Erdemli	Rural	9.7	(Koçak <i>et al.</i> , 2007)
Greece	Athens	Urban	41	(Karanasiou <i>et al.</i> , 2009)
	Thessaloniki	Industrial-urban	37	(Salameh <i>et al.</i> , 2015; Tolis <i>et al.</i> , 2015)
	Pentelli	Suburban	23.8	(Pateraki <i>et al.</i> , 2012)
	Votanikos (Athens center)	Industrial	24.7	
	Tourlos (Aegina)	Background	19.2	
Spain	Escuelas Aguirre (Madrid)	Urban	34	(Artiñano <i>et al.</i> , 2003)
	Iturrama and Plaza Cruz (Pamplona)	Urban	15 and 17	(Aldabe <i>et al.</i> , 2011)
	Castillo de Bellver (île)	Suburban	20	(Pey, Querol, and Alastuey, 2009)
	Barcelona (downtown)	Urban	18.7	(Pennanen <i>et al.</i> , 2007)
Italy	Lecce	Urban	19	(Cuccia <i>et al.</i> , 2013)
	Corso Firenze (Genoa)	Urban	13	(Perrone <i>et al.</i> , 2011)

3.2.2. Major Elements (ME), Soluble Ions (SI), Total Carbon (TC) and Trace elements (TE)

The composition of FP and UFP in inorganic ions, metals and total carbon was determined by ion chromatography (F⁻, Cl⁻, NO₃⁻, SO₄²⁻, PO₄³⁻, NH₄⁺, Na⁺, Mg²⁺, K⁺ and Ca²⁺), ICP-AES (Al, Ba, Cr, Cu, Fe, Mn, P, Pb, Sr, V, and Zn), ICP-MS (Ag, As, Cd, Ce, Co, La, Nb, Ni, Rb, Sb, Sc, Se, Sn, Te, and Ti) as well as by the elemental microanalysis (Total C). FP analyses were performed directly on the particles (powder) while those of UFP were performed on the filters retaining these particles. Quartz fiber filter have high levels of certain insoluble elements, making it impossible to retrieve certain contents associated with UFP. Some species such as Ca, Na, Mg and K are present at high levels in the blank quartz fiber filter; these impurities appear insoluble in water and in this case, the UFP contents of the corresponding water-soluble ions can be interpreted (Ca²⁺, Na⁺, Mg²⁺ and K⁺). Some species are present in the filters at relatively high and variable levels not allowing their quantification such as Al.

The result interpretations will incorporate three chemical species groups:

- Major Elements (ME): Al, Fe, P, Ti and Total carbon (TC).
- Water-soluble Ions (SI): F^- , Cl^- , NO_3^- , PO_4^{3-} , SO_4^{2-} , Na^+ , NH_4^+ , K^+ , Mg^{2+} and Ca^{2+} .
- Trace Elements (TE): Ag, As, Ba, Cd, Ce, Co, Cr, Cu, La, Mn, Nb, Ni, Pb, Rb, Sb, Sc, Se, Sn, Sr, Te, V and Zn.

TE elements will be considered altogether as a global trace elements concentration. The detailed analysis of this group will be discussed later in this part.

The concentrations of inorganic ions, total carbon and major and trace elements in individual samples, fine and quasi-ultrafine, for every sampling period are shown in Figures 3.8A and B respectively (detailed tables are in Appendices 15 and 16). The average concentrations of inorganic ions, major elements, TC and trace elements (in ng/m^3) were determined in individual FP samples (FP-I and FP-R) and UFP samples (UFP-I and UFP-R) (Figure 3.8). The measured mean concentrations in FP-I, FP-R, UFP-I and UFP-R are presented in Table 3.8. The distributions of these species (mean concentrations) in FP and UFP samples are shown in Figure 3.9 (contribution of every element in the total mass of all the adsorbed elements on $PM_{2.5}$).

3.2.2.1 Major elements and water soluble ions

The relative composition of individual FP-I, UFP-I, FP-R and UFP-R samples depended on the sampling periods and exceptional weather conditions. In fact, the second sampling period was affected by sandstorm condition, leading to higher ME (Al, Ca^{2+} , Fe) concentrations (FP-R2), similarly to Malaguti observations in 2015 (Malaguti, 2015) (Figure 3.8).

In the industrial site, the $NO_3^- > SO_4^{2-} > TC > Ca^{2+}$ are the most abundant species in the FP-I samples with 76% as a total mass average followed by Na^+ , Al, Fe, Mg^{2+} , and K^+ . The dominant species in the UFP-I are the same but in another order of abundance: $SO_4^{2-} > TC > NO_3^- > Ca^{2+}$ with a total average of 83%, followed by Na^+ , Fe, K^+ , and Mg^{2+} (Table 3.8 and Figure 3.9). The total carbon constitutes 23% of FP-I and 29% in UFP-I. In addition, carbon concentration was higher in UFP than in FP for all the sampling periods (between 1504 and 3293 ng/m^3 in FP-I vs 2804 and 6861 ng/m^3 in UFP-I) (Appendix 15). Concerning the secondary inorganic ions, a dominance of the nitrate ions is observed for all the FP-I samples, it accounted for 22% of total analyzed components of $PM_{2.5}$, and a dominance of the SO_4^{2-} ions is observed for all the UFP-I samples with an average contribution of 35% of the total analyzed components. Therefore, the secondary ions highly contribute in the total analyzed $PM_{2.5}$ average: 38% in the FP-I [NO_3^-] > [SO_4^{2-}] > [NH_4^+] and 51% in the UFP-I [SO_4^{2-}] > [NO_3^-] > [NH_4^+].

In the rural site, NO_3^- , total C, Ca^{2+} , and SO_4^{2-} constitutes 76% of the analyzed species in the FP-R. The dominant species in the UFP-R are the SO_4^{2-} , total C, NO_3^- , and Ca^{2+} and accounts for 81% of the analyzed species (Table 3.8 and Figure 3.9). The total carbon accounts for 19% of FP-R and 30% of UFP-R. The carbon concentration was higher in the quasi-ultrafine particles than in fine

particles in FP-R1, FP-R3 and FP-R4 periods (3.5, 2.9 and 2.8 times respectively). Concerning the secondary inorganic ions, a dominance of the nitrate ions is observed for all the FP-R samples, it accounted for 26% of total analyzed components of $PM_{2.5}$, and a dominance of the SO_4^{2-} ions is observed for all the UFP-R samples with a contribution of 40% of the total analyzed components. SI highly contribute in the total analyzed mass average, 40% in the FP-R ($[NO_3^-] > [SO_4^{2-}] > [NH_4^+]$) and 56% in the UFP-R ($[SO_4^{2-}] > [NH_4^+] > [NO_3^-]$). Therefore, UFP contain the highest proportion of secondary inorganic particles which is consistent with the literature (Ansari and Pandis, 1999; Schlesinger and Cassee, 2003; Seinfeld and Pandis, 2012). The contribution of major elements such as Ca^{2+} and Fe in the UFP is lower than in the FP for the two sites.

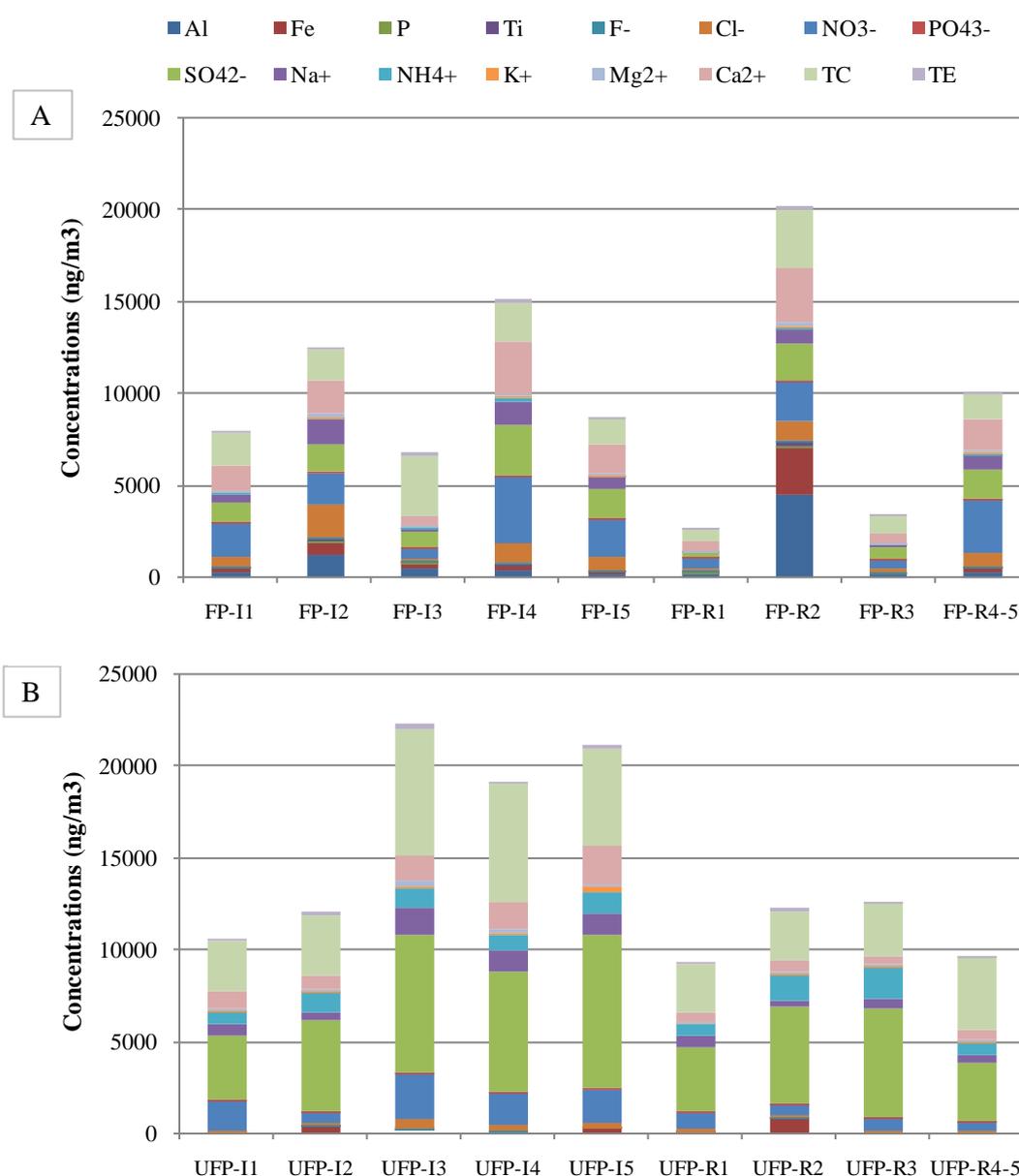


Figure 3.8: Concentrations of inorganic ions, total carbon (TC) and major and trace elements (TE) in individual samples, fine (A) and quasi-ultrafine (B), from 12 February till 28 April 2014 at Zakroun (industrial influence) and Kaftoun (rural influence)

Table 3.8: Mean concentrations of inorganic ions, major elements, total C and trace elements (in ng/m³) in FP (FP-I and FP-R), UFP samples (UFP-I and UFP-R) and in the composite samples (FP-I and FP-R in µg/g) collected in Zakroun (industrial influence) and Kaftoun (rural influence)

	Mean concentrations of individual samples (ng/m ³)				Composite samples ((µg/g)	
	FP- I	FP- R	UFP- I	UFP- R	FP-I	FP-R
F⁻	12.7	6.2	62	35.5	1336	968
Cl⁻	593	333	335	158	48211	36479
NO₃⁻	2085	1405	1925	705	170196	193315
PO₄³⁻	12.6	3.0	30.2	11.1	1052	327
SO₄²⁻	1552	778	6468	4224	118776	86212
Na⁺	668	385	1101	501	54347	43369
NH₄⁺	75	11.7	940	1032	3680	1175
K⁺	58	35.4	148	59	4831	4780
Mg²⁺	91	57	169	69	7418	6736
Ca²⁺	1554	889	1487	494	115354	109028
Ag	0.02	0.01	0.00	0.00	2.1	1.7
Al	384	247	-	-	33468	41719
As	0.14	0.04	0.02	0.01	9.4	10.7
Ba	5.7	3.3	13.5	11.9	464	546
Cd	0.04	0.01	0.00	0.00	2.6	2.1
Ce	0.39	0.25	0.05	0.07	28.8	38.2
Co	0.16	0.09	0.01	0.01	12.7	14.7
Cr	0.70	0.37	7.2	3.0	84	98
Cu	55	17.3	61	13.4	5145	3946
Fe	260	166	162	51	19597	25985
La	0.37	0.19	0.04	0.03	27.2	28.8
Mn	3.8	2.7	3.4	1.1	322	482
Nb	0.10	0.07	0.03	0.03	8.7	13.6
Ni	1.6	0.64	0.29	0.13	102	103
P	26	15.3	20.4	9.4	2354	2558
Pb	10.3	2.4	13.5	5.6	839	573
Rb	0.37	0.24	0.05	0.06	30.2	39.3
Sb	0.43	0.19	0.03	0.02	24.5	20.5
Sc	0.07	0.04	0.01	0.01	9.2	13.2
Se	1.1	0.71	0.15	0.12	70.5	70.2
Sn	0.41	0.12	0.06	0.01	32	21
Sr	10.5	6.4	13.2	4.7	424	498
Te	0.41	0.15	0.020	0.015	19.2	15.6
Ti	26.4	17.2	3.3	3.9	2086	2688
V	2.9	1.3	12.7	4.5	203	209
Zn	11.6	4.3	45.2	17.3	910	831
TC	2185	1054	5323	3194	104667	107535

* Second sampling period discarded

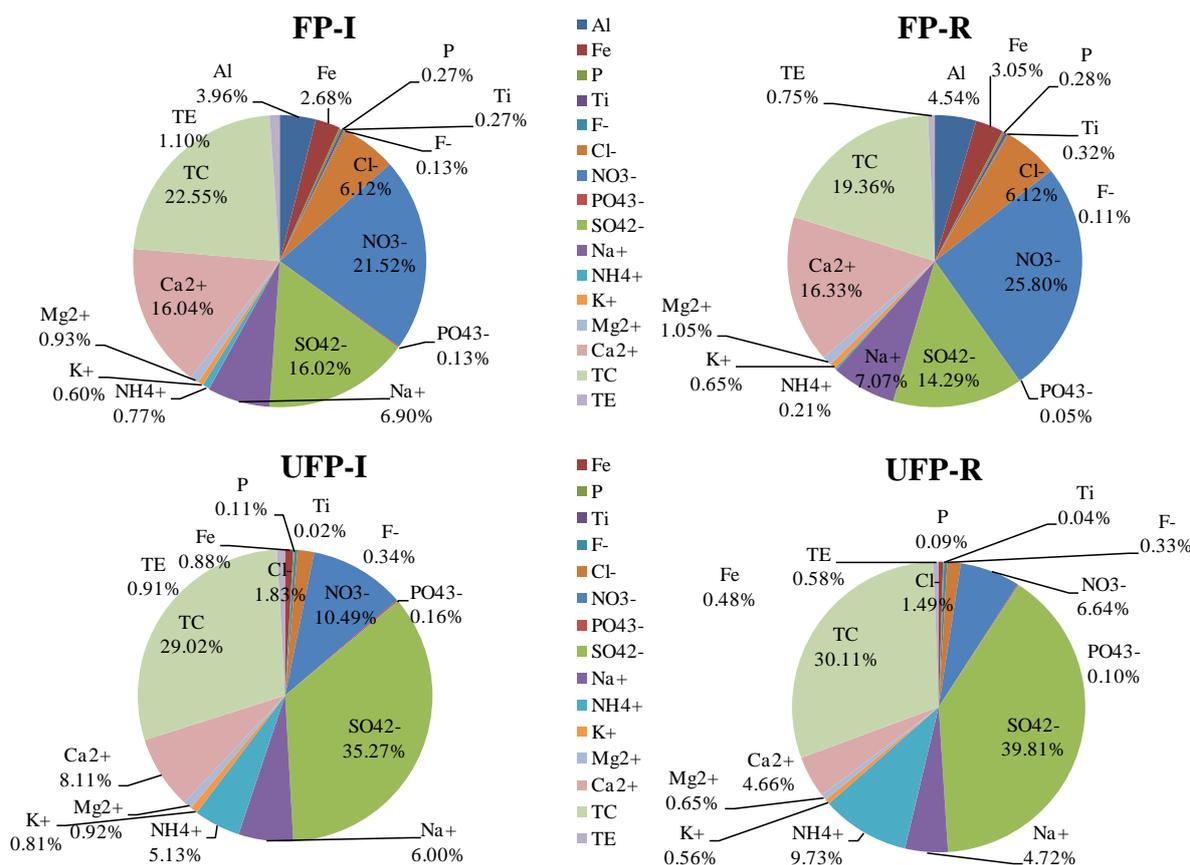


Figure 3.9: Distribution of inorganic ions, total carbon and major and trace elements in FP and UFP samples, from 12 February till 28 April 2014 at Zakroun (industrial influence) and Kaftoun (rural influence)

SO_4^{2-} , NO_3^- , and NH_4^+ are mainly formed in the atmosphere by gas to particle conversion from their precursor gases (SO_2 , NO_x and NH_3). The major source of NO_x is the combustion phenomenon by engines, in particular using diesel, but it is also known that the combustion of fuels at high temperatures in cement kilns results in the release of NO_x emissions (Mousavi *et al.*, 2014). In the Eastern coast of the Mediterranean Sea, SO_4^{2-} is known to originate from long-range transport from distant sources especially from the highly populated Southern and Eastern Europe areas (Luria *et al.*, 1989). Nevertheless, the local scale emission could not be neglected as sulfate can also be found in raw materials or results from the oxidation of local SO_2 emission (MoE/UNDP/ECODIT, 2011; Waked A. *et al.*, 2012) or/and as a sea-salt ion. SO_2 is emitted from the power plants, generators and heavy vehicles (buses and trucks). It is also generated in cement plants from the processing of pyrite and sulfur in raw materials, the kiln operation, and the combustion of sulfur-bearing compounds in coal, oil, and petroleum coke (Waked A. *et al.*, 2012). Therefore the high concentrations of SO_4^{2-} could also be partly explained by the industrial activities present in the sampling areas. Nevertheless, considering the kinetics of sulfates formation from SO_2 , the origin of SO_4^{2-} in this study area could be more likely related to long range transport or local emission from raw materials rather than oxidation of local SO_2 emission.

Nitrate (NO_3^-) concentrations were higher in FP than UFP. This is explained by the low presence of NH_4^+ emission source in the Mediterranean Basin, which will not be sufficient to establish a charge compensation nor neutralization with NO_3^- and SO_4^{2-} . The relatively low NH_4^+ concentrations could partly be explained by the low emission rates of ammonium precursors in the region, and/or because of the instability of ammonium salts in particulate form in relatively warm season (Galindo *et al.*, 2008). As shown by Schaap *et al.* (2004), the partial volatilization of ammonium nitrate is significant when temperature exceeds 20°C and its partial decomposition during sampling can also not be neglected. In this case, the neutralization of NO_3^- and SO_4^{2-} species could be obtained by their association with other cations, such as Ca^{2+} , Na^+ and Mg^{2+} , without excluding other transition metal ions (Arruti *et al.*, 2011; Hodzic *et al.*, 2006). When considering the neutralization of NH_4^+ with NO_3^- and SO_4^{2-} , a clear cation deficit is evidenced (Figure 3.10a). The consideration of Ca^{2+} and K^+ cations has been investigated in Figure 3.10b and 3.10c, to equilibrate the cation deficit. For UFP, neutralization is completed ($R^2 = 0.98$, ratio = 0.939 and intercept close to 0). For $\text{PM}_{2.5-0.3}$, the correlation and the intercept are also satisfying ($R^2 = 0.99$; intercept = 2.696) but the ratio between anions and cations is less than 1 (0.69) and indicating an anion deficit. According to Qu *et al.* (2008) and Noguchi and Hara (2004), who studied particle composition in Asia region, missing anion equivalents could be related to the unmeasured HCO_3^- and CO_3^{2-} , which are components usually presented as coarse particles in source regions. Moreover, Lebanese soils are also known to be rich in CaCO_3 (Verheye, 1973), which is used in cement plant rotary kiln and can be originated for the quarries in the proximity of the sampling sites (Gibbs *et al.*, 2011). Therefore, discarding the CO_3^{2-} species in the ion balance can be the reason for the anion deficit in the coarse $\text{PM}_{2.5-0.3}$ fraction. The predominance of the nitrate in FP can finally be explained by its association with Ca^{2+} species, mainly present in this fraction, as also reported in several studies performed in hot climate such as that of Mediterranean countries (Borgie *et al.*, 2016; Hodzic *et al.*, 2006; Kouyoumdjian and Saliba, 2006).

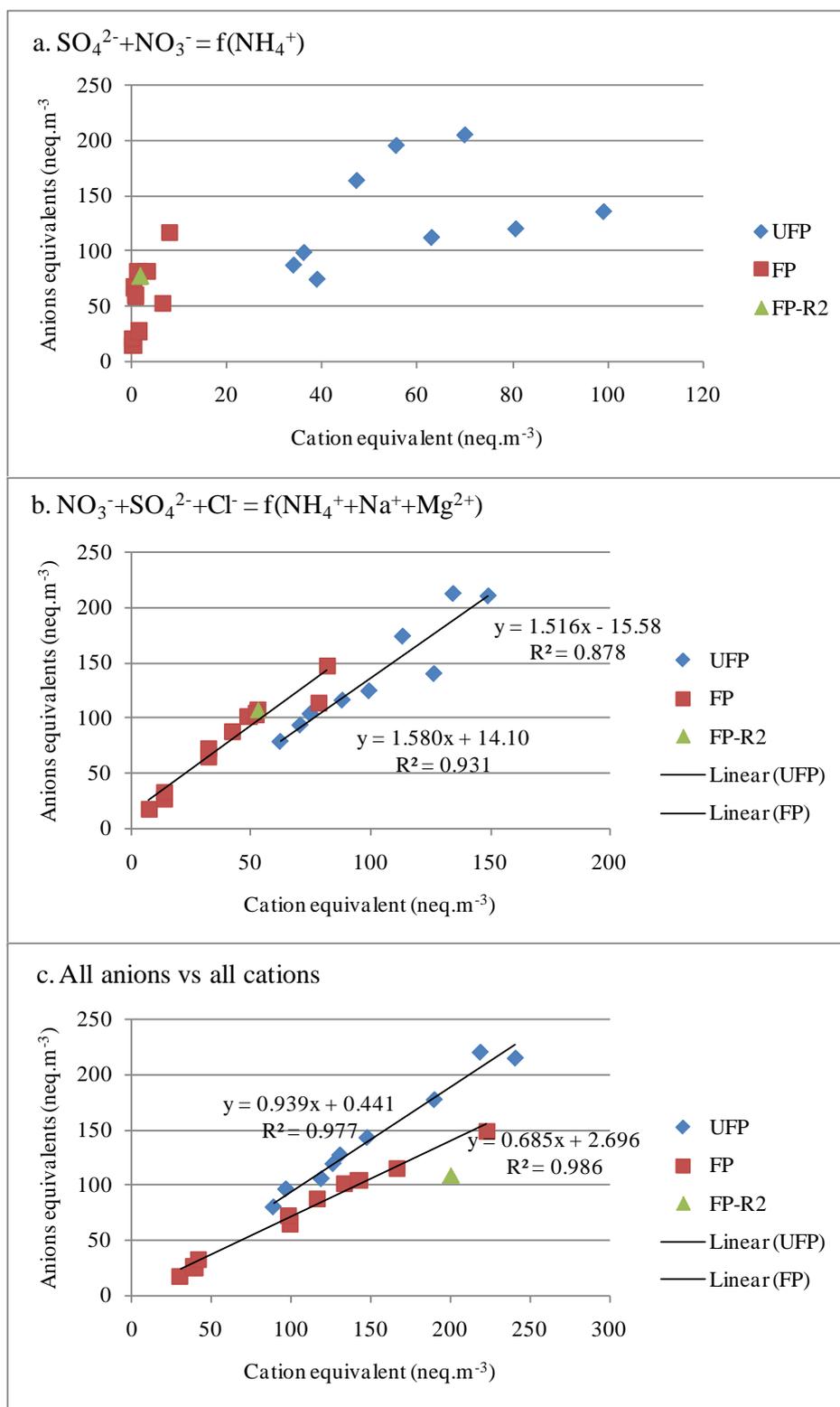


Figure 3.10: Ion balance evaluation between water soluble cations and anions in FP ($\text{PM}_{2.5-0.3}$) and UFP ($\text{PM}_{0.3}$), considering (a) NO_3^- , SO_4^{2-} and NH_4^+ , (b) NO_3^- , SO_4^{2-} , Cl^- and NH_4^+ , Na^+ , Mg^{2+} , and (c) all analyzed anions and cations

The marine influence in the FP-I and FP-R samples was showed by the presence of high levels of Na^+ , Cl^- , and Mg^{2+} as well as K^+ and SO_4^{2-} in a lesser extent (typical sea-salt ions). This

influence is less significant in UFP-I and UFP-R, consistent with the literature that describes marine aerosols as mainly large particles. Freshly emitted sea salts carry a certain Cl⁻ load that is related to Na⁺ concentration ($[Cl^-] = 1.789 [Na^+]$; 1.789 is the mass ratio Cl/Na in sea water) (Seinfeld and Pandis, 2012). When the fresh sea salt aerosol passes over an atmosphere loaded with anthropogenic emissions (such as NO_x and SO₂) that transform into acidic species, a Cl⁻ depletion can occur resulting from the volatilization of HCl due to reaction of nitric acid (HNO₃) and sulphuric acid (H₂SO₄), coming from the nitrogen oxides and sulfur dioxide respectively, with NaCl. The aerosols referred to as “fresh sea salt” at the beginning becomes an “aged sea salt” after these chemical changes (Cesari *et al.*, 2016). FP-I1-I2-I3-I4-I5 have a [Cl⁻]/[Na⁺] ratio of 0.977, 1.35, 0.81, 0.826 and 0.966 respectively. FP-R1-R2-R3-R4-5 have a [Cl⁻]/[Na⁺] ratio of 0.967, 1.321, 0.913 and 0.839 respectively. Obtaining a [Cl⁻]/[Na⁺] ratio lower than that found in seawater is a known phenomenon indicating the aged character of the sampled sea salt and a deficit of Cl⁻ relatively to Na⁺. This situation has been already observed at other remote coastal sites in the Mediterranean area (Kishcha *et al.*, 2011; Koulouri *et al.*, 2008; Malaguti, 2015; Sciare *et al.*, 2005).

K⁺ may also be released into the atmosphere from vegetation and biomass burning and is usually associated to carbon soot particles. Cl⁻ ions can also be generated by some anthropogenic activities like combustion related emissions (biomass and incineration). Combustible wastes of industry and consumers can be used in cement kilns to supplement traditional carbonaceous fuels. The four most common wastes burned in cement kilns are used automobile and truck tires, blended liquid and solid hazardous wastes, used oil, and combustible nonhazardous solid wastes (Greer, 2003). The concentrations of Al and Fe are higher in the fine particles than in the ultrafine particles. Al and Fe, as well as Ca²⁺, Mg²⁺, K⁺, PO₄³⁻, and SO₄²⁻ are also soil components, and it is not surprising to find them mainly in the fine fraction as such particles originate from mechanical process. It should also be noted that most of these species are constituents of the raw materials used in cement plants or fertilizer industries. Calcium carbonate (CaCO₃) is used in cement plant rotary kiln (Gibbs *et al.*, 2011). Mg²⁺ can also arise from dust storm episode (Shahsavani *et al.*, 2012). PO₄³⁻ is known to originate from rocks grinding and other processes in cement plants and phosphate fertilizer industry (Kfoury *et al.*, 2009).

The second period of sampling (FP-I2 and FP-R2) was characterized by higher elements concentrations especially Al and Fe, which can be explained by the local sandy wind occurring from 28/02/2014 to 07/03/2014 (Figure 3.8A). In fact, wind-driven suspension of particles from surface soils and desert produces natural dust contributing to the aerosol (Viana *et al.*, 2008). The main elements present in dust are Al, Si, Ca and Fe. According to Koçak *et al.* (2012), Malaguti, (2015), and Marconi *et al.* (2014). Al concentrations greater than 1 µg/m³ and Ca/Al ratio values (equal to 1) indicate the presence of Saharan dust in the second sampling period. This is correlated with backward trajectory that shows air masses coming from Africa through Egypt.

Therefore when comparing the industrial and the rural particles, the mean concentration of $PM_{2.5}$ in the industrial atmosphere ($36.5 \mu\text{g}/\text{m}^3$) is higher than in rural areas ($22.8 \mu\text{g}/\text{m}^3$) without taking into consideration the second sampling period impacted by sandy winds. The dominant species in the FP-I and in FP-R are the NO_3^- , TC, Ca^{2+} and SO_4^{2-} with higher concentrations in FP-I than in FP-R during all the sampling periods except the second one (Figure 3.8). The concentrations of Al and Fe are higher in FP-I samples than in FP-R samples except FP-I2. Regarding secondary inorganic ions, their levels are higher in the FP-I than in the FP-R samples. However, the same order of abundance of these ions exists in the two sites with $[\text{NO}_3^-] > [\text{SO}_4^{2-}] > [\text{NH}_4^+]$. The industrial site being closer to the sea, the fine particles in Zakroun have higher levels of Cl^- , Na^+ and Mg^{2+} (sea-salt origin). K^+ concentrations were higher in FP-I samples than FP-R samples except the second period (FP-I2). Ti concentration is higher in FP-I than FP-R which is related to the abrasion of mobile part of motor vehicles (brakes, tires) (Gietl *et al.*, 2010). All the measured species are higher in UFP-I than in UFP-R. The dominant species in the UFP-I are SO_4^{2-} , TC, NO_3^- , and Ca^{2+} and in UFP-R are SO_4^{2-} , TC, NH_4^+ and NO_3^- . Regarding secondary inorganic ions, their levels are higher in the UFP-I than in UFP-R and SO_4^{2-} has the higher concentration, with $[\text{SO}_4^{2-}] > [\text{NO}_3^-] > [\text{NH}_4^+]$. These high values may be explained by the expanded use of diesel in Lebanon in buses, trucks and generator sets (use to alleviate the frequent power cuts) whose combustion leads to the formation of carbonaceous soot rich in sulfur. The high values of the total carbon in both sites may be explained by the expanded use of diesel in Lebanon in buses, trucks and generator sets (use to alleviate the frequent power cuts) whose combustion leads to the formation of carbonaceous soot rich in sulfur.

In the composite samples, few differences are observed between the major components detected in both sites. In both FP-I and FP-R composite samples, NO_3^- , SO_4^{2-} , Ca^{2+} and TC followed by Na^+ , Al, and Cl^- are the most abundant species and account for 64.5% and 61.8% of the mass of PM respectively. After these species, Fe and Mg^{2+} account for 2.7% and 3.3% in FP-I and FP-R respectively, followed by Mg^{2+} (0.7%), K^+ (0.5%), Cu (0.5% and 0.4% for FP-I and FP-R composites respectively), NH_4^+ (0.4% and 0.1%), Ti (0.2% and 0.3%), F⁻ (0.1%), PO_4^{3-} (0.1% and 0.03%) (Table 3.8). In particular, PO_4^{3-} , four times more concentrated in FP-I composite, originates from rocks grinding and other processes in cement plants and phosphate fertilizer industry (Kfoury *et al.*, 2009). Therefore, the presence of such type of industries in the industrial sampling area can explain the observed results. The total carbon content, high in both sites and slightly higher in the industrial site, could be linked to the combustion of fuel oil. Al and Fe concentrations, as well as Ti, were higher at the rural site and could be related to higher contribution of crustal particles in FP-R composite than in FP-I composite.

The sources of these species were explained above, however this difference between the two sites could be explained by many factors that: i) favor the formation of particles; ii) explain their higher concentrations; iii) show the origin of elements encountered in the industrial site compared to

the rural site: industrial activities (NO_x , SO_2 , Ca^{2+} and PO_4^{3-} emissions); burning fuels due to dense traffic, generators and industrial power plants; vegetation and biomass burning (K^+ , Cl^-)...

3.2.2.2 Trace elements

The distribution of trace elements in FP and UFP samples (average concentrations of individual samples) is presented in Figure 3.11. Concentrations of TE are higher in samples collected in the industrial site compared to the rural one, and also higher in the UFP compared to the FP (UFP-I: 170.3 ng/m^3 ; UFP-R: 62.0 ng/m^3 ; FP-I: 106.2 ng/m^3 ; FP-R: 40.8 ng/m^3)

In FP-I, Cu, Zn, Sr, Pb followed by Ba, Mn, V and Ni are the dominant species and account for 95% of the total concentration of trace elements (as defined in 3.2.2.). In UFP-I, Cu, Zn, Ba, Pb, Sr, V followed by Cr, Mn are the dominant species and account for 99.6% of the total concentration of trace elements. In the FP-R sample, Cu, Sr, Zn followed by Ba, Mn, Pb and V are the dominant species (92% of TE) while in UFP-R, the dominant species are Zn, Cu, Ba, followed by Pb, V and Cr (90% of TE).

Pb, Cu, Zn and Sb are related to the abrasion of mobile part of motor vehicles (brakes, tires, ...) (Gietl *et al.*, 2010). Tire rubber is rich in Zn (Adachi and Tainosho, 2004) as well as in Cu, Pb, Mn, Co, Ni and Cd (Thorpe and Harrison, 2008). In cement plants and phosphate fertilizer complexes, raw materials, fossil fuel and waste fuel cause significant emissions of heavy metals such as lead, cadmium. Tires used as combustible waste in cement plants could be the reason of the presence of some of these heavy metals at Zakroun (Greer, 2003). Moreover, the main sources of Ni and V are the burning of fossil fuels and oils (Al-Momani *et al.*, 2005a; Pacyna, 1984).

When comparing industrial and rural FP and UFP, much higher concentrations are found at the industrial influenced site; this can be explained by numerous direct and indirect sources of TE in this site. To account for the contribution of industrial emissions as TE composition in the collected FP-I and UFP-I samples, the Industrial Impact (I.I.) was calculated using Equation 3.1 (Puxbaum *et al.*, 2004). The I.I. was calculated in Zakroun (industrial site), considering Kaftoun as a reference site (rural background) and using elemental concentrations in FP-I and FP-R, and in UFP-I and UFP-R respectively.

$$I.I. (\%) = (I.L. - R.L.) \times \frac{100}{I.L.} \quad \text{Equation 3.1}$$

Where:

I.I. is the industrial impact (in %);

I.L. is the industrial level (the concentration at the industrial site (Zakroun) in ng/m^3);

R.L. is the rural level (the concentration at the rural reference site (Kaftoun) in ng/m^3).

The obtained results are listed in Table 3.9. The results indicate that in FP-I, high industrial impact (> 50%) can be observed for Pb, Cd, Sn, As, Cu, Te, Zn, Ni, Ag, V, Sb compared to relatively less but still significant impact for La, Cr, Co, P, Ba, Sr, Sc, Ce, Rb, Ti, Se, Mn and Nb. In UFP-I,

high industrial impact can be observed for Cd, Sn, Cu, Mn, V, Sr, Zn, Pb, Cr, Ni, As compared to relatively less but still significant impact for Sb, Se, Te, La, Ba, Ag and Co.

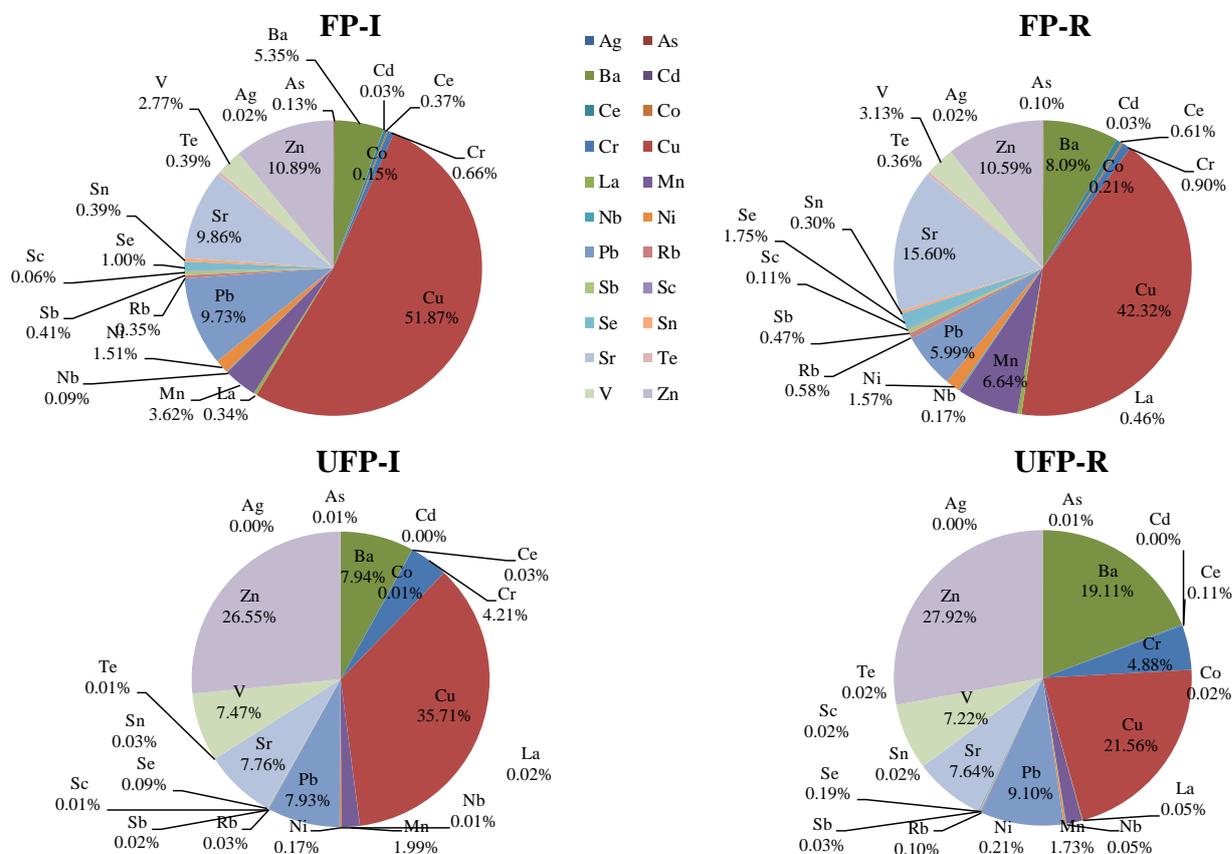


Figure 3.11: Distribution of trace elements in FP and UFP samples (average concentrations of individual samples)

Table 3.9: Industrial impact in Zakroun (industrial influenced site) and considering Kaftoun as a reference site (rural background) using elemental concentrations

Industrial Impact (I.I.) (%)	Ag	As	Ba	Cd	Ce	Co	Cr	Cu	La	Mn	Nb
	FP	59	70	42	70	36	46	48	69	49	30
UFP	7	51	12	88	-	5	58	78	16	68	-
Industrial Impact (I.I.) (%)	Ni	Pb	Rb	Sb	Sc	Se	Sn	Sr	Te	V	Zn
	FP	60	76	36	56	36	33	70	39	65	63
UFP	55	58	-	38	-	21	80	64	21	65	62

3.2.2.3 Comparison between the composition of PM_{2.5} collected in the industrial and rural influenced sites and Lebanese and Mediterranean sites PM_{2.5}

Table 3.10 shows the average concentrations of sea-salt major ions, secondary inorganic ions, other major elements and total carbon (ng/m³) in the PM_{2.5} collected in Zakroun, Kaftoun and other Mediterranean sites. In our case, PM_{2.5} was considered as the sum of FP and UFP.

When first comparing sea-salt major ions (Na⁺, Mg²⁺ and Cl⁻) in our study sites to different Mediterranean sites, higher mean concentration were found in Zakroun (industrial influenced site) and

Kaftoun (background rural site) than the other sites including industrial sites or densely populated urban areas affected by road traffic in the Mediterranean basin. The mean concentration of Na^+ in Zakroun (1769 ng/m^3) and in Kaftoun (886 ng/m^3) were higher than in sites in all the compared sites. Our concentrations were lower than those found in Erdemli (Turkey, 3095 ng/m^3) which is located directly on the seashore (Koçak *et al.*, 2007). Compared to other studies in Lebanon, Na^+ concentrations in Zakroun were higher than those in Chekka (1079 ng/m^3), in Sin El-Fil (1183 ng/m^3) and HaretHrek (1060 ng/m^3) (Borgie *et al.*, 2016; Kfoury *et al.*, 2009; Saliba *et al.*, 2010). The mean concentration of Mg^{2+} in Zakroun (259 ng/m^3) was higher than different urban and industrial Mediterranean sites but lower than in Erdemli (Turkey, 489 ng/m^3). Cl^- in Zakroun (929 ng/m^3) was higher than many Mediterranean sites except in Chekka (Lebanon, 1278 ng/m^3), some Egyptian sites ($9200\text{-}13000 \text{ ng/m}^3$), Erdemli (Turkey, 5492 ng/m^3), Pentelli, Finokalia and Crete (Greece, 1530, 1460, 1509 ng/m^3 respectively). These ions are highly dependent on the distance of the sampling site to the sea and the wind direction. It is a major contributor to the coarse fraction (Salameh *et al.*, 2015). The Cl^- ions can also be generated by some anthropogenic activities like combustion related emissions (biomass and incineration) and, in colder regions, a part of Cl^- ions could be neutralized by ammonium ions (Ledoux *et al.*, 2006). The Mg^{2+} can arise from dust storm episode (Borgie *et al.*, 2016) and can also be found in raw materials used in cement plants or fertilizer industries (Shahsavani *et al.*, 2012).

Ammonium concentrations in the Mediterranean sites appear relatively constant (between 1210 and 2168 ng/m^3) except for some sites in Egypt (Cairo) where the concentration can reach 8700 ng/m^3 and in Thessaloniki where the concentration is about 3800 ng/m^3 . The NH_4^+ concentrations in Zakroun and in Kaftoun were 1015 and 1044 ng/m^3 respectively, lower than in Mediterranean sites. Nitrates (NO_3^-) and sulfates (SO_4^{2-}) average concentrations in the industrial site, Zakroun, (4010 and 8021 ng/m^3 respectively) are both higher than those observed in other comparison sites including industrial sites or densely populated urban areas affected by road traffic in the Mediterranean basin. Exceptions are observed for nitrates in Chekka (6182 ng/m^3) and Venice (5400 ng/m^3), and for sulfates at two urban sites in Lebanon (8659 ng/m^3 in Sin El Fil and 8530 ng/m^3 in Haret Hreik) where similar concentrations were observed. The sulfates might be due to: i) an enhanced photochemical activity, which increase the oxidation of local SO_2 from cement production activity and its conversion rate to sulfate (Khoder, 2002; Mihalopoulos *et al.*, 2007); ii) an increased emissions from raw materials containing sulfates in the industries; iii) the stagnation of air masses over the Mediterranean Basin (Rodríguez *et al.*, 2004). Conversely, nitrate aerosols are more sensitive to temperature and the shift in equilibrium from gas-phase ammonia and nitric acid to particulate phase ammonium nitrate is favored at lower temperatures and its major source is combustion phenomena (Cesari *et al.*, 2016; Salameh *et al.*, 2015). NO_3^- concentrations could be high in our study sites due to the combustion of fuels at high temperatures in cement kilns results in the release of NO_x emissions (Mousavi *et al.*, 2014). The relatively low NH_4^+ concentrations could partly be explained by the lower emission rates

of ammonium precursors in the region, and/or because of the instability of ammonium salts in particulate form in relatively warm atmospheres (Galindo *et al.*, 2008; Hodzic *et al.*, 2006) as discussed previously. Cement factories emit NO_2 which is unstable in gaseous form and might react with sea salts and/or other gases like SO_2 (gas-particle conversion reactions) to form NO_3^- in a high humidity media (Seinfeld and Pandis, 2012). Nevertheless, due to the kinetics, the most probable explanation for this high level of atmospheric nitrate can be in the presence of secondary atmospheric aerosols, carried within air masses by long range transport mechanisms and accumulating in the regional atmosphere.

Previous studies demonstrated that the mass ratio of $\text{NO}_3^-/\text{SO}_4^{2-}$ is an indicator of the relative importance of stationary versus mobile sources in air pollution (Gao *et al.*, 2011). High $\text{NO}_3^-/\text{SO}_4^{2-}$ mass ratios have been measured in southern California, with 2 in downtown Los Angeles and 5 in Rubidoux, which was due to lower coal usage (Kim *et al.*, 2000); However in Chinese cities (e.g. Beijing, Shanghai), lower ratios had been reported as a result of the wide use of sulfur-containing coal (Yao *et al.*, 2002). In this study, the mean mass ratio of $\text{NO}_3^-/\text{SO}_4^{2-}$ was 0.49 in Zakroun. These values are similar to those reported in the study done in Jinan, China by Gao *et al.* where the mean mass ratio of $\text{NO}_3^-/\text{SO}_4^{2-}$ was 0.44 (Gao *et al.*, 2011) and other studies done in other cities in China. The above findings indicated that stationary sources such as industry emissions play an important role in atmospheric particle concentrations in Zakroun, even though the vehicular emissions cannot be ignored.

The average concentration of Al is higher in all the other sites than in $\text{PM}_{2.5}$ collected in Kaftoun and Zakroun (Table 3.10) except in Egypt. The Fe concentration was higher in $\text{PM}_{2.5}$ collected in Zakroun when compared to other sites, except in Escuelas Aguirre (Spain), Crete and Thessaloniki (Greece) and in Sin El Fil (Lebanon). The mean concentration of Ca^{2+} is higher in $\text{PM}_{2.5}$ collected in Zakroun when compared to other sites, and in Kaftoun is close to that in Greece (Pentelli; 2030 ng/m^3). The origin of calcium in Lebanon, like other Mediterranean sites (Al-Momani, 2003; Anatolaki and Tsitouridou, 2009), is considered to be mainly of natural sources due to calcareous nature of the soil (Abdel-Rahman and Nader, 2002). These high concentrations could be due to a larger contribution of crustal and re-suspended dust at the industrial site, especially in the warm and dry season, due to the presence of unpaved roads located nearby the site. In addition, different contribution of biomass burning could also play a role, considering that K^+ is a tracer of biomass burning. Specifically, fugitive and channeled emissions from cement plants as well as dust emissions from the extraction and transportation of mineral raw materials are responsible for elevated calcium concentration (Galindo *et al.*, 2011). The mean concentration of P and Ti is high in our site compared to other Mediterranean sites. In general, Ti is found in crustal dusts therefore its high concentration could be explained by the re-suspension of dust by the movement of vehicles on the roads (Wedepohl, 1995). Emissions from: dust and sea-salt transport, volcanic eruptions, biogenic sources and

combustion of fossil fuels, biofuels and biomass, contribute about 5% of total phosphorus emissions. Wang *et al.* showed that estimates of phosphorus emissions from combustion suggest that anthropogenic emissions represent more than 50% of atmospheric sources of phosphorus (Wang *et al.*, 2015). In our study, phosphate fertilizer industries could have an impact on P concentration. As mentioned before, calcium carbonate (CaCO_3) is used in cement plant rotary kiln so this can explain the increase of Ca^{2+} concentration in Zakroun, the industrial site. The concentrations of crustal elements (Al and Fe) are high because wind-driven suspension of particles from surface soils and desert gives rise to natural dust contribution to aerosol (Viana *et al.*, 2008).

Furthermore, average concentration of TC in Zakroun (7509 ng/m^3) was close to those found in Sin El-Fil (7439 ng/m^3), an urban background site in Italy (Lecce; 7100 ng/m^3) and two residential sites in Palestine (7400 and 7100 ng/m^3) both influenced by road traffic, but was higher than those found in background or suburban sites in Italy, Greece and Spain. It could suggest the influence of road traffic on the composition and the concentration of $\text{PM}_{2.5}$ in Zakroun. These high values may be explained by the expanded use of diesel in Lebanon in buses, trucks and generator sets. However, all the other comparison sites (urban and residential with higher population density than Zakroun) have higher total carbon contents.

The average concentrations of trace elements (ng/m^3) in $\text{PM}_{2.5}$ collected in Kaftoun, Zakroun and other Mediterranean sites are presented in Table 3.11. Regarding trace elements, Cu, Zn, Pb and Sr had the highest average concentrations compared to other trace elements. Concentrations of As, Ba, Cd, Co, Cr, Mn, Nb, Ni, Pb, Rb, Sb, Sn, V and Zn in Zakroun and Kaftoun samples ranged between those obtained at the other sites in the Mediterranean basin, while the average concentration of Cu and Sr was much higher in our site. According to trace elements emission sources, elaborated before, this difference is due to the abrasion of mobile part of motor vehicles (Cu, Zn, Mn, Pb, Cd), re-suspension of dust by the movement of vehicles on the roads (Ba), burning of fossil fuels and oils (Ni and V). Therefore, these elements are emitted by the wear of vehicle tires, explaining their highest levels in urban sites and in those with higher population density than our site. For example Sin El Fil and Bourj Hammoud have higher Zn concentration (47.3 and 100 ng/m^3) compared to Zakroun. These latter are very dense urban cities and the very important road traffic could explain the highest Zn values.

3.2.3 Crystalline phases, individual particle composition and morphology

After detecting major and trace elements by ICP, IC and CHNS-O, the XRD and SEM-EDX were used in order to investigate how detected species are associated within the particles. FP-I and FP-R composites were tested to identify the crystalline phases and different morphologies that can give us an idea about the origins of these particles.

Table 3.10: Average concentrations of sea-salt major ions, secondary inorganic ions, other major elements and total carbon (ng/m³) in PM_{2.5} collected in Zakroun, Kaftoun and other Mediterranean sites

Country	City	Characteristics of the site	Sea-salt ions			Secondary inorganic ions			Other majors elements					Total carbon	Studies	
			Mg ²⁺	Na ⁺	Cl ⁻	NO ₃ ⁻	SO ₄ ²⁻	NH ₄ ⁺	Al	Fe	P	Ti	Ca ²⁺	K ⁺		TC
Lebanon	Zakroun	Industrial	259	1769	929	4010	8021	1015	384	422	47	30	3042	206	7509	This study
	Kaftoun	Background	127	886	491	2110	5002	1044	247	217	25	21	1383	94	4248	
	Sin El-Fil	Urban	165	1183	850	3242	8659	1802	535	639	42.5	110	2349	145	7439	(Borgie <i>et al.</i> , 2016)
	Chekka	industrial-urban	148	894	1278	6182	4048	1726	522	386	-	37	2134	113	-	(Kfoury <i>et al.</i> , 2009)
	HaretHreik	Urban	210	1060	840	1270	8530	1740	-	-	-	-	2900	250	-	(Saliba <i>et al.</i> , 2010)
Egypt	Al Zamalek	Residential	-	300	13000	2700	6000	8700	100	200	-	-	-	400	19000	(Abu-Allaban <i>et al.</i> , 2002)
	El Qualaly	Traffic	-	400	9200	3000	6700	6800	100	500	-	-	-	500	36200	
	Helwan	Residential	-	300	1400	1700	4000	1700	0	100	-	-	-	300	13200	
	Kaha	Background	-	200	10300	3200	4800	7900	100	100	-	-	-	700	16100	
Palestine	Jerusalem-East	Residential, traffic	-	-	-	920	4400	-	-	-	-	-	-	7400	-	(Sarnat <i>et al.</i> , 2010)
	Hebron	Residential, traffic and light industries	-	-	-	910	3800	-	-	-	-	-	-	7100	-	
	Nablus	Residential and commercial	-	-	-	1010	4300	-	-	-	-	-	-	13800	-	
Jordan	Amman	Residential and commercial	-	-	-	1020	4500	-	-	-	-	-	-	8600	-	
Turkey	Erdemli	Urban	489	3434	5492	1857	4953	850	-	351	-	27.1	1560	203	-	(Mustafa Koçak <i>et al.</i> , 2007)
	Istanbul	Urban	-	-	340	1720	4270	1240	-	460	-	-	-	-	-	(Szigeti <i>et al.</i> , 2013)
Italy	Lecce	Urban	100	400	100	200	5590	2100	-	-	-	-	600	400	7100	(Perrone <i>et al.</i> , 2011)
	Lecce (downtown)	Urban	34	389	206	421	2826	769.6	-	14	-	-	-	157	4248	(Cesari <i>et al.</i> , 2016)
	Corso Firenze	Urban	-	-	-	-	-	-	-	-	6	7	-	-	-	(Cuccia <i>et al.</i> , 2013)
	Genoa	Urban background	-	100	-	500	3600	1400	-	124	-	-	-	60	3400	(Salameh <i>et al.</i> , 2015)
	Venice	Urban background	-	200	-	5400	3400	2300	-	357	-	-	-	400	5800	
Greece	Pentelli	Suburban	190	-	1530	1110	4620	1250	-	-	-	-	2030	520	4580	(Pateraki <i>et al.</i> , 2012)
	Votanikos	Industrial, traffic	70	-	800	1330	5190	1300	-	-	-	-	850	350	6260	
	Tourlos	Coastal	80	-	700	760	5180	1210	-	-	-	-	750	480	2830	

	(Aegina)	background														
	Athens	Urban, residential and traffic	-	-	80	176	1380	-	101	212	-	-	-	-	-	(Karanasidou <i>et al.</i> , 2009)
	Finokalia	Marine background site	160	1080	1460	980	4410	1540	-	-	-	-	440	310	-	(Bougiatioti <i>et al.</i> , 2013)
	Athens (downtown)	Urban	20.4	92	9	1224	7268	2168	-	-	-	-	451	99.5	-	(Pennanen <i>et al.</i> , 2007)
	Crete	Urban background	186	1375	1509	1731	5478	1617	-	598	-	43	1298	189	-	(Koulouri <i>et al.</i> , 2008)
	Thessaloniki	Industrial- urban	7	290	390	2400	3960	3800	741	864	-	3	1430	140	-	(Tolis <i>et al.</i> , 2015)
Spain	Iturrama	Urban	-	-	80	1630	2070	1240	-	80	24.4	6.31	-	-	4040	(Aldabe <i>et al.</i> , 2011)
	Plaza de la Cruz	Traffic	-	-	110	2060	2070	1220	-	90	11.4	4.11	-	-	5110	
	Castillo de Bellver	Suburban	-	-	400	900	3900	2000	-	-	11	7	-	-	3600	(Pey <i>et al.</i> , 2009)
	Escuelas Aguirre	Urban	-	-	-	1330	3800	1410	-	570	34	21	-	-	-	(Artiñano <i>et al.</i> , 2003)
	Barcelona (downtown)	Urban	50.5	765	475	3366	3441	1515	-	-	-	-	142	99.1	-	(Pennanen <i>et al.</i> , 2007)
	Barcelona	Urban	-	300	-	1000	2800	1000	-	155	-	-	155	-	4200	(Salameh <i>et al.</i> , 2015)
France	Marseille	Urban background	-	100	-	1700	2200	1500	-	265	-	-	-	100	7900	

Using XRD, several similar crystalline phases were identified in both FP-I and FP-R composite samples (Figure 3.12): Calcite (CaCO_3 , JCPDS: 00-005-0586), Gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 00-033-0311), Halite (NaCl , 00-005-0628), Nitratine (NaNO_3 , 00-036-1474), Quartz (SiO_2 , 00-046-1045), and an aluminosilicate phase that could be Chloritoid ($\text{Fe}_{1.81}\text{Mg}_{0.27}\text{Al}_{3.92}\text{Si}_2\text{O}_{10}(\text{OH})_4$, 01-070-0843, or $\text{Fe}_{1.77}\text{Mg}_{0.15}\text{Al}_{3.84}\text{Fe}_{0.16}\text{Si}_2\text{O}_{10}(\text{OH})_4$, 01-083-1362). SiO_2 can have a crustal origin or can be derived from cement factories. In fact, cement is produced from geological materials that contain CaO , SiO_2 , Al_2O_3 and Fe_2O_3 in certain proportions according to the formulation of cement (Smadi *et al.*, 2009). The presence of intense diffraction lines of CaCO_3 is compatible with the calcareous soil in Lebanon and could also be due to its use as raw materials in cement plants. The presence of halite in the PM can be explained by the proximity to the sea. Nitratine is used in fertilizer industries but can also result from the ageing of fresh sea salts. Chloritoid is a silicate mineral of metamorphic origin, it is a rock-forming mineral found in Al-rich metapelitic rocks (Koch-Müller *et al.*, 2000).

The scanning electron microscopy coupled with energy dispersive X-ray (SEM-EDX) analysis of collected PM provides information about the association between elements even if they are not in a crystallized state. In both FP-I and FP-R composite samples, particles with different sizes and morphologies were detected (Figure 3.13 and Figure 3.14). In the two sites, aluminosilicates particles, mainly consisting of Si, Al, Ca, Fe, Ti and Mg were observed and found flat and angular or coarse with rounded or softly eroded contours. Most of these are seen in agglomerates. Aluminosilicates PM originate mainly from the soil. Ca-S rich particles characterized with an almost constant proportion of these two elements (2/3 Ca and 1/3 S), corresponding probably to the gypsum particles ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) as shown by XRD, were also identified rather flat and angular on the two sites. The formation of this mineral may arise from the heterogeneous oxidation of SO_2 on the surface of existing mineral particles, which could be present due to the combustion of fossil fuels and biomass burning. These compounds are also known to have natural origins as a result of water evaporation from sea spray (Dehoog *et al.*, 2005) but also originated from raw materials used in cement production. Si and Ca rich particles probably as SiO_2 (quartz) and CaCO_3 (calcite) were frequently detected on both sites as shown by XRD. Particles rich in Fe, Ti, and Cu were also identified on sites under industrial and rural wind influence. For the industrial site, particles composed of P and Ca, are observed and could be related to the presence of monocalcium phosphate or calcium fluorophosphates minerals that correspond to raw materials used in the manufacture of phosphate fertilizers. This is confirmed by the higher concentrations of F^- , PO_4^{3-} , Ca^{2+} and SO_4^{2-} found in FP-I compared to FP-R. It should also be noted that different types of carbonaceous soot particles are present in the industrial sample originating from combustion processes (Witt *et al.*, 2010).

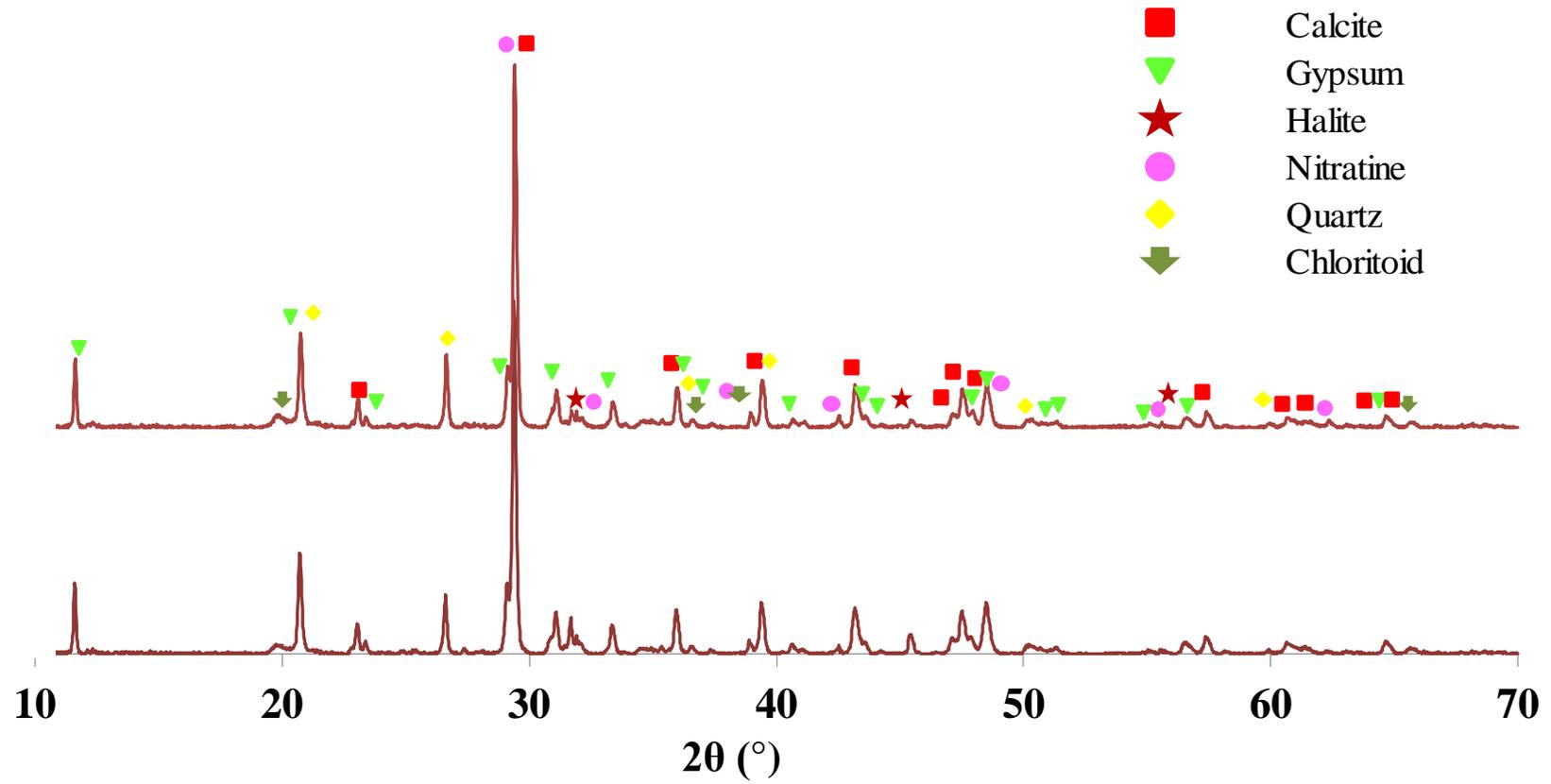


Figure 3.12: X-ray Diffraction patterns of PM_{2.5} from Zakroun (FP-I) and Kaftoun (FP-R)

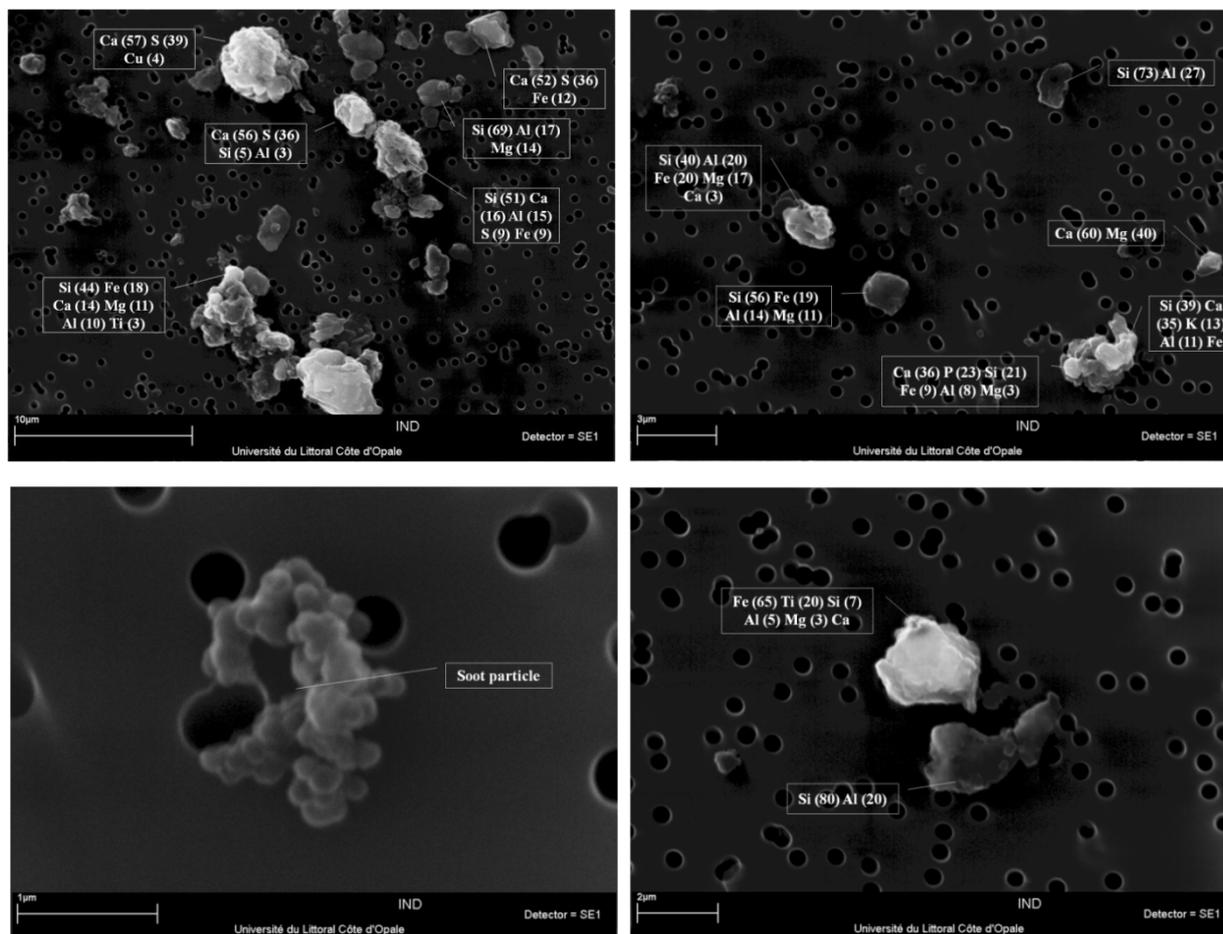


Figure 3.13: Scanning Electron Microscopy coupled with energy dispersive X-ray analysis of the industrial fine PM (FP-I)

The composition of each particle is given as mass percentage between brackets.

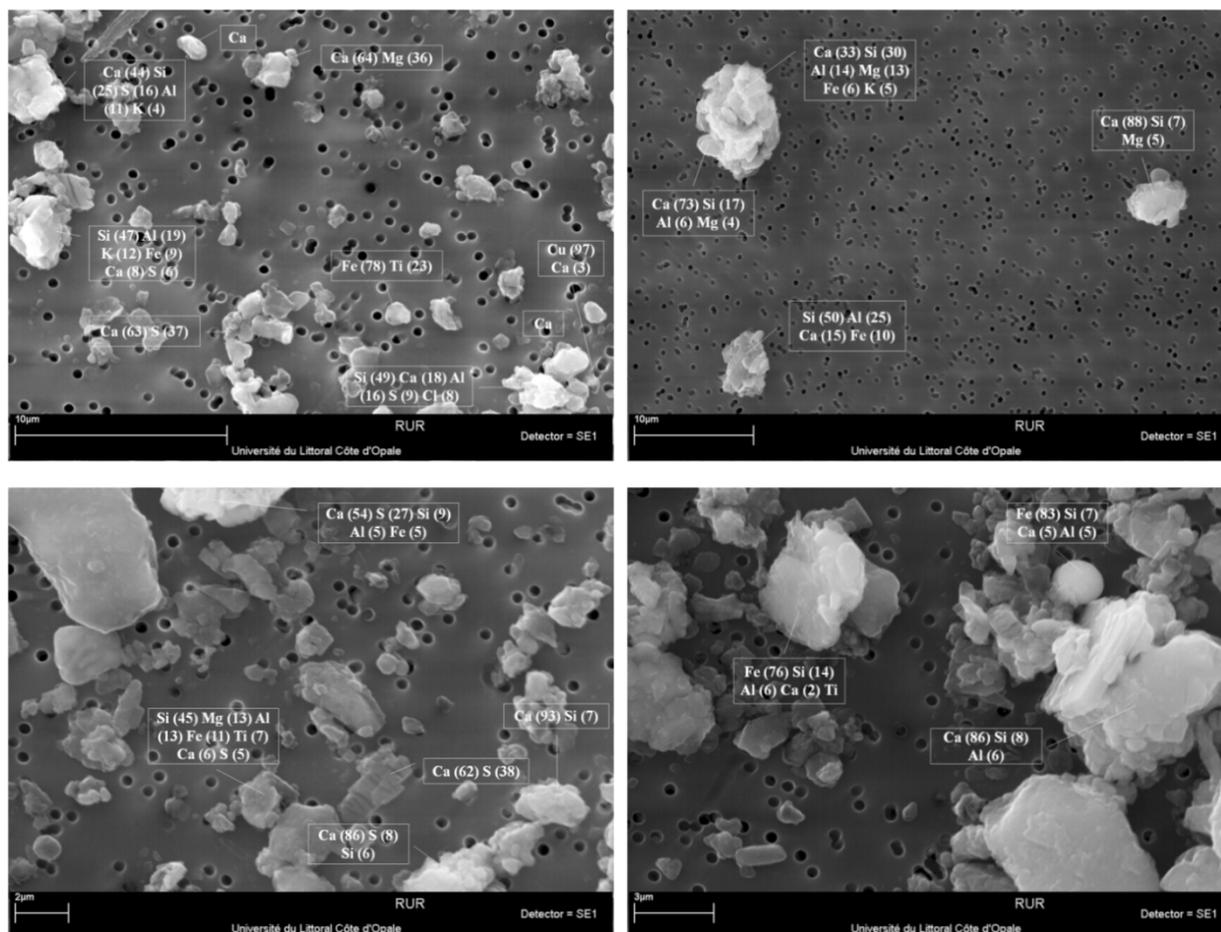


Figure 3.14: Scanning Electron Microscopy coupled with energy dispersive X-ray analysis of the rural fine PM (FP-R)

The composition of each particle is given as mass percentage between brackets.

3.2.4 Polycyclic Aromatic Hydrocarbons, paraffins, PCDD/Fs and PCBs

The analyses of Polycyclic Aromatic Hydrocarbons (PAHs) and paraffins in fine (composites) and ultrafine (individual samples) particles collected in Zakroun and Kaftoun, were performed by GC-MS.

3.2.4.1 PAHs concentrations

The concentrations of the 16 priority PAHs listed by the United States Environmental Protection Agency were investigated: Acenaphthene (Ac), Acenaphthylene (Acl), Anthracene (Ant), Benz[a]anthracene (B[a]An), Benzo[b]fluoranthene (B[b]Fl), Benzo[k]fluoranthene (B[k]Fl), Benzo[a]pyrene (B[a]P), Benzo[g,h,i]perylene (B[ghi]Pe), Chrysene (Chr), Dibenz[a,h]anthracene (DiB [a,h]An), Fluoranthene (Fla), Fluorene (Flu), Indeno[1,2,3-cd]pyrene (InPy), Naphthalene (Nap), Phenanthrene (Ph) and Pyrene (Pyr).

PAHs are ubiquitous organic pollutants originated mainly from incomplete combustion of organic materials (Borrás *et al.*, 2009; Liu *et al.*, 2015). Song *et al.* showed that many of them are

toxic and potentially carcinogenic at relatively low levels (Song *et al.*, 2012). Due to their low vapor pressure, some PAHs are present at ambient temperature in air, both as gas and associated with particles. The lighter PAHs (2 and 3 cycles) are found almost exclusively in gas phase whereas the heavier PAHs (5 and 6 cycles) are almost totally adsorbed on particles (Borgie *et al.*, 2016; Cecinato *et al.*, 2014; Tobiszewski and Namieśnik, 2012).

PAHs concentrations in FP-I, FP-R, UFP-I and UFP-R are shown in Table 3.12 and the average concentrations are represented in Figure 3.15. The InPy, B[ghi]Pe, DiB[a,h]An, Chr, B[b]Fl, and B[a]An were the dominant PAHs found in both FP samples with higher concentrations in the FP-I sample. The main difference in concentrations between the FP-I and FP-R appears for the InPy, B[b]Fl, B[a]An, Chr and B[ghi]Pe. The total PAHs mass concentrations were higher in FP-I (13 $\mu\text{g/g}$) than in FP-R (9 $\mu\text{g/g}$). When converted to atmospheric concentrations, the sum of the PAHs in FP-I (0.2 ng/m^3) and in FP-R (0.11 ng/m^3) were similar to those found in $\text{PM}_{2.5-0.3}$ at industrial and rural sites such as Dunkirk (France, 0.29 ng/m^3) and Rubrouck (France, 0.14 ng/m^3) respectively in northern France (Cazier *et al.*, 2016) and in Beirut, Lebanon (rural site, 0.217 ng/m^3) (Borgie *et al.*, 2015).

The major compounds in all UFP-Is are InPy > B[ghi]Pe > DiB[a,h]An > B[b]Fl > B[a]An > B[a]P > B[k]Fl > Flu. The major compounds in all UFP-Rs are InPy > B[ghi]Pe > B[b]Fl > B[a]P > B[a]An > Fla (Appendix 17). PAHs concentrations were higher in UFP-I2 and UFP-I3 compared to the other samples. The main difference in concentrations between the UFP-I and UFP-R appears for the DiB[a,h]An, B[k]Fl, InPy, B[b]Fl, B[a]An, Chr and B[ghi]Pe.

The PAHs concentrations are 20 times higher in $\text{PM}_{0.3}$ (total PAHs = 4.63 and 2.04 ng/m^3 in UFP-I and UFP-R respectively) than in $\text{PM}_{2.5-0.3}$ (total PAHs = 0.2 and 0.11 ng/m^3 in FP-I and FP-R respectively). This is probably due to larger surface areas of UFP compared to FP making UFP a more suitable adsorption medium for organic compounds (Di Filippo *et al.*, 2015; Gupta *et al.*, 2011).

The mentioned PAHs are emitted by numerous combustion sources, including motor vehicles (mainly gasoline vehicles), biomass burning, coal combustion and industrial processes. The InPy is associated with diesel and also gasoline emissions (Boström *et al.*, 2002; Riddle *et al.*, 2007). Therefore, Chen *et al.* investigated the PAHs generated in a waste-tire pyrolysis process and the dominant PAHs were InPy, DiB[a,h]An and B[a]P (Chen *et al.*, 2007). The use of scrap tires as combustible in cement factories (Greer, 2003) is also suspected and could be the reason behind the large difference in InPy concentrations between the two sites (FP and UFP). This hypothesis is reinforced by our previous results showing high proportion of characteristic metals related to tires composition at the site under industrial influence. The B[ghi]Pe is known to be the main tracer for gasoline emissions (Li *et al.*, 2003; Wang *et al.*, 2009). The B[b]Fl and B[k]Fl were reported to be strongly related to diesel and gasoline motor vehicles in urban environments (Carreras *et al.*, 2013).

High molecular weight PAHs have been described as tracers of gasoline, while light PAHs such as Phe, Fla and Pyr have been found predominantly in diesel vehicle emissions (Amador-Muñoz *et al.*, 2010; Pietrogrande *et al.*, 2014).

In order to determine the sources of PAHs in the FP and UFP samples, the reference ratios of PAHs were calculated and compared with typical ones used as source indicators in the literature (Table 3.13) (Cazier *et al.*, 2011; Cecinato *et al.*, 2014; De La Torre-Roche *et al.*, 2002; Masclet *et al.*, 1986; Ravindra *et al.*, 2008; Yunker *et al.*, 2002). In fact, despite the physicochemical transformations occurring during the life of an atmospheric particle, some selected PAHs concentrations ratios can remain constant during the transport in the atmosphere from the emission source. This is especially the case of isomers which have similar physicochemical properties and could be affected by oxidation or photochemical phenomenon in an identical way (Guillon, 2011; Robinson *et al.*, 2006). The atmospheric oxidation causes systematic changes in the ambient ratios with increasing photochemical age. Consequently, ratios such as Fla/(Fla+Pyr) and InPy/(InPy+B[g,h,i]Pe) are of interest due to the identical photo-stability of such PAHs. On the contrary, PAHs ratios such as Anth/(Anth+Phe) or B[a]An/(B[a]An+Chr) must be considered with care, since the concerned isomers have different detachment reactivities.

In both FP and UFP, the InPy/(InPy+B[ghi]Pe) ratio showed the influence of diesel burning in the two sites, and is also compatible with wood burning in the industrial site. Nevertheless, this last suggestion should be taken with care considering that this ratio is also affected by the highest concentration of InPy probably related to tire pyrolysis. The ratio Flu/(Flu+Pyr) showed the influence of petrogenic sources and it is an indicator of gasoline combustion in the two sites. Moreover, the ratios B[a]P/(B[a]P+Chr), B[a]P/B[ghi]Pe and InPy/B[ghi]Pe showed the contribution of the transport sector and the diesel combustion. These ratios are interesting due to the photo-stability of these PAHs. The ratio Fla/(Fla+Pyr) showed the possible influence of cement plant raw material, paved roads, soil dust and diesel combustion. The ratio Chr/(Chr+B[a]P) is compatible with the influence of the industries and fertilizer production and house heating (FP). According to these results, an influence of diesel and gasoline emissions on the composition of PAHs was demonstrated at the two sampling sites, with a lesser influence of gasoline at the rural site. In Lebanon, gasoline is used as fuel for private cars while diesel is used in buses, trucks and generator sets (use to alleviate the frequent power cuts). Particularly on the site under industrial influence, ratios could show a contribution of cement factories activities (tires burning), fertilizer production and heavy fuel used in the energy sector.

From the concentrations data it was also possible to calculate the ratio between combustion-related PAHs (CPAHs: Fla, Pyr, B[a]An, Chr, B[b]Fl, B[k]Fl, B[a]P, InPy, B[ghi]Pe) and total PAHs (TPAHs). This ratio is an indicator of the extent in which combustion sources contribute to PM. Typical CPAH/TPAH reference values are 0.43 for non-urban areas, 0.7 for an urban area and 0.96 for a highly industrialized area (Gogou *et al.*, 1996). CPAH/TPAH values obtained for FP-I and FP-R

are very close, 0.84 and 0.82 respectively. Thus, the FP-I and FP-R have the characteristic of an urban area influenced by industries.

Table 3.12: PAHs concentrations in the composite fine particles (FP-I and FP-R) and quasi-ultrafine particles (UFP-I and UFP-R) collected under industrial and rural influences expressed in $\mu\text{g/g}$ and in pg/m^3

	Composite samples				Average of individual samples	
	FP-I	FP-R	FP-I	FP-R	UFP-I	UFP-R
	$(\mu\text{g/g})$		(pg/m^3)		(pg/m^3)	
Naphthalene	0.14	0.17	2.1	2.1	-	-
Acenaphthylene	-	-	-	-	-	-
Acenaphthene	-	-	-	-	-	-
Fluorene	0.023	0.024	0.36	0.29	255	-
Phenanthrene	-	-	-	-	200	82
Anthracene	-	-	-	-	251	-
Fluoranthene	0.99	0.92	15.5	11.2	248	164
Pyrene	0.85	0.87	13.4	10.5	212	145
Benz[a]Anthracene	0.44	0.38	7.0	4.6	368	240
Chrysene	1.5	1.2	24.2	14.3	242	154
Benzo[b]fluoranthene	1.3	0.41	19.8	5.0	437	256
Benzo[k]fluoranthene	-	-	-	-	287	23
Benzo[a]pyrene	0.69	0.63	10.8	7.6	365	254
Dibenz[a,h]anthracene	1.8	1.4	28.6	16.5	535	-
Indeno[1,2,3-c,d]pyrene	3.0	1.5	46.9	18.6	628	377
Benzo[g,h,i]perylene	1.8	1.6	28.7	19.6	601	350

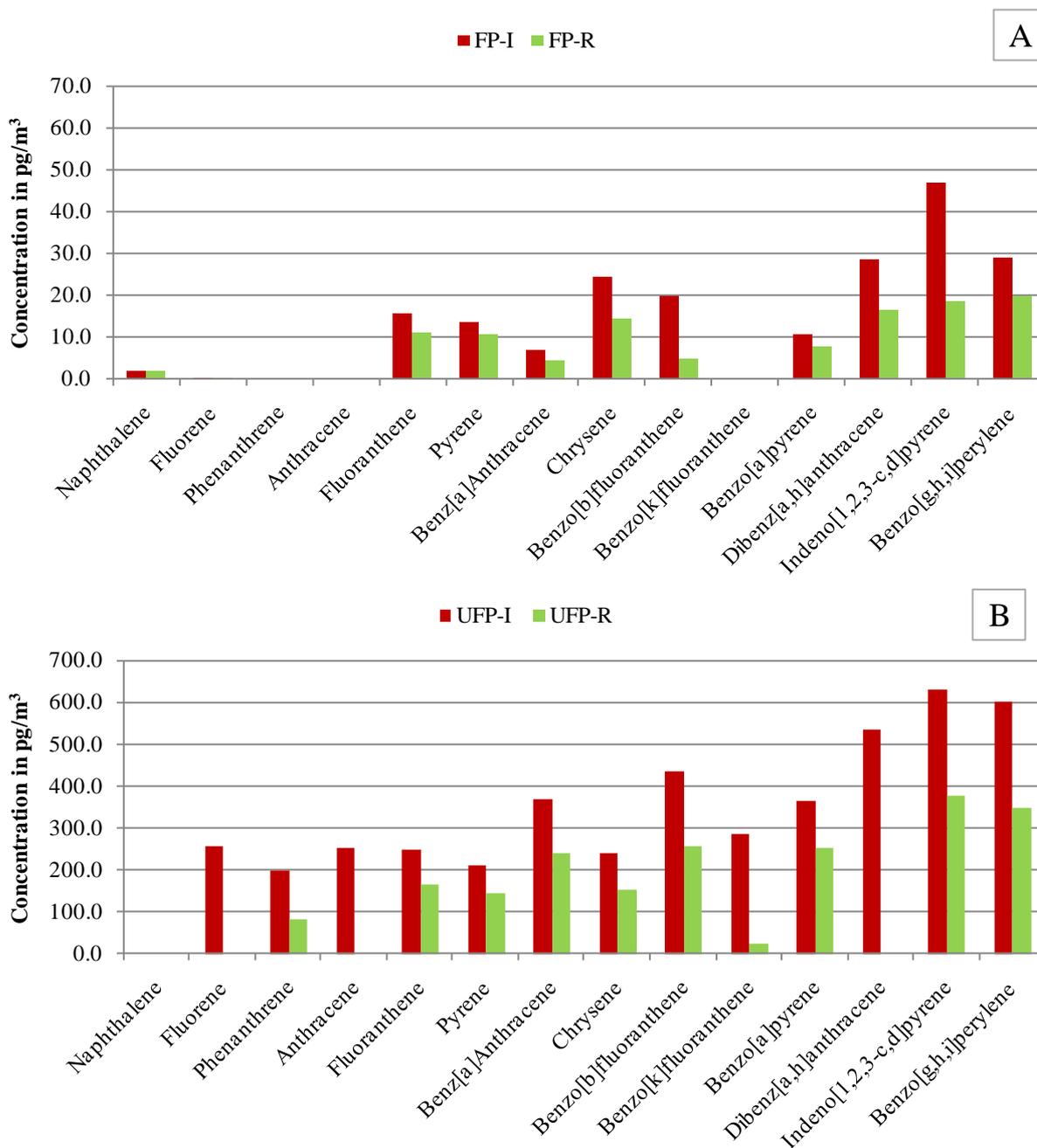


Figure 3.15: PAHs concentrations in FP and mean concentrations in UFP (period 2 excluded) under industrial and rural influence

Table 3.13: PAH ratios calculated for PM_{2.5-0.3} (FP-I and FP-R) and PM_{0.3} (UFP-I and UFP-R) samples collected in Zakroun and Kaftoun - comparison with typical PAH ratios for different sources from the literature

Ratios	FP-I	FP-R	UFP-I	UFP-R	Potential Source According to Literature				
InPy / (InPy+B[ghi]Pe)	0.620	0.487	0.511	0.519	0.18: Cars ^(a)	0.37: Diesel ^(a)	0.56: Coal ^(a)	0.62: Wood burning ^(a)	0.35-0.70: Diesel burning ^(a)
					0.27-0.48: Urban air ^(g)	0.52- 0.69: Grasses combustion ^(g)	0.51: Road dust ^(g)		
Flu/ (Flu+Pyr)	0.026	0.027	0.546	0	>0.5: Diesel ^(a)	<0.5: Gasoline ^(a)	<0.4: Petrogenic ^(b)	0.4-0.5: Fossil fuel ^(b)	>0.5: Grass, wood, coal combustion ^(b)
B[a]P/ (B[a]P+Chr)	0.308	0.348	0.601	0.622	0.5: Diesel ^(a)	0.73: Gasoline ^(a)			
B[a]P/ B[ghi]Pe	0.375	0.389	0.607	0.726	0.5-0.6: Traffic emission ^(a)	0.6-1.7: Refinery ^(c,d)	>1.25: Brown coal or lignite ^(a)	1.3: vehicles ^(d)	0.4: catalysed car ^(f)
InPy/ B[ghi]Pe	1.632	0.950	1.045	1.079	<0.4: Gasoline ^(a)	~1: Diesel ^(a)			
Pyr/ B[a]P	1.245	1.378	0.582	0.569	0.3-0.8: Heating ^(d)	~1: Gasoline engine ^(a)	1.5: Petrol ^(d)	~10: Diesel engine ^(a)	1.32: Catalysed car ^(f)
Fla/ Pyr	1.154	1.063	1.171	1.135	0.5: Refinery ^(d)	0.6: Vehicular ^(a)	~1: coke oven ^(e)		
Fla/ (Fla+Pyr)	0.536	0.515	0.539	0.532	0.54: Electroplating industries ^(f)	0.53: Cement plant raw material ^(f)	0.52: Paved roads ^(f)	0.52: Soil Dust ^(f)	0.50: Electrostatic precipitator ^(f)
					0.41- 0.67: Wood combustion ^(g)	0.42: Road dust ^(g)	0.61: Bush fire ^(g)	0.24 : Power production ^(f)	
B[a]An/ (B[a]An+B[a]P)	0.393	0.375	0.502	0.486	0.40: Paved roads dust ^(f)	0.36: Oil fuelled house heating ^(f)			
Chr/ (Chr+B[a]P)	0.692	0.652	0.399	0.378	0.69: Industries ^(f)	0.68: Fertiliser production ^(f)	0.65: House heating ^(f)	<0.65: Vehicles ^(f)	

^(a)(Ravindra *et al.*, 2008), ^(b)(De La Torre-Roche *et al.*, 2009), ^(c)(Mandalakis *et al.*, 2002)

^(d)(Masclat *et al.*, 1986), ^(e)(Cazier *et al.*, 2011), ^(f)(Cecinato *et al.*, 2014), ^(g)(Yunker *et al.*, 2002)

3.2.4.1.a Comparison of the composition in PAHs between the industrial and rural influenced PM_{2.5} (FP+UFP) and Lebanese and Mediterranean sites PM_{2.5}

Significantly higher concentration of total PAHs was in the industrial influenced PM_{2.5} (4.8 ng/m³) compared to the rural influenced PM_{2.5} (2.2 ng/m³). Compared to other sites, concentration of 3.82 to 9.81 ng/m³ were measured in southern China (Wei *et al.*, 2011), 464 ng/m³ in Turkey (Akyüz and Cabuk, 2008) or from 0.92 to 13 ng/m³ in Tuscany, Italy (Martellini *et al.*, 2012). Observed concentrations are nevertheless higher than 0.7 ng/m³ found near freeway in Beirut, Lebanon (Daher *et al.*, 2013).

For the comparison of every PAH in Zakroun and Kaftoun with the other Mediterranean sites, Nap, Acl, Ac, Ph, Ant, and B[k]F were excluded because they were not detected in one of the two fractions.

Fluorene concentrations in the industrial influenced PM_{2.5} (0.2557 ng/m³) were higher than in all the tested Mediterranean sites such as Florence (Italy, urban, 0.014-0.05 ng/m³) (Martellini *et al.*,

2012), Kozani (Greece, urban, 0.07 ng/m³) (Evangelopoulos *et al.*, 2010), Athenes (Greece, urban, 0.03 ng/m³) (Andreou and Rapsomanikis, 2009b), in different urban sites in Spain (0.19-0.0267 ng/m³ in Madrid and 0.03 ng/m³ in Escuelas Aguirre Madrid) (Barrado *et al.*, 2012; Mirante *et al.*, 2013), and in Sin El Fil (Lebanon, urban, 0.0095 ng/m³) (Borgie, 2014).

Fluoranthene concentrations at the industrial site (0.264 ng/m³) were close to that in Zonguldak (Turkey) in summer (0.3 ng/m³) (Akyüz and Cabuk, 2008), Florence in winter (0.24 ng/m³), CIEMAT Madrid in winter (0.2264 ng/m³), lower than in Zonguldak in winter (21.2 ng/m³), in Athenes (2.75 ng/m³), in Kozani (0.53 ng/m³) but higher than in Sin El fil (0.0446 ng/m³). Fluoranthene concentrations at the rural site (0.175 ng/m³) was similar or higher than in some urban sites such as Escuelas Aguirre Madrid (0.133 ng/m³) and CIEMAT Madrid in summer (0.1792 ng/m³). Pyrene, Benzo[a]Anthracene and Chrysene concentrations in PM_{2.5} at Zakroun are 0.226, 0.375 and 0.266 ng/m³ and at Kaftoun are 0.155, 0.245, and 0.169 ng/m³ respectively. The concentrations of these 4 rings PAHs are higher than in Madrid (0.1977, 0.0246, 0.0689 ng/m³) and in Sin El Fil (0.0618, 0.0413 and 0.0636 ng/m³), close to Florence (0.26, 0.34 and 0.4 ng/m³) and Kozani (0.45, 0.41 and 0.25 ng/m³); but lower than in Zonguldak during winter (21.6, 14.8 and 18.7 ng/m³).

The concentrations of the 5 rings PAHs, B[b]Fl, B[a]P and Dib[a,h]An in industrial influenced PM_{2.5} are 0.456, 0.375 and 0.675 ng/m³ respectively and in the rural influenced PM_{2.5} are 0.261, 0.261 and 0.396 ng/m³ respectively. These concentrations were higher than those found in some Mediterranean sites such as Sin El Fil (0.1773, 0.0518 and 0.0333 ng/m³), close to some sites such as Florence (0.54, 0.47 and 0.41 ng/m³) and Kozani (0.57, 0.38, 0.09 ng/m³) but higher concentration were found in Zonguldak in winter (16, 15.7 and 1.1 ng/m³).

The concentrations of the 6 rings PAHs, InPy and B[g,h,i]Pe, were 0.564 and 0.63 ng/m³ respectively in the industrial influenced PM_{2.5} and 0.017 and 0.369 ng/m³ in the rural influenced PM_{2.5}. These concentrations are very high in our samples and higher than all the sites in the Mediterranean sites and in Sin El Fil in Lebanon (0.1483 and 0.3189 ng/m³). Higher concentrations of these 2 PAHs were found in Zonguldak (13.6 and 9.8 ng/m³), Athenes (1.83 and 6.05 ng/m³) and in Torre Sarca (2.68 and 3.32 ng/m³) (Akyüz and Cabuk, 2008; Andreou and Rapsomanikis, 2009b; Gualtieri *et al.*, 2009). The highest concentrations of all PAHs were observed in an urban site in Turkey (Zonguldak) during the winter. This site is characterized by the presence of coal mines used in winter for central heating and domestic which explains the high levels of PAHs in this site.

PAHs related to diesel and gasoline motor vehicles in urban environments and tracers for gasoline emissions are higher in sites with higher number of population.

3.2.4.2 Paraffins concentrations

Paraffins, also known as linear alkanes, were analyzed in FP and UFP collected under industrial and rural influences. The mean concentrations were calculated from all the industrial and all rural samples except samples extracted during the second sampling episode (UFP-I2 and UFP-R2).

In FP-I, C33> C35> C34> C28> C31> C30 were dominant whereas in FP-R, C33> C35> C31> C32> C34> C37 were dominant (Figure 3.16). C27> C29> C25> C28> C30> C31> C26 were dominant in UFP-I whereas in UFP-R, C27> C29> C25> C28> C23> C26> C30 were dominant. There is a contribution of petroleum source certified by the presence of C₃₅-C₄₀ molecular weight region (Hsieh *et al.*, 2000). Higher concentrations of these dominant species were present in the industrial particles. There is a contribution of petroleum source in all the samples certified by the presence of C₃₅-C₄₀ molecular weight region (Hsieh *et al.*, 2000). This contribution was also shown by the predominance of C₂₃-C₂₉ paraffins related to emissions of petrochemical activities in both sites (Simoneit, 2002).

Higher paraffin concentrations were present in the quasi-ultrafine particles compared to the fine particles. Total even carbon number n-alkanes were 6.81 and 5.05 ng/m³ in FP-I and FP-R, and 9.44 and 6.07 ng/m³ in UFP-I and UFP-R. Total odd carbon number n-alkanes were 7.91 and 9.01 ng/m³ in FP-I and FP-R, and 13.7 and 10.35 ng/m³ in UFP-I and UFP-R, respectively. Therefore, total paraffin concentrations were higher in UFP, 23.14 and 16.42 ng/m³ in UFP-I and UFP-R, than in FP-I and FP-R, 14.72 and 14.05 ng/m³ respectively. Total paraffins concentrations were higher in UFP-I3 compared to the other UFP samples (Figure 3.17).

Two diagnostic measures, the carbon maximum number (C_{max}) and carbon preference index (CPI) have been commonly used to evaluate the sources for n-alkanes (Chen *et al.*, 2014). C_{max} is a general indicator of the relative contributions of two n-alkane sources. That is, higher values for C_{max} indicate that the n-alkanes primarily originated from terrestrial plants, especially epicuticular waxes while lower C_{max} values are more consistent with n-alkanes from anthropogenic sources (Andreou and Rapsomanikis, 2009a). C_{max} values ranged from C₃₃ to C₃₅ in FP-I and FP-R showing the contribution of natural sources in both samples (Figure 3.16). In all the UFP-I and UFP-R, the C_{max} values ranged from C₂₇ to C₂₉ showing the contribution of anthropogenic sources in all the ultrafine samples (Figure 3.17).

The Carbon preference index (CPI) can be used as a diagnostic parameter for describing the emission sources during sampling (Simoneit, 1986). The CPI is defined as the sum of the concentrations of the odd carbon number n-alkanes divided by the sum of the concentrations of the even carbon number n-alkanes. It is useful to determine the degree of biogenic versus petrogenic input (Simoneit and Mazurek, 1982). The CPIs for the n-alkanes are often calculated in several ways.

First, CPIs were calculated using two equations which were proposed by Bray and Evans (1961) and Cooper and Bray (1963): Overall CPI₂₁₋₃₅ = $(\sum C_{21-C_{33}} + \sum C_{21-C_{35}}) / (2 \times \sum C_{20-C_{34}})$ and High CPI₂₅₋₃₅ = $(\sum C_{25-C_{33}} + \sum C_{27-C_{35}}) / (2 \times \sum C_{26-C_{34}})$. The Overall CPI₂₁₋₃₅ and High CPI₂₅₋₃₅ values were 1.079 and 1.070 for FP-I, and 1.765 and 1.777 for FP-R. In UFP-I, the Overall CPI₂₁₋₃₅ and High CPI₂₅₋₃₅ values were 1.637 and 1.538 and in UFP-R, 2.122 and 2.147. When plotting High CPI values versus Overall CPI values (Bakhtiari *et al.*, 2009) for FP and all UFPs, two groups are evidenced

(Figure 3.18). Considering paraffins composition, $PM_{2.5}$ (FP-I and UFP-I) collected under industrial influence clearly show an anthropogenic origin and mainly come from petrogenic source. However, background rural $PM_{2.5}$ (FP-R and UFP-R) paraffin mainly come from mixed natural and anthropogenic sources and the contribution of petrogenic sources appears less marked. The conclusion was also the same when considering the calculation of CPIs proposed by Chen *et al.* (Chen *et al.*, 2014).

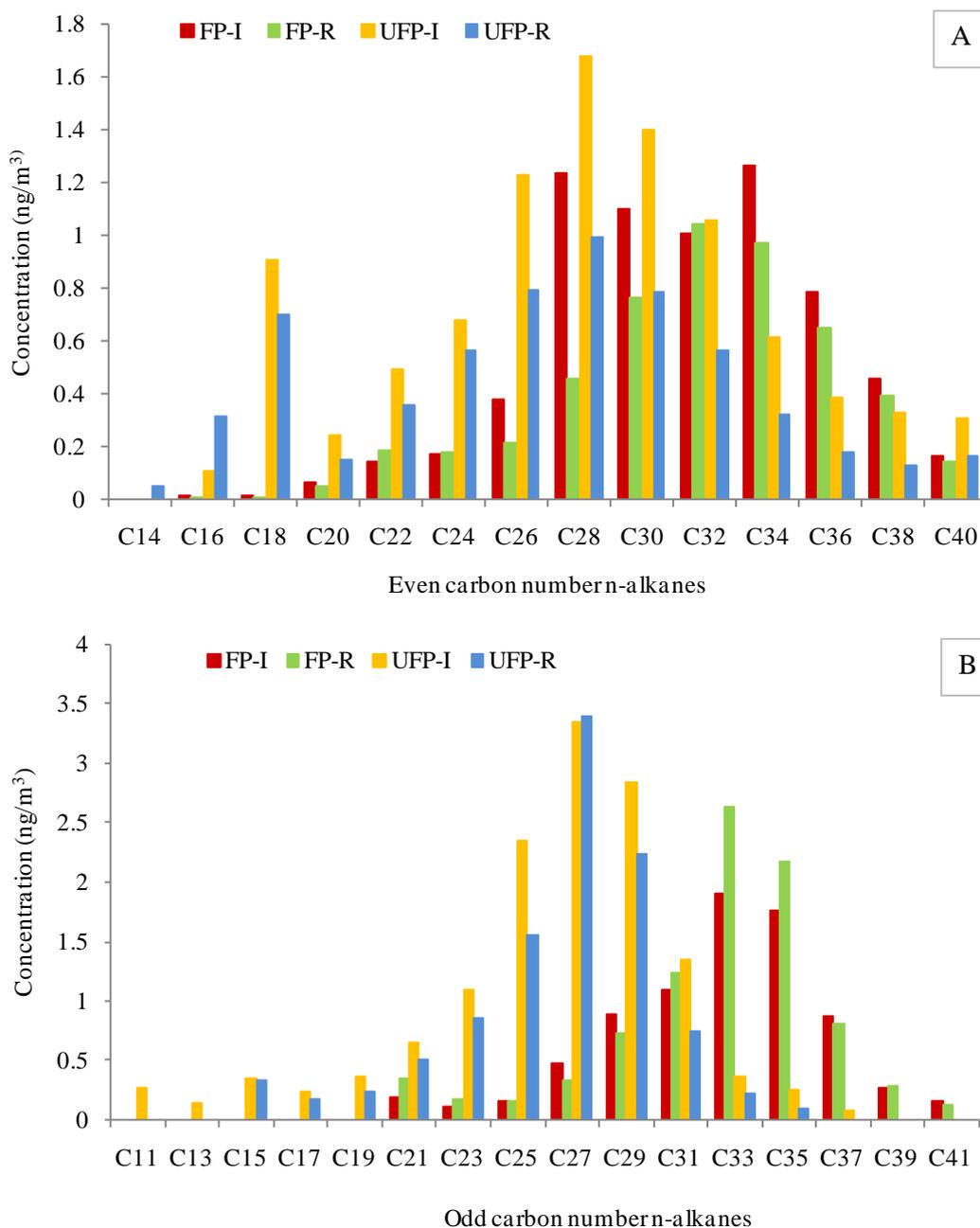


Figure 3.16: Concentrations of paraffins in $PM_{2.5-0.3}$ (FP) and $PM_{0.3}$ (UFP) samples collected under industrial (-I) and rural (-R) influences (A: odd carbon number n-alkanes, B: even carbon number n-alkanes)

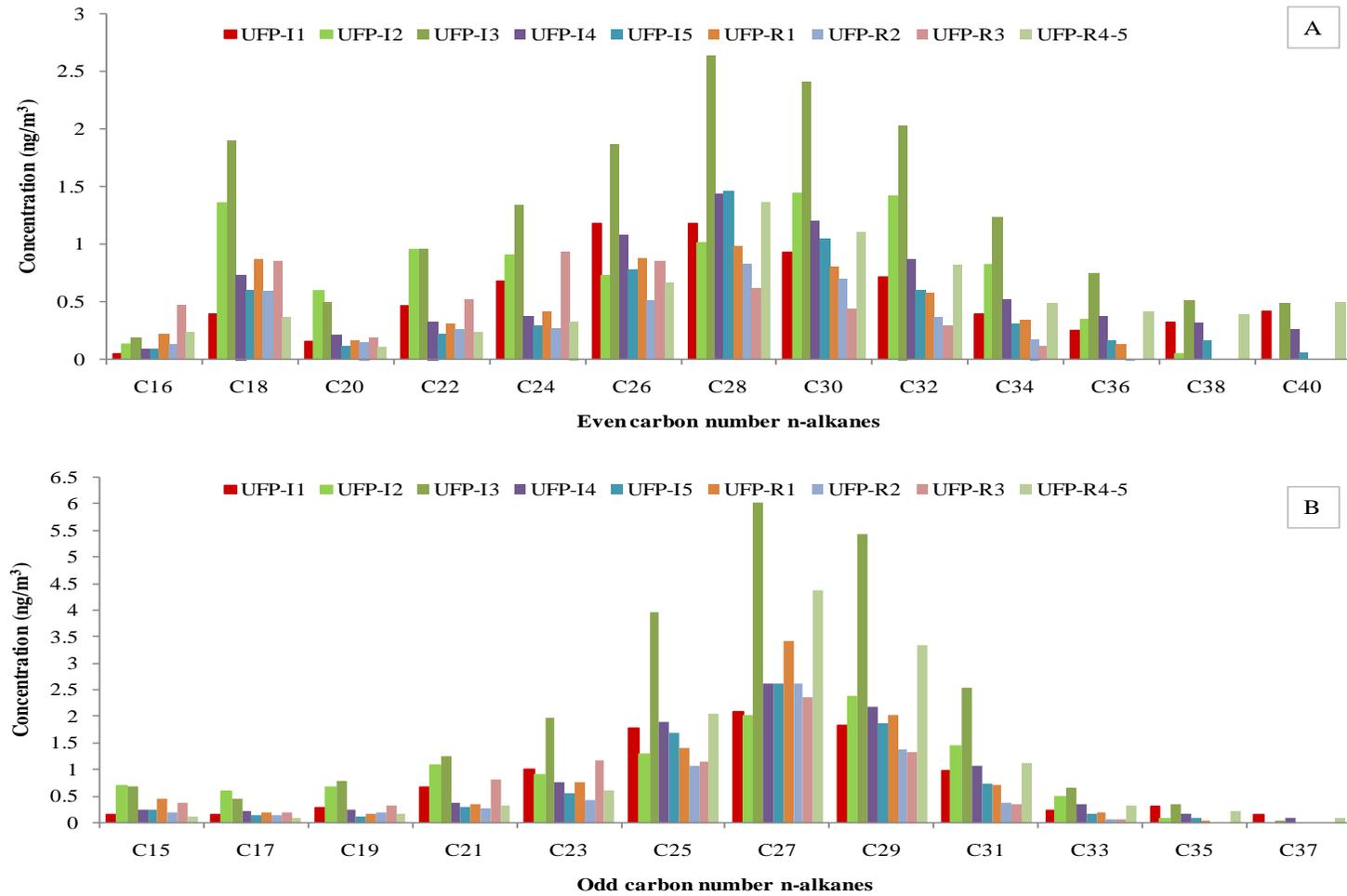


Figure 3.17: Concentration profiles of paraffins for individual tested UFP samples collected under industrial (UFP-I) and rural (UFP-R) influences (A: odd carbon number n-alkanes, B: even carbon number n-alkanes)

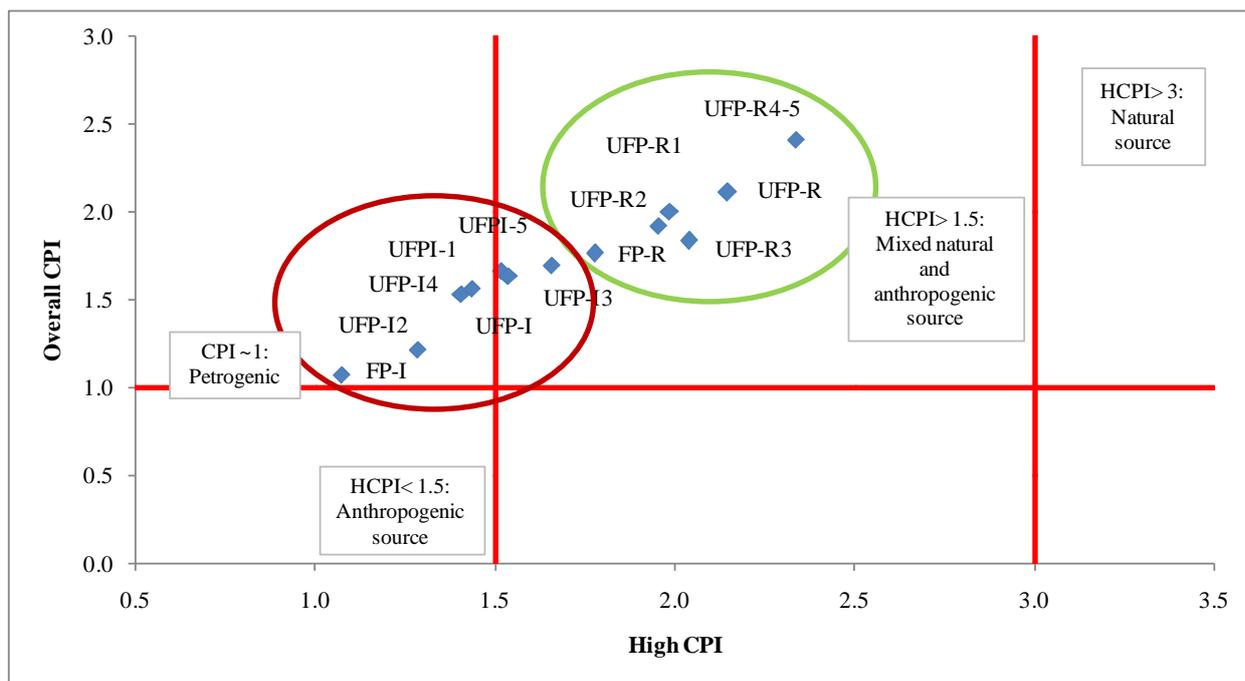


Figure 3.18: Source identification for n-alkanes in UFP and FP samples using Overall CPI_{21-35} and High CPI_{25-35}

3.2.4.3 Dioxins, furans and Dioxin-Like-PCBs

Only Composite samples of PM collected under industrial (FP-I) and rural (FP-R) influences were characterized for dioxins (PCDDs), furans (PCDFs) and Dioxin-Like polychlorinated biphenyls (DL-PCBs).

3.2.4.3.a Dioxins, furans and DL-PCBs concentrations

PCDD, PCDF and PCBs are found at higher concentrations in the industrial influenced particles compared with the rural influenced particles (Table 3.14). The dioxins 1,2,3,4,6,7,8-HpCDD and OCDD had the highest concentration levels and their concentrations were much higher at the industrial influenced site. Under rural influence, these two dioxins were the only to be detected. These emissions can be due to emissions from solid waste incineration (Chao *et al.*, 2004) and uncontrolled domestic waste burning (Gullett *et al.*, 2001) at a local scale. The furan 1,2,3,4,6,7,8 HpCDF had the highest concentration level in both sites but much higher at the industrial influenced site. This can be related to the influence of combustion activities at the industrial site. In addition, the raw materials in cement plants may contain chlorine in trace amounts that could contribute to the formation of HCl or the precursors of D/Fs. As said before, the most common wastes burned as supplement traditional carbonaceous fuels in cement kilns are used or rejected automobile and truck tires, blended liquid and solid hazardous wastes, used oil, and combustible nonhazardous solid wastes (Greer, 2003). These wastes may contain sulfur, nitrogen, and/or chlorine that could contribute to the formation of SO_2 ,

NO_x, NH₃, HCl, or D/Fs (Greer, 2003). PCB 118 and PCB 105 had the highest concentrations level at both sites. Industries, waste incinerators and traffic are the main DL-PCB emitters (Guéguen *et al.*, 2011). Therefore, these three different sources could be responsible for the higher levels of DL-PCBs in industrial particles.

3.2.4.3.b Toxicity evaluations of the mixture of dioxins, furans and DL-PCBs

Given the high number of congeners that show various degrees of toxicity, a synthetic indicator, International Toxic Equivalent Quantity (I-TEQ), was developed to characterize the global toxicity linked to dioxins. In airborne particulates, dioxins are generally present in mixtures containing many congeners, each having a specific degree of toxicity. A toxicity coefficient is attributed to each congener depending on its activity related to that of the most toxic dioxin (e.g., 2,3,7,8-TCDD, Seveso dioxin). Among PCDD and PCDF chlorinated congeners in the lateral position (2, 3, 7 or 8) are the most toxic. To calculate the toxic equivalent (expressed in I-TEQ) of a mixture of congeners, the contents of the 17 most toxic compounds (dioxins and furans) are first multiplied by their respective toxicity coefficient, and then added together. The TEQ calculation considers only congeners of dioxins and furans. However, WHO also added 12 DL-PCBs.

Using the Toxic Equivalent Factor (TEF) provided by WHO 2005 (Berg *et al.*, 2006), I-TEQ_{OMS} were 224.66 and 0.25 fg TEQ/m³ in FP under industrial and rural influence respectively. Therefore much higher toxicity was found in the industrial influenced site. It is important to note that the determined TEQ does not reflect the full amount of PCDD, PCDF and PCB in the atmosphere since they also exist in the gaseous state not associated to particles (Mandalakis *et al.*, 2002). The proportion of congeners in the gaseous phase tends to decrease with the increase of the number of linked chlorine atoms (penta, hexa, hepta and octa forms). Dioxins are, in the majority, in particulate form (Lee and Jones, 1999; Park and Kim, 2002). Nevertheless, dioxins like 2,3,7,8TCDD or 1,2,3,7,8 PeCDD show high toxicity factors. Thus, dioxins in a gaseous form represent nearly 55% of the total toxicity of dioxins in ambient air. Mandalakis *et al.* estimated that furans in a gaseous form represent 67% of the total toxicity of furans in ambient air (Mandalakis *et al.*, 2002) but the total toxicity of gaseous dioxins and furans could be between 2 and 3 times higher than particulate dioxins and furans.

When comparing I-TEQ in Zakroun (FP-I) to other sites, the value in Zakroun is within the range typically found in Cologne (Germany, 240 fg TEQ/m³), higher than other European towns such as Toulouse 2004 (France, 26-50 fg TEQ/m³), Halluin 2007 (France, 52 fg TEQ/m³), and lower than in Manchester (UK, 410 fg TEQ/m³). Compared to Mediterranean cities, the value in Zakroun was higher than in Athens (Greece, 42-73 fg TEQ/m³) (Lohmann and Jones, 1998). In Lebanon, there is no limit for dioxins and furans in ambient air because the risk of contamination by inhalation is deemed low in comparison with food. At an international level, the Environment Minister in Ontario (Canada) recommends an air quality of 5 pg I-TEQ/m³. The concentrations found in Zakroun and Kaftoun are

inferior to this recommendation even if the concentration is extrapolated to take into account the gaseous part of dioxins and furans.

Table 3.14: Dioxins (PCDDs), furans (PCDFs) and Dioxin-Like compounds (DL-PCBs) concentrations in PM_{2.5-0.3} samples collected under industrial (FP-I) and rural (FP-R) influences

< DL= under the detection limit

pg/g	FP-I	FP-R	FP-I	FP-R
	pg/g		fg/m ³	
2,3,7,8 TCDD	< DL	< DL	< DL	< DL
1,2,3,7,8 PeCDD	584	< DL	9.2	< DL
1,2,3,4,7,8 HxCDD	2204	< DL	34.6	< DL
1,2,3,6,7,8 HxCDD	18112	< DL	284	< DL
1,2,3,7,8,9 HxCDD	12162	< DL	191	< DL
1,2,3,4,6,7,8 HpCDD	103590	721	1626	8.8
OCDD	81639	1014	1282	12.38
Total PCDD	218287	1735	3427	21.1
2,3,7,8 TCDF	1033	< DL	16	< DL
1,2,3,7,8 PeCDF	6569	< DL	103	< DL
2,3,4,7,8 PeCDF	8448	< DL	133	< DL
1,2,3,4,7,8 HxCDF	14994	< DL	235	< DL
1,2,3,6,7,8 HxCDF	12550	< DL	197	< DL
2,3,4,6,7,8 HxCDF	11828	< DL	186	< DL
1,2,3,7,8,9 HxCDF	3140	< DL	49	< DL
1,2,3,4,6,7,8 HpCDF	37669	1081	591	13.1
1,2,3,4,7,8,9 HpCDF	6508	< DL	102	< DL
OCDF	5754	8759	90	106
Total PCDF	108494	9841	1703	120
PCB 81	< DL	< DL	< DL	< DL
PCB 77	356	344	5.6	4.2
PCB 123	54	19.9	0.85	0.24
PCB 118	3955	129	62	15.8
PCB 114	186	< DL	2.9	< DL
PCB 105	2215	764	34.8	9.3
PCB 126	110	< DL	1.7	< DL
PCB 167	457	< DL	7.2	< DL
PCB 156	1253	261	19.7	3.2
PCB 157	248	< DL	3.9	< DL
PCB 169	< DL	< DL	< DL	< DL
PCB 189	295	< DL	4.6	< DL
Total PCB	9128	2688	143	32.7

3.3 Conclusion

The individual FP and UFP samples collected in the industrial (Zakroun) and rural (Kaftoun) influenced sites and the prepared composite FP were used for the physicochemical analysis.

During the whole sampling period, and after discarding the second sampling period characterized by a local sandy wind, the mean concentration of $PM_{2.5}$ was estimated at 36.5 and 22.8 $\mu\text{g}/\text{m}^3$ in Zakroun and Kaftoun respectively. The quasi ultrafine fraction ($PM_{0.3}$) represents 50% of $PM_{2.5}$, at both sites. The industrial site is influenced by numerous direct and indirect sources of fine particles such as industrial activities (cement factories, phosphate fertilizer industries...) and burning fuels (dense traffic, generators, and industrial power plants). All these factors explain the higher concentration of fine particles encountered in the industrial influenced site compared to the rural background site.

The dominant species are the NO_3^- , total C, Ca^{2+} and SO_4^{2-} in the FP-I and in FP-R (76% and 81% of the total analyzed species), and SO_4^{2-} , TC, NO_3^- , NH_4^+ and Ca^{2+} , in UFP-I and UFP-R. All the considered ions showed higher content in UFP-I than in UFP-R. Particularly, The highest Ca^{2+} and PO_4^{3-} contents in FP-I, could be related the presence of a cement plant and a fertilizer company close to the sampling site.

Regarding the chemical association between these species, several similar crystalline phases were identified in both particles from the industrial and rural influenced sites such as calcite, gypsum, halite, nitratine, quartz, and an aluminosilicate phase that could be chloritoid. These results were confirmed by SEM-EDX analysis which additionally, only in FP-I, showed the presence of particles containing Ca and P that could be ascribable to mono-calcium phosphate or fluorophosphates, raw materials used in the fertilizer industry.

In addition, the total carbon appears in high concentration in the FP-I, 2185 ng/m^3 compared to in the FP-R, 1054 ng/m^3 ; this could be linked to the predominance of combustion source as it was evidenced by the PAHs and paraffins detailed analysis using characteristic ratios.

For trace metals, the industrial impact was evidenced at Zakroun sampling site for all trace metals and especially for Pb, Cd, Sn, As, Cu, Mn, Zn, Sr, Pb, Ni and Ag. Several sources could be involved to explain this result, such as abrasion of mobile part of motor vehicles (Cu, Zn); re-suspension of dust by the movement of vehicles on the roads (Ba); burning of fossil fuels and oils (Ni and V). The contribution of characteristic elements related to tires composition is also evidenced (Cu, Pb, Mn, Co, Ni and Cd and Zn) (Adachi and Tainosho, 2004; Thorpe and Harrison, 2008).

PAHs concentrations were much higher in $PM_{0.3}$ (total PAHs= 4.63 and 2.04 ng/m^3 in UFP-I and UFP-R respectively) than in $PM_{2.5-0.3}$ (total PAHs= 0.2 and 0.11 ng/m^3 in FP-I and FP-R respectively), in confirming that UFP are a preferred medium for organic compounds absorption compared to FP. InPy, B[ghi]Pe, DiB[a,h]An, Chr, B[b]Fl, and B[a]An were the dominant PAH found in both FP samples and in higher concentrations in the FP-I sample with a main difference for InPy,

B[b]Fl, B[a]An, Chr and B[ghi]Pe. The major compounds in UFP collected at both sites are InPy, B[ghi]Pe, DiB[a,h]An, B[b]Fl, B[a]An with a main difference for the DiB[a,h]An, B[k]Fl, InPy, B[b]Fl content in UFP-I.

As evidenced using source characteristic ratios, the detected PAHs could be originated from numerous combustion sources, including motor vehicles (mainly gasoline vehicles), biomass burning, coal combustion and industrial processes.

Analysis of paraffin results was done considering Carbon Preference Index. The anthropogenic and petrogenic contribution in PM collected in the industrial influenced site was confirmed. PM collected in the rural background site, Kaftoun, possesses a mixed natural and anthropogenic pattern, with a weaker petrogenic contribution.

Dioxins and furans, PCDD, PCDF and PCBs, were found at far much higher concentrations in the industrial influenced particles compared with the rural influenced particles. The contribution of combustion source is clearly evidenced at Zakroun. It is well known that high amount of dioxins are emitted during waste combustion and the question regarding the nature of the combustible used in such industries should be asked.

To conclude, several physicochemical characteristics showed the presence of markers of industries in Zakroun area compared to Kaftoun, especially cement factories activities, production of phosphate fertilizers, and combustion phenomena related to diesel and gasoline emissions, biomass or other sources. One important point that should be clarified concerns the use of non-conventional combustible as several results in this study suggest that domestic waste (high dioxins concentrations), or used tires (metal industrial impact, PAHs concentrations, in particular Indeno[1,2,3-cd]pyrene) could be used as combustible.

CHAPTER 4

TOXICOLOGICAL ANALYZES OF FINE PARTICULATE MATTER (PM_{2.5-0.3})

*In this chapter, the mutagenic and genotoxic effect of fine particulate matter (PM_{2.5-0.3}) are assessed. The genotoxicity mechanisms involved are also studied. It is divided into two parts. The first part includes a detailed explanation of the materials and methods used to assay the genotoxic effects of industrial influenced fine particles composite (FP-I) and rural influenced fine particle composite (FP-R) on bacterial strains (*Salmonella typhimurium* and *Escherichia coli*) and on human bronchial cells (BEAS-2B). In the second part, the results are presented and discussed.*

4.1 Materials and Methods

4.1.1 Ames fluctuation test

4.1.1.1 Principle

Mutagenic materials may be hazardous due to their ability to induce cancerous transformation of normal cells. The Ames fluctuation test is based on the most generally used and validated bacterial reverse-mutation test, known as the 'Ames Test'. The Ames test, also known as the *Salmonella*/microsome test and bacterial reverse mutation test, is an assay that is used to detect a broad range of mutagens that cause genetic mutations. The Classical Ames test was developed by Dr. Bruce Ames in 1971. Ames test is used usually to evaluate the mutagenicity of pure chemicals and complex environmental mixtures (Maron and Ames, 1983). It is a bacterial reverse mutation test that uses amino-acid requiring strains of *Salmonella typhimurium* or *Escherichia coli* to detect point mutations (substitution, addition or deletion). The test employs mutant strains of *Salmonella typhimurium*, carrying mutation(s) in the operon coding for histidine biosynthesis. A back mutation may restore the functional capability of the bacteria to synthesize histidine (Figure 4.1). Revertant bacteria are detected by their ability to grow in the absence of histidine (Mortelmans and Zeiger, 2000). L-histidine is an essential α -amino acid that is synthesized by *Salmonella* (Sheppard, 1964). In a histidine deficient medium, colonies or other evidence of bacterial growth such as broth turbidity is not observed. When these bacteria are exposed to mutagenic agents, under certain conditions, reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs. The test depends on genetically modified strains of *Salmonella typhimurium*, such as TA98, TA102 and YG1041 (Table 4.1).

The *Salmonella* strain TA98 was selected in this study due to its high sensitivity and its wide usage (Jolibois and Guerbet, 2005; Jolibois *et al.*, 2003; Margolin *et al.*, 1981; McGeorge *et al.*,

1985). This strain detects frameshift mutagens (Maron and Ames, 1983). It is mostly sensitive to PAHs especially after metabolic activation. TA98 strain possesses the *rfa* mutation that allows large molecules to penetrate the cells due to a partial loss of the lipopolysaccharide of the bacteria cell wall and *uvrB* mutation that decreases the DNA excision repair, responsible of HAPs adducts repair. This repair pathway takes place after exposure to UV light or certain mutagens. Therefore, the *uvrB* mutation strains are more responsive to a variety of mutagens. This deletion extends to other genes such as the gene for biotin synthesis thus biotin should be added to the medium. *Salmonella* TA98 strains have the R-factor plasmid pKM101 that codes for the error-prone DNA repair enzymes and an ampicillin resistance gene. The strains that contain this plasmid, easily express a mutation, repair the DNA damage and are resistant to ampicillin (Levine *et al.*, 1994). *Salmonella* strain TA102 detects base-pair substitutions mutagens. These strains are mostly sensitive to compounds acting via oxidative stress. TA102 possesses the *rfa* mutation, the R-factor plasmid pKM101, and the pAQ1 plasmid that carries a tetracycline resistance gene (Ames *et al.*, 1973). The *Salmonella* strain YG1041, developed by Watanabe *et al.*, is a plasmid-modified TA98 derivative (Watanabe *et al.*, 1989). pNM13 is a derivative of plasmid pACYC184, which carries both the nitroreductase and acetyltransferase genes of *S. typhimurium*. This metabolically enhanced tester strain shows extreme sensitivity to nitroarenes and/or aromatic amines such as 2-nitrofluorene, 1-nitropyrene and p-nitrophenetole (Hagiwara *et al.*, 1993). The kanamycin-resistance gene was inserted into pNM13 and the resulting plasmid, pYG233, was introduced into *S. typhimurium* TA98. Therefore, YG1041 is resistant to both kanamycin and ampicillin and is a nitroreductase (NR) and an O-acetyltransferase (OAT) overproducing strain.

Table 4.1: *Salmonella typhimurium* genetically modified strains used in Ames fluctuation test

	TA98	YG1041	TA102
Type of mutation	frameshift	frameshift	substitution
Sensitivity to mutagens	PAHs (+S9)	nitro-aromatic compounds (-S9)	compounds acting via oxidative stress

Traditionally, reverse-mutation assays have been performed using agar plates, known as “pour plate”, “plate-incorporation” or “agar-overlay” assays. An alternate assay performed entirely in liquid culture is the “Fluctuation test”. It was originally devised by Luria and Delbruck (1943) and modified by Hubbard *et al.* (1984). The Ames fluctuation test is a miniaturized screening microplate version of the classical test based on colorimetric analysis (Hubbard *et al.*, 1984). In this test, bacteria are exposed to different concentrations of the tested sample, in a medium containing sufficient histidine to support approximately two cell divisions. The presence of a pH indicator allows the detection of the presence or absence of a mutation. Within few days, mutated cells will grow into colonies and their metabolism will lower the pH of the medium resulting in a color change from purple to yellow. Positive controls are used to test the efficiency of the assay and they vary according to the bacterial strains used and whether the sample was subjected to metabolic activation with S9

liver enzymes. The samples are suspended in 96-microplates and a colorimetric assay is performed (Jolibois and Guerbet, 2006; Legault *et al.*, 1994). The Ames fluctuation test is used for predicting possible carcinogenicity and for the detection of oxidative mutagens (Beaune *et al.*, 1985; Hakura *et al.*, 1999; Hakura *et al.*, 2005; Levin *et al.*, 1982).

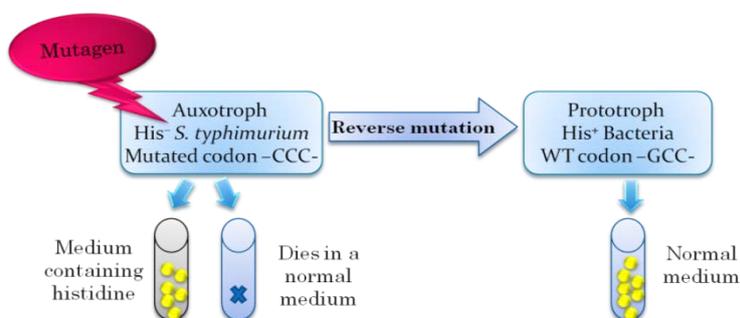


Figure 4.1: Principle of the Ames fluctuation test

4.1.1.2 Chemicals, media, solvents, and buffer preparation

The following solutions and chemicals were used from the EBPI's Muta-ChromoPlate™ Kit: Davis-Mingioli salts (concentrated), D-glucose, bromocresol Purple, D-Biotin, L-histidine, sterile distilled water, growth medium (Luria broth), 100% DMSO and 10% DMSO in saline.

- Positive controls:

The positive controls used for the Ames fluctuation test using the TA98 and YG1041 are: 3 mg/mL 2-Nitrofluorene (2-NF) in 10% DMSO-saline (without activation) and 100 µg/mL 2-Amino-Anthracene (2AA) in 100% DMSO (with activation). The positive controls used with TA102 are: 1 ng/mL Mitomycin C (Sigma-Aldrich) in 100% DMSO (without activation) and 100 µg/mL 2AA in 100% DMSO (with activation) (EBPI's SOS-CHROMOTEST™ Kit).

- S9 activation mixture:

Aliquots of the S9 fractions (1 mg/mL) were prepared from the stock solution of S9 liver enzymes (Invitrogen) (20 mg/mL) by diluting it with the storage buffer. This buffer consisted of 6 g of monopotassium phosphate (KH₂PO₄) (HiMedia), 11 g of dipotassium phosphate (K₂HPO₄) (HiMedia), 0.372 g of Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), and 0.154 g of dithiothreitol (DTT) (Sigma-Aldrich), dissolved in 500 mL of distilled water. A mixture of 19 mL of the storage buffer and 1 mL of the S9 liver enzymes was divided to aliquots of 1 mL and stored at -80°C.

The S9 mix consists of a salt solution composed of 1.65 M potassium chloride (KCl) (HiMedia) and 0.4 M magnesium chloride hexahydrate (MgCl₂•6H₂O) (HiMedia). The S9 activation mixture (10 mL) was prepared by mixing 0.05 mL of 1 M glucose 6-phosphate (G6P) (Sigma-Aldrich), 0.15 mL of 0.1 M nicotinamide adenine dinucleotide phosphate (NADP) (Sigma-Aldrich), 2.5 mL of 0.4 M tris buffer (pH= 7.4), and 6.1 mL of Luria broth. This mixture was mixed with

0.2 mL of the salt solution and one vial (1 mL) of the S9 fractions to a final concentration of 10% (v/v). S9 fractions were removed from -80°C and thawed on ice a couple of hours before the assay.

- Fluctuation media or reaction mixture:

The reaction mixture (30 mL) was prepared by adding 21.62 mL of Davis-Mingioli salts, 4.75 mL of D-glucose, 2.38 mL of Bromocresol Purple, 1.19 mL of D-Biotin and 0.06 mL of L-Histidine.

4.1.1.3 Bacterial preparation

S. typhimurium TA98 and TA102 were provided with the EBPI's Muta-ChromoPlate™ Kit. *S. typhimurium* YG1041 strain was kindly provided by Dr. Veronique André (EA 4651 ABTE). Luria broth medium (+Ampicillin) was used for the culture of the *Salmonella* TA98 and TA102 strains. Brain Heart Infusion Broth medium (+Ampicillin and Kanamycin) was used for the culture of the *Salmonella* YG1041 strain. Strains were stored in 60% glycerol in aliquots of 1.6 mL at -20°C in cryogenic vials. About 18 hours before the assay, the vial was thawed at room temperature, and an inoculum of 1 mL of the vial was added to 5 mL of Luria broth medium. The bacterial culture was kept overnight in an incubator at 37°C. The quality and quantity of the bacterial culture was inspected through subculture on Mueller-Hinton agar (MHA), MacConkey's Agar (MAC) and Brain-heart infusion medium (BHI), Gram staining, and analytical profile index-strip testing (API-strip testing).

4.1.1.4 Sample preparation

Industrial and rural influenced PM_{2.5-0.3} samples (FP-I and FP-R respectively) were dissolved in 10% DMSO and distilled water to a concentration of 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and 0.625 µg/mL. Final DMSO concentration did not exceed 0.5%.

4.1.1.5 Standard procedure for Ames test

For each sample tested, seven tubes are prepared according to Table 5.2. The final volume in each tube was 20 mL including 5 µL of the overnight bacterial culture which was added to all tubes except the blank. Samples were tested with *Salmonella* strains TA 98, TA102, YG1041, with and without activation.

A stock solution of 0.4 mg of PM_{2.5} with 10% DMSO (150 µL) and 17.350 µL of sterile distilled water was prepared. To prepare the 10 µg/mL solution, 17.5 mL of sterile water were added to the stock solution and mixed very well. Serial 2-fold dilutions were performed in order to have different concentrations of FP-I and FP-R: 10 µg/mL (2 µg/well), 5 µg/mL (1 µg/well), 2.5 µg/mL (0.5 µg/well), 1.25 µg/mL (0.25 µg/well) and 0.625 µg/mL (0.125 µg/well).

A volume of 2.5 mL of the reaction mixture (fluctuation media) were added to the prepared tubes to have a final volume of 20 mL. 5 µL of the bacterial suspension from the culture grown over

night were added to all the tubes with the exception of the reaction blank tube (5 μ L of growth media). A background tube was prepared by adding 2.5 mL volume from the reaction mixture, 17.5 mL of distilled water and 5 μ L of bacteria. The positive control tube was prepared by adding 2.5 mL from the reaction mixture, 17.4 mL of distilled water, 0.1 mL of the positive control and 5 μ L of the bacterial culture.

With metabolic activation, 2 mL of the prepared activation mixture and 15.5 mL of distilled water or diluted sample were added to 2.5 mL of the reaction mixture and 5 μ L of bacterial culture.

A volume of 200 μ L from the prepared mixture was dispensed into 96-well microplates using a multi-channel pipette. The plates were then covered with a lid, individually wrapped with an aluminum foil, and sealed in Ziploc air-tight bags. They were then placed in an incubator at 37°C for 3-5 days.

Table 4.2: Set-Up of the Ames fluctuation assay (EBPI, 2009)

Treatment Plate	-S9			+S9			Fluctuation Media (mL)	Positive Control (mL)	Bacteria (mL)
	Distilled water (mL)	Diluted Sample (mL)	S9 Mix (mL)	Distilled water (mL)	Diluted Sample (mL)	S9 Mix (mL)			
Blank	17.5	-	-	15.5	-	2	2.5	-	-
Background	17.5	-	-	15.5	-	2	2.5	-	0.005
Positive Control	-	17.4	-	-	15.4	2	2.5	0.1	0.005
PM _{2.5} 10 μ g/mL	-	17.5	-	-	15.5	2	2.5	-	0.005
PM _{2.5} 5 μ g/mL	-	17.5	-	-	15.5	2	2.5	-	0.005
PM _{2.5} 2.5 μ g/mL	-	17.5	-	-	15.5	2	2.5	-	0.005
PM _{2.5} 1.25 μ g/mL	-	17.5	-	-	15.5	2	2.5	-	0.005
PM _{2.5} 0.625 μ g/mL	-	17.5	-	-	15.5	2	2.5	-	0.005

4.1.1.6 Expression of the Results

Revertant mutants secreted an acid which changed the pH of the well. The modification was revealed using bromocresol purple, a pH indicator that changes from purple in a basic medium to yellow in an acidic medium. Hence, yellow plates indicate the presence of back-mutation and histidine production. Yellow, partially yellow, or turbid wells are considered positive, whereas purple wells are scored negative (Jolibois and Guerbet, 2005). The background plates show the spontaneous reversion mutation that occurs without the addition of the sample. For this reason, plates were compared to their background plates. The sample was considered genotoxic if the number of the positive plates was significantly greater than the number of positive plates in its corresponding background plate. The Mutagenicity Ratio (MR) corresponds to the ratio of the positive wells in the sample plates to the positive wells in the background plates (Jolibois and Guerbet, 2005) (Equation 4.1). The results were tested statistically by the *Chi*-squared test (χ^2).

$$\text{MR} = \frac{\text{number of positive wells in treated plates}}{\text{number of positive wells in negative control plates}} \quad (4.1)$$

4.1.2 SOS Chromotest

4.1.2.1 Principle

The SOS Chromotest was first developed by Quillardet *et al.*, 1982. It is a quantitative colorimetric bacterial test that detects induction of the SOS genes involved in DNA repair in *Escherichia coli*. Damage to the bacterium's DNA results in a set of genetic responses, known as the SOS responses and "damage inducible" (*din*) genes. This test was first described in details by Quillardet and Hofnung (Quillardet and Hofnung, 1993).

E. coli PQ37, also called *E. coli* K-12, is a genetically engineered strain that carries a *sfiA::lacZ* fusion (GC4436). The SOS gene repair promoter region of the genome, responsible for activating the SOS genes, is linked to the β -*gal* gene responsible for the production of the β -galactosidase (β -gal) enzyme assayed in this test. This tester strain has a deletion for the normal *lac* region, responsible of the synthesis of the β -galactosidase, thus the β -galactosidase activity is strictly dependent on *sfiA* expression (Quillardet *et al.*, 1982; Huisman and D'Ari, 1981). *sfiA* gene is involved in cell-division inhibition and is part of 17 or more genes that make up the SOS error-prone DNA repair system. The PQ37 strain carries other genetic mutations: a *uvrA* mutation that renders the strain deficient in excision repair and a *rfa* mutation that makes the lipopolysaccharide of this bacteria deficient and therefore allows better diffusion of large chemicals into the cell. It also contains *PHO^c* gene enabling continuous production of alkaline phosphatase (PAL) which is tested as a control for general protein synthesis. The bacterial genome also carries a gene for resistance to ampicillin which ensures test strain selectivity.

In a non-induced cell, the Lex A repressor prevents the expression the *sfiA/lacZ* operon fusion. Hence upon exposure to a damaging agent, the SOS signal is induced producing the activation of the protein *RecA*. The protein *RecA* cleaves the *LexA* repressor which controls the *sfiA* expression thus allowing the expression of β -galactosidase. The active protease cleaves the *lexA* product (LexA) at an -alanine-glycine- peptide bond to yield two proteolytic cleavages (Walker, 1984). As LexA is cleaved, it loses its repressing effects on many gene loci such as *sfiA* (also known as *sulA*), *uvrA*, *umuC*, *umuD*, and *dinD*, as well as *recA* and *lexA*. Since in *E. coli* PQ37, the *sfiA* locus is fused with the *lacZ* operon, the cleavage of LexA will activate the *sfiA::lacZ* system, which will in turn lead to the production of the *lacZ* product β -galactosidase. The production of the β -galactosidase allows the colorimetric evaluation of the genotoxic activity of a suspected genotoxin quantitatively, since the more it is produced, the more the SOS response, and thus the more the DNA damage. Figure 4.2 demonstrates the principle of the SOS chromotest.

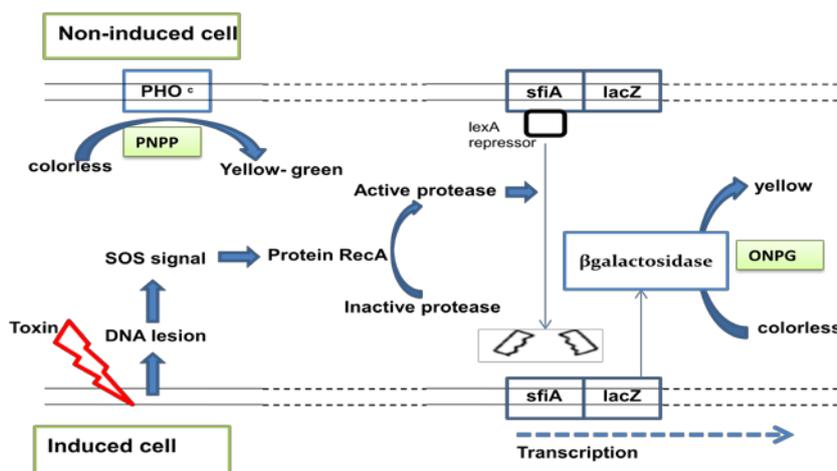


Figure 4.2: Principle of the SOS Chromotest, a colorimetric bacterial assay for genotoxins (modified from Quillardet *et al.*, 1985)

4.1.2.2 Chemicals, media, solvents, and buffer preparation

The following solutions and chemicals are used from the SOS-CHROMOTEST™ Kit (EBPI, USA): the growth medium for the bacteria strain (Luria broth), the diluent (10% DMSO in saline), the blue chromogen solution, the dried alkaline phosphatase substrate and the stop solution.

- Positive controls:

The positive controls used for the SOS chromotest are: 10 µg/mL 4-Nitro-Quinoline-Oxide (4-NQO) in 10% DMSO-saline (without activation) and 100 µg/mL 2AA in DMSO (with activation) (EBPI SOS-CHROMOTEST™ Kit).

- Preparation of the S9 activation mixture:

The S9 mix for the Ames fluctuation test was prepared as indicated in the *paragraph 4.1.1.2*.

4.1.2.3 Culture medium and maintenance of the tester strains

E. coli PQ37 bacteria were kindly provided by Dr. Montserrat Llagostera Casas (Universitat Autònoma de Barcelona, Spain). La broth (Luria + ampicillin) medium was used for the incubation of *E. coli* PQ37. Sterile distilled water (50 mL) was added to 500 mg of grinded Ampicillin to obtain a final concentration of 10,000 µg/mL stock solution. The stock solution was dispensed into 1 mL aliquots and stored at -20°C. The culture medium was prepared by adding 2 mL Ampicillin (10 000 µg/mL) to 1 L of Luria broth.

4.1.2.4 Bacterial preparation

E. coli PQ37 was stored in 60% glycerol at -20°C in aliquots of 1.6 mL in cryogenic vials. About 18 hours before the assay, the vial was thawed at room temperature, and 50 µL were dispensed into 5 mL of La medium. The culture was left overnight in an incubator at 37°C. The quality and quantity of the bacteria were monitored by spreading the cultures on MHA and MAC plates, Gram

staining, and API-strip testing. The following day, the OD₆₀₀ reached an optical density of around 0.15-2, the culture was then diluted with either L-medium (if without metabolic activation), or with the S9 mix (if with metabolic activation), to obtain an OD of 0.05 at 600 nm (+/-20).

4.1.2.5 PM samples preparation

A stock solution was prepared by dissolving the samples of collected PM in 10% DMSO in saline to a concentration of 22 mg/mL (2.2 mg in 100 µL 10% DMSO) in order to have a final concentration of 2000 µg/mL in the first tested well. Stock solution was homogenized by ultrasonication for 5 minutes to obtain maximum dispersion of particles.

4.1.2.6 Standard procedure of the SOS Chromotest

PM samples concentrations were tested in triplicates. Using a 96 well micro-plate, 10 µL of the diluent solution were dispensed to all the wells of the assays except for the wells of the undiluted sample and the wells of the positive control (2A, 2B, 2C and 9D, 9E, 9F) as shown in Appendix 18.

10 µL of the stock solution were added to the wells of the undiluted sample (2A, 2B, 2C). To prepare 14 two-fold dilutions, 10 µL were removed from well 2A and dispensed into well 2D, mixed well and then 10 µL were removed from 2D into 3A. A two-fold dilution was performed until well 9A-B-C, 10 µL were removed from these 3 wells and discarded. The same procedure was applied for the replicates. 10 µL of the properly diluted 4-NQO or 2AA were dispensed in the wells 9D, 9E, 9F.

100 µL of the diluted bacteria were dispensed to each well of the micro-plate that will be used in the assay except the wells of the blank machine (1A, 1B, 1C). In these 3 wells, 100 µL of Luria broth were added instead. The micro-plate was incubated at 37°C for 2 hours. During this time, bacteria are exposed to the particles. In order to simultaneous test the activity of β-galactosidase and alkaline phosphatase, after the two hour of incubation, a chromogenic substrate was added. The blue chromogen solution (to test β-gal) was transferred to the dry alkaline phosphatase substrate and mixed well. 100 µL from this mixture were added into all the wells. Then, the plate was incubated at 37°C for 60 to 90 minutes until a green color appears. After the color changes, 50 µL of the stop solution were added to each well of the plates and the absorbance (optical density) was read at 600 nm to measure genotoxic activity and at 405 nm to determine the viability of bacteria.

4.1.2.7 Expression of the Results

After recording the absorbance values of all the wells, the mean was computed for each triplicate set of wells. From the mean, the enzyme units were calculated for the β-gal and PAL assay of each sample (Equation 4.2a and b) (Quillardet *et al.*, 1985).

$$\text{Enzyme units} = 1000 \times \frac{\bar{A}_{600}}{t} \quad (4.2a)$$

$$\text{Enzyme units} = 1000 \times \frac{\bar{A}_{405}}{t} \quad (4.2b)$$

Where:

\bar{A}_{600} is the mean of the triplicates' OD at 600 nm for the β -gal values.

\bar{A}_{405} is the mean of the triplicates' OD at 405 nm for the PAL values.

t is the time of incubation after adding the substrates until the termination.

The genotoxic activity was assessed by the genotoxicity factor (R_c), which is the ratio of the enzyme units of the β -gal assay of a sample to the enzyme units of the PAL assay of this sample (Equation 4.3).

$$R_c = \frac{\bar{A}_{600}^{\beta} \times t_p}{\bar{A}_{405}^p \times t_{\beta}} = \frac{\text{Enzyme Units}^{\beta}}{\text{Enzyme Units}^p} \quad (4.3)$$

Where

\bar{A}_{600}^{β} is the mean optical density of the sample's β -gal assay at 600 nm.

\bar{A}_{405}^p is the mean optical density of the sample's PAL assay at 405 nm.

t_{β} is the time of incubation of the β -gal assay.

t_p is the time of incubation of the PAL assay.

The Induction Factor (IF) compares the SOS response induced at a certain concentration to the SOS response induced with the negative control (the solvent). The Induction Factor (IF) allows the comparison between different samples and experiments and assesses whether the sample at a certain concentration is genotoxic or not. The IF is calculated by dividing the genotoxic activity of a sample at a certain concentration (R_c) by the genotoxic activity of the solvent R_0 (Equation 4.4).

$$\text{IF} = \frac{R_c}{R_0} \quad (4.4)$$

Where

R_c is the genotoxic activity measured in the sample at a certain concentration.

R_0 is the genotoxic activity measured in the solvent control (diluent).

A sample is considered as genotoxic or SOS repair inducer if it has an IF value of 1.5 or more, if the β -galactosidase activity increases significantly compared to the negative control, and if a dose-dependent effect is found. A sample having an induction factor >1.5 was considered as slightly genotoxic, having an $1.5 < \text{IF} < 2$ as moderately genotoxic and an $\text{IF} > 2$ as highly genotoxic.

4.1.3 Cell culture and cell exposure to $PM_{2.5-0.3}$

4.1.3.1 Cell line and culture conditions

The toxicity study of the industrial influenced fine particles (FP-I) and rural influenced fine particles (FP-R) was performed on BEAS-2B cells. These cells, an Ad12-SV40 hybrid virus-transformed human bronchial epithelial cell line, were purchased from European Collection of Cell Cultures (ECACC, Wiltshire, UK; reference, 95102433) and were originally derived from normal bronchial epithelial human cells obtained from the autopsy of noncancerous individual. Therefore, these cells are immortalized and non-mutated for TP53 locus while retaining the characteristics of epithelial bronchial cells whose metabolic characteristics have been already proved (Garcia-Canton *et*

al., 2013; Reddel *et al.*, 1988). Cells should be cultured in CellBIND® surface plastic flasks in order to adhere. These cells retain the ability to undergo squamous differentiation in response to serum therefore it should be cultured in a specific medium. It should be cultured at a density lower than 80% of confluence, in order to prevent their differentiation. Cytogenetic studies have highlighted the absence of major anomalies until the 12th cell passage and the appearance of anomalies after the 21th passage (Ohnuki *et al.*, 1996).

4.1.3.2 Cell culture

BEAS-2B cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in the LHC-9 medium (Gibco). According to the type of the analysis, Corning® CellBIND® plates or flasks were used for culture as follow:

- 96-well microplates for cytotoxicity tests,
- 96-well microplates for Fluorescence-based Assays (black- 96 wells plates) for In-cell western test,
- pre-treated 6-well culture plates for flow cytometry analysis
- 75 cm² culture flasks for other analyses.

BEAS-2B cells were plated and grown to near confluence. Cells were subcultured weekly (1500-3000 cells/cm²). To avoid phenotypic changes affecting responsiveness to chemicals, all the cells derived from the same initial cell culture were not passaged more than five passages. Depending on the incubation time and the type of the analysis, BEAS-2B were seeded at different density and exposed to different PM concentrations, and different methods of recovery and preservation were then used (*Paragraph 4.1.3.3*). Cells were seeded 24 h before exposure to PM and culture medium was then removed and replaced by fresh medium. PM solutions were sonicated for 10 min immediately before exposure in order to maximize particles dispersion.

4.1.3.3 Cell exposure and sampling

4.1.3.3.a Cytotoxicity tests

BEAS-2B cells were seeded in 96-well culture microplates (Corning CellBIND® ThermoFisher Scientific, Illkirch, France) at a density of 2×10^4 cells/200 µL containing 3.75; 7.5; 15; 30; 60; 120; 180; 240 µg/cm² of PM and incubated in a humidified atmosphere (37°C, 5% CO₂, 100% humidity). Cytotoxicity tests were performed at 24h, 48h, or 72h. Therefore, six plates were used for every sample of PM_{2.5-0.3}: three plates for the lactate dehydrogenase activity (LDH) - mitochondrial dehydrogenase activity (MDH) test, and three plates for measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU test). Nonexposed cells were used as negative controls and Triton X-100 (4%, v/v)- exposed cells as positive controls (Appendix 19). Accordingly, 8 replicas were chosen as controls with only media (200 µL of medium), 16 replicas were chosen as controls with only cells (200 µL of cells), 8 replicas per PM concentration as exposed cells, 4 replicas as positive controls

(cells and Triton), and 4 replicas negative control of PM (LHC-9 medium + PM at highest concentration $240 \mu\text{g}/\text{cm}^2$). After 24, 48 or 72 h of incubation, the incorporation of BrdU and the activities of MDH and LDH were evaluated, following the manufacturer's instructions.

After determining the cytotoxicity of $\text{PM}_{2.5-0.3}$ and to assess their toxicological effects, BEAS-2B cells were exposed to defined concentrations (3.75 and $15 \mu\text{g}/\text{cm}^2$) of particles in either 75 cm^2 flasks, 96 wells microplates or 6-well plates according to the parameters to be studied.

4.1.3.3.b The study of genes expression (RT-qPCR) and proteins expression (Western Blot)

Depending on the exposure time (24, 48, or 72 h), BEAS-2B cells were cultured in CellBIND[®] surface cell culture flasks (75 cm^2 ; Corning[®]) at different densities (5×10^6 , 2.5×10^6 , or 1.25×10^6 cells/flask/20 mL of LHC-9 respectively) and incubated at 37°C in humidified atmosphere ($5\% \text{ CO}_2$, 90% humidity). In order to expose BEAS-2B cells for 24, 48, or 72 h, each of the two $\text{PM}_{2.5-0.3}$ samples, industrial or rural, was suspended in LHC-9 medium and then added to cell culture supernatant at two concentrations (3.75 and $15 \mu\text{g}/\text{cm}^2$) without renewing the culture media. PM solutions were warmed and sonicated for 5 min immediately before exposure, in order to maximize particles dispersion. For each exposure time, 4 flasks were chosen at random as negative controls (only with LHC-9), 4 flasks/concentration/PM sample (industrial or rural) as exposed cells, and 4 flasks as positive controls ($1 \mu\text{M}$ B[a]P-exposed cells).

After exposure, the supernatant was removed, adherent cells were detached and centrifuged (200 g , 5 min , 4°C). PBS (1 mL) was added to the pellet, the mixture was centrifuged (200 g , 5 min , 4°C), cell-free supernatants were collected and cell pellets were immediately stored at -80°C until DNA/RNA/proteins extraction by NucleoSpin[®] TriPrep (Macherey-Nagel).

4.1.3.3.c Measurement of DNA adducts by ^{32}P post-labeling method

BEAS-2B cells were seeded in CellBIND[®] surface cell culture flasks (75 cm^2 ; Corning[®]) at a density of 1.5×10^6 cells/ 20 mL and incubated for 24 h, at 37°C , in humidified atmosphere containing $5\% \text{ CO}_2$. $\text{PM}_{2.5-0.3}$ samples (industrial or rural) were suspended in LHC-9 medium and then added to cell culture supernatant at two concentrations (3.75 and $15 \mu\text{g}/\text{cm}^2$) without renewing the culture media and incubated for 72 hours. For each exposure time, 3 flasks were chosen at random as negative controls (only with LHC-9), 3 flasks/concentration/PM sample as exposed cells, and 3 flasks as positive controls ($1 \mu\text{M}$ B[a]P-exposed cells).

After 72 h of exposure, the supernatant was removed and adherent cells were washed with 10 mL PBS (0.01 M ; $\text{pH} = 7.2$). After removing the washing solution, $400 \mu\text{L}$ of a lysis solution (Guanidinium thiocyanate (GITC)) and $600 \mu\text{L}$ of PBS were added. Cells were scraped from the flasks and the cell culture supernatants were stored at -80°C until DNA extraction by DNeasy[®] Blood and Tissue Kit (Qiagen).

4.1.3.3.d Histone 2AX phosphorylation (γ H2AX) quantification by flow cytometry

BEAS-2B cells were seeded in CellBIND[®] 6-well culture plate (Corning[®]) at a density of 1.5×10^5 cells/well in humidified atmosphere containing 5% CO₂ (1 mL of cell culture). PM_{2.5-0.3} samples were suspended in cell culture medium, added to cell culture supernatant at two concentrations (3.75 and 15 $\mu\text{g}/\text{cm}^2$) without renewing the culture media and incubated for 72 hours. Non-exposed cells were used as negative controls, B[a]P (1 μM)-exposed cells and H₂O₂ (50, 100, 200 μM)-exposed cells as positive controls. For each exposure time, 2 wells were chosen for every condition. 1 mL of H₂O₂ was added to the cells 20 min before the test.

At the end of exposure time (72 h), cells were washed with pre-warmed PBS (1 mL/well) and then collected by trypsinization (0.5 mL/well of trypsin and incubated for 3 minutes). LHC-9 medium (0.5 mL) was then added. The mixture was transferred to a centrifuge tube and centrifuged for 5 min at 150g, 4°C. Free-cells supernatants were removed and 1 mL of 4% formaldehyde (in PBS 0.01 M; pH= 7.2) was added. Cells were fixed for 20 min at room temperature, chilled on ice for 1 min and centrifugated as before. Cell- free supernatants were removed and cell pellets were then resuspended in 900 μL of ice-cold methanol (90%), incubated on ice for 30 min to allow cells permeabilization, and stored at -20°C until further use.

4.1.3.3.e Histone 2AX phosphorylation (γ H2AX) quantification by In-Cell Western

BEAS-2B cells were seeded in 96-well microplates for Fluorescence-based Assays (black- 96 wells plates) at a density of 3×10^4 cells/200 μL in humidified atmosphere containing 5% CO₂ (100 μL /well). Each of the two PM_{2.5-0.3} samples was suspended in cell culture medium, added to cell culture supernatant at two concentrations (3.75 and 15 $\mu\text{g}/\text{cm}^2$) without renewing the culture media and incubated for 72 hours. Non-exposed cells were used as negative controls, B[a]P (1 μM)-exposed cells as positive control. For each exposure time, 16 micro-wells were chosen for every condition. At the end of exposure time (72 h), cells were washed with pre-warmed PBS (200 μL /well) and fixed with 200 μL of 4% paraformaldehyde in PBS at room temperature (RT) for 20 minutes. Paraformaldehyde was neutralized with 200 μL of 20 mM NH₄Cl and the wells were then washed with PBS to be used directly to quantify γ H2AX by In-Cell Western.

4.1.4 PM_{2.5-0.3} cytotoxicity

4.1.4.1 Principle

The cytotoxicity of the PM_{2.5-0.3} was determined using three complementary methods:

- measuring of membrane integrity by testing the activity of extracellular lactate dehydrogenase (LDH) in cell-free culture supernatants (Cytotoxicity Detection Kit LDH, Roche Diagnostics)
- assessing cell viability by testing the activity of the mitochondrial dehydrogenase (MDH) in cells (Cell Proliferation Reagent WST-1, Roche Diagnostics)

- testing the extent of cell proliferation by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) in cells (Cell Proliferation ELISA BrdU, Roche Diagnostics)

The LDH activity test is a colorimetric assay for the quantification of cell lysis and death. This test is based on measuring the activity of extracellular LDH, a stable cytosolic enzyme released from the cytosol of damaged cells into the culture medium. The LDH activity is determined in an enzymatic test: in the first step, the nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH/H^+ by the conversion of pyruvate to lactate catalyzed by LDH released from the cells and in the second step, the catalyst (diaphorase) transfers two hydrogen atoms from NADH/H^+ to the yellow tetrazolium salt which is reduced to a red formazan (Appendix 20). Formazan concentration, measured spectrophotometrically, is proportional to the amount of the released lactate dehydrogenase.

The MDH test is a colorimetric assay for the quantification of cell proliferation and cell viability. It is based on the cleavage of the WST-1 tetrazolium salt by DHM in living cells to form the formazan (dark red). This latter's concentration, measured spectrophotometrically, is proportional to the activity of MDH and therefore to the mitochondrial activity and cell viability.

BrdU incorporation is measured by ELISA, using anti-BrdU antibodies coupled to peroxidase, and revealed by the addition of a chromogenic substrate. The amount of BrdU was measured by spectrophotometry and its concentration is proportional to the cell proliferation.

4.1.4.2 Protocol

Two plates are prepared for each sample: one for LDH-MDH test and another for BrdU test. BrdU reagent (20 μL) was added to the cells in the BrdU plate 24 hours before running the experiment. Where indicated, Triton X-100 (4%) was added to the cells 15 minutes prior to the start of the assay.

At the end of the exposure time (24, 48 or 72 h), 100 μL of the supernatant were removed with a multichannel pipette from the LDH/MDH plate to a new plate. The original plate, containing the cells, was used to test WST1 and the new plate, containing the supernatant, was used to the determination of extracellular LDH activity.

- Lactate dehydrogenase (LDH) activity:

The cell-free culture supernatant was incubated with 100 μL /well of reaction mixture from the "cytotoxicity detection kit (LDH)" (Roche Applied Science, Germany) following the manufacturer's instructions. After 15 min, 30 min, 60 min of incubation in the dark, the absorbance of the formed formazan was measured spectrophotometrically at 490 nm wavelength (reference wavelength: 700 nm) using a microplate reader (Multiskan spectrum, Thermo Fisher Scientific, Saint Herblain, France).

- Mitochondrial dehydrogenase (MDH) activity:

WST-1 reagent kit (Roche Applied Science, Germany) was added to the plate with cells and incubated at 37°C in the dark according to the manufacturer's instructions. The absorbance of the formed formazan was measured at 440 nm (reference wavelength: 700 nm) using a microplate reader after 2, 3 and 4 hours of incubation.

- 5-bromo-2-deoxyuridine (BrdU) incorporation:

The determination of BrdU incorporation was performed on the second microplate. The labeling medium containing the non-incorporated BrdU was first removed by tapping off the plates. The cells were then fixed and permeabilized by adding 200 µL /well of FixDenat and incubated for 30 min at room temperature. This solution was then removed thoroughly by tapping and the cells were incubated with 100 µL/well of anti-BrdU- peroxidase antibody for approx. 90 min at 15-25°C in the dark. The antibody conjugate was removed by tapping and the wells were rinsed with 200 µL of washing solution. After removing the washing solution, 100 µL/well of chromogenic substrate solution was added and incubated for 15, 30, 45 min in the dark until color development is sufficient for photometric detection. The reaction was stopped by adding 25 µL (1 M) sulfuric acid and incubating for 1 min on the shaker at 300 rpm in the dark. The absorbance was measured at 370 nm (reference wavelength: 492 nm) before stopping the reaction and at 450 nm (reference wavelength: 690 nm) after stopping the reaction.

4.1.4.3 Analysis

The absorbance values at the reference wavelength were subtracted from those obtained at the wavelength of interest for each well. The lack of signal in the wells without cells was verified, and then the difference in optical density was plotted against the concentration of PM_{2.5-0.3}. The results are expressed as mean of the eight biological replicates. Results were expressed by concentration generating 10% of cytotoxicity or 10% of inhibition of proliferation (IC₁₀) and their 95% confidence interval was calculated by concentration-response curve fitting using Hill equation model (Khinkis *et al.*, 2003).

4.1.5 DNA/RNA/Proteins purification by NucleoSpin® TriPrep and quantification

One of the most important aspects in the isolation of DNA, RNA, and protein is to prevent their degradation during the isolation procedure. After thawing the pellets of exposed cells, 350 µL of lysis solution (RP1) and 10 µL of reducing agent (DTT) were directly added. In order to clear the lysate, filtration of the sample was done using the NucleoSpin® Filter in a collection Tube (2 mL) and centrifuged for 1 min at 11,000g (20°C). A volume of 350 µL of ethanol (70%) was added to the filtrate. The lysate was loaded in a new collection tube with NucleoSpin® TriPrep Column and

centrifuged for 30 s at 11,000g. RNA and DNA are bound to the column membrane and proteins are in the flow-through.

- For DNA and RNA purification, the column membrane was transferred to a new collection tube. Two washes of silica membrane were done by adding 500 μ L of DNA Wash and centrifuging for 1 min at 11,000g. After discarding the flowthrough, the washed membrane was transferred to a new collection tube and 100 μ L of DNA Elute was added directly onto the membrane and incubated for 3 min. Then, the tubes were centrifuged for 1 min at 11,000g. These tubes contain the DNA elute solution. The membranes were transferred into new collection tube and 95 μ L of rDNase reaction mixture were added directly onto the center of the silica membrane of the column and incubated at room temperature for 15 min. The columns were first washed by adding 200 μ L of RA2 buffer (inactivates the rDNase) followed by centrifugation for 30 s at 11,000g. Then 600 μ L of RA3 buffer was added before centrifugation for 30 s at 11,000g. The third wash consisted of 250 μ L of RA3 buffer followed by centrifugation for 2 minutes at 11,000g. RNase-free H₂O (60 μ L) was then added to the membrane prior to centrifugation at 11,000g for 1 min to elute the RNA to the collection tube.

- For protein purification, 450 μ L of protein precipitator were added to the tubes for 10 minutes at room temperature and then centrifuged for 5 min at 11,000g. The supernatant was removed by decanting and 500 μ L of 50% ethanol were added to the pellet and the tube was centrifuged for 1 min at 11,000g. After removing the supernatant, the lid was kept open for 10 min in order to dry the precipitate at room temperature. Urea (100 μ L) was added to the pellets and incubated for 3 min at 95-98°C for complete protein denaturation.

- DNA and RNA were quantified and stored at -20°C until further use. Proteins were quantified using the Bicinchoninic Acid Protein Assay (BCA method) (Sigma-Aldrich) and stored at -80°C until further use.

The Bicinchoninic Acid (BCA) Protein Assay relies on the formation of protein-Cu²⁺ complexes under alkaline conditions, followed by reduction of the Cu²⁺ to Cu⁺. This reduction is catalyzed by the peptide bond and certain amino acids (cysteine, cystine, tryptophan and tyrosine). The amount of the formed Cu⁺ is proportional to the amount of proteins present. BCA then form colored complexes with the Cu⁺, whose concentration can be determined by spectrophotometry. It is possible to assay the proteins contained in the samples by comparing their absorbance at 562 nm to a series of beef albumin serum standards (BSA).

The Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich) was used to assay the protein by optimizing the protocol (Lepers, 2013). BSA solution serial concentrations (800; 600; 400; 200; 100; 50 and 0 μ g/mL) were prepared from a stock initial concentration of 1 mg/mL. 25 μ L/well of the standards and samples (diluted at 1/5 and 1/10) were then placed in duplicates in a 96-well microplate. A mix BCA solution was prepared by mixing Bicinchoninic Acid (BCA) and copper (II) sulfate pentahydrate (50/1, v/v). 200 μ L of this reagent were added to the samples and the plate was

incubated for 30 min at 37°C with shaking. The absorbance of the solutions in each well was determined at 562 nm using the Multiskan reader. The linearity of the regression line corresponding to the range of BSA was checked ($R^2 > 0.95$). The resulting equation from the standard graph was used to calculate the concentrations of proteins in each sample.

4.1.6 DNA extraction by DNeasy[®] Blood and Tissue Kit

After thawing the cells exposed to PM_{2.5-0.3}, DNA adducts were measured by ³²P post-labelling (Paragraph 4.1.3.3.c), DNA extraction was performed using the DNeasy[®] Blood and Tissue Kit (Qiagen). Proteinase K (20 µL) was added and the samples were incubated in a water bath at 56°C for 10 minutes. RNase A (1 µL) was then added to the samples and incubated at room temperature for 15 minutes. A volume of 200 µL of ethanol (96-100%) was added and mixed thoroughly by vortexing. The mixture was transferred to a DNeasy mini spin column placed in a 2 mL collection tube and centrifuged for 1 min at 8,000g. The flow-through was discarded and the column was transferred to a new collection tube. AW1 buffer (500 µL) was added and centrifuged for 1 min at 8,000g, and then 500 µL of AW2 buffer were added and centrifuged for 3 min at 20,000g. After transferring the column to the final tube, 200 µL of the AE buffer were added, incubated 1 min at room temperature, and centrifuged for 1 min at 8,000g. The DNA elute solutions were quantified and then stored at -20°C until further analysis.

4.1.7 PAHs-metabolizing enzyme and DNA repair enzymes genes expressions by RT-qPCR

Gene expression profiles of *CYP1A1* (PAHs-metabolizing enzyme), *OGG1* (DNA glycosylase involved in the repair of 8-OHdG), *NTH1* (DNA glycosylase involved in the repair of 8-OHdG), *APE1* (DNA endonuclease involved in the repair of 8-OHdG), *NUDT1* (8-oxodGTPase involved in the repair of 8-OHdG), *DNMT1* (involved in the repair of DNA methylation), *MGMT* (involved in the repair of DNA methylation), *XPA* (involved in the repair of DNA adducts), and *XRRC1* (involved in the repair of DNA strand breaks) genes expressions were studied using the TaqMan[®] Gene Expression Cells-to-CT[™] Kit and predefined TaqMan[®] Gene Expression Assays.

4.1.7.1 Principle

Real-time quantitative polymerase chain reaction method (qPCR), preceded by a step of Reverse Transcription (RT) of the mRNA (RT-qPCR), is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. TaqMan assay, the used quantitative detection system, is adopted for both the quantification of mRNAs and for detecting variation. *More details are shown in Appendix 21.*

4.1.7.2 Protocol

Genes expressions were studied using the TaqMan® Gene Expression Cells-to-CT™ Kit (Life Technologies) according to the manufacturer's instructions, in exposed BEAS-2B cells to two concentrations of PM_{2.5-0.3} (3.75 and 15 µg/cm²) or B[a]P for 24, 48 and 72 hours of incubation (Lepers, 2013). RNA was extracted using the *NucleoSpin® TriPrep kit* and quantified. RT was performed using the High Capacity cDNA Reverse Transcript kit (Applied biosystem). Extracted RNA (2 µg) was used and the volume was adjusted using RNase free H₂O to 10 µL of each RNA sample. For every sample, Reverse transcription (RT) mix was prepared as follow: 2 µL of RT buffer (10x), 2 µL of Random primer (10x), 0.8 µL of dNTP (100 mM), 1 µL of enzyme superscribe reverse transcriptase, 1 µL/20 samples RT of RNase inhibitor and 6.8 µL of nuclease free water.

10 µL of the RT Mix were added to 10 µL of RNA samples in a 1.5 mL eppendorf tube. RT was performed using the thermal cycler 7500 Fast Real-Time PCR System (Life Technologies) programmed as follows: 60 min at 37°C (reverse transcription), 5 min at 95°C (inactivation) and holding at 4°C. RT products were then diluted by adding 180 µL per tube (to obtain a 200 µL final volume) of RNase free water. RT samples were stored at 4°C.

The PCR was performed in a 96-well microplate by adding 2.5 µL of cDNA and 7.5 µL reaction mixture containing: 5 µL of TaqMan® Fast Universal PCR Master Mix (2X), 0.5 µL of the target gene and 2 µL of RNase free water. Samples were tested in duplicates for every target gene and the reference gene (18S RNA: Hs99999901_s1). The plate was then covered with an adhesive film (Life Technologies, St Aubin, France) and centrifuged briefly using a microplate centrifuge (Eppendorf France SAS, Le Pecq, France). The PCR was performed on the same device as RT, configured in "Fast" mode, according to the following protocol:

- 20 s at 95°C (activation of Taq polymerase)
- 40 cycles of amplification: 3s at 95°C (denaturation) and 30s at 60°C (hybridization-elongation and data collection).

4.1.7.3 Analysis

The amplification results were visualized in real time and expressed in the form of ΔR_n (difference between the intensity of fluorescence emitted by the reporter and the fluorescence background) based on the number of amplification cycles. For each gene, the threshold cycle (C_t), defined as the number of amplification cycles for a significant increase in fluorescent intensity beyond the baseline, was determined using the 7500 Sequence Detection Software Ver.2.0.3 (Life Technologies, St Aubin, France). The calculations were then carried out according to the $2^{-\Delta\Delta C_t}$ method described by Livak and Schmittgen (Livak and Schmittgen, 2001). The C_t of a sample depends on the initial number of mRNA copies. If the number is high, it requires a smaller number of amplification cycles to reach the fluorescence threshold, and therefore a smaller C_t .

The relative quantitation (RQ) of target mRNA, which corresponds to the differences in target mRNA levels in exposed cells (i.e., PM_{2.5-0.3}-exposed cells or B[a]P-exposed cells) *versus* in non-exposed cells, was corrected by the level of endogenous ribosomal 18S RNA used as the reference gene. It was calculated using the following equation: $RQ = 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct(\text{target gene}) - Ct(18S \text{ RNA})$, and $\Delta(\Delta Ct) = \Delta Ct(\text{exposed cells}) - \Delta Ct(\text{non-exposed cells})$. The results are expressed as median form [Q1, Q3] of four biological replicates.

4.1.8 Measure of DNA adducts by ³²P post-labeling method

This assay was performed in collaboration with Dr. Véronique ANDRÉ (University of Caen, Normandy, EA 4651 ABTE).

4.1.8.1 Principle

³²P-postlabeling analysis is an ultrasensitive method for the detection and quantification of carcinogen-DNA adducts. Among the numerous organic compounds detected in the collected particles, a number of PAH, including B[a]P after metabolic transformation (BPDE), are capable of forming bulky adducts in the DNA of exposed cells. These larger adducts can be detected by ³²P-postlabeling analysis. It consists of four principal steps: i) enzymatic digestion of DNA to nucleoside 3'-monophosphates; ii) enrichment of the adduct fraction of the DNA digest; iii) 5'-labeling of the adducts by transfer of ³²P-orthophosphate from [γ -³²P] ATP mediated by polynucleotide kinase (PNK); iv) chromatographic or electrophoretic separation of the labeled adducts or modified nucleotides and quantitation by measurement of their radioactive decay. Thus only the nucleotide carrying bulky adducts are marked and can therefore be detected after separation by thin layer chromatography (TLC).

4.1.8.2 Protocol

The protocol employed (Figure 4.3) was based on the standardized method described by Reddy and Randerath (1986) and applied to BEAS-2B cells exposed (or not) for 72 hours to PM_{2.5-0.3} at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$ or to B[a]P. A positive control (calf thymus DNA exposed to B[a]P-7,8-diol-epoxide (BPDE)) and a negative control (without any DNA) were included.

5 μg of DNA (extracted by DNeasy[®] Blood and Tissue Kit) were digested by micrococcal nuclease and spleen phosphodiesterase (SPD) for 3.5 hours. Enrichment of DNA adducts was obtained by treatment with nuclease P1 (NP1) for 30 min at 37°C and stopped by adding 1.8 mL of Tris-base. Adduct labeling step was carried out for 30 min at 37°C in the presence of T4 PNK and 30 μCi of ³²P-cATP.

Separation of adducts was achieved by multidirectional PEI-cellulose Thin Layer Chromatography (TLC) (12 cm x 12 cm) (Macherey-Nagel, Hoerd, France). Four migration solvents were successively used: i) 1 M sodium phosphate, pH 6.8 (overnight); ii) 4.5 M lithium formate, 8.5 M urea, pH 3.5 (3 hours); iii) 0.6 M lithium chloride, 0.5 M Tris-HCl, 8.5 M urea, pH 8 (3 hours);

iv) 1.7 M sodium phosphate, pH 5 (overnight). Autoradiograms (Kodak XOMat film) were exposed to TLC-plates for 24 h at -80°C with intensifying screens.

4.1.8.3 Analysis

Every sample was processed in triplicates. Two samples with $3\ \mu\text{mol}$ of dAp were labeled and diluted to 1 mL final. From these samples, three deposits of $4\ \mu\text{L}$ were separated on TLC sheets. After counting, dAp mean values served to estimate the labeling of $5\ \mu\text{g}$ of dNp. Spot areas on TLC plates were excised and the radioactivity was quantified using a scintillation counter (Cerenkov mode). Results were expressed as Relative Adduct Levels (RAL): $\text{RAL} = \text{cpm}_{\text{adducts}} / [(\text{cpm}_{\text{dAp}}/0.012) \times 3240 \times 5] = 1.22 \times 10^{-6} \text{ cpm}_{\text{adducts}}/\text{cpm}_{\text{dAp}}$, with: 0.012 pmol of dAp/deposit, 3240 = pmol of dNp in $1\ \mu\text{g}$ DNA, and $5\ \mu\text{g}$ of DNA by sample. The above-described protocol confer to the ^{32}P -postlabeling method a sensitivity of about 1 adduct/ 10^9 nucleotides, which will, therefore, be sufficiently sensitive for its application to a wide range of cell and/or tissue samples from human populations exposed to ambient air pollution.

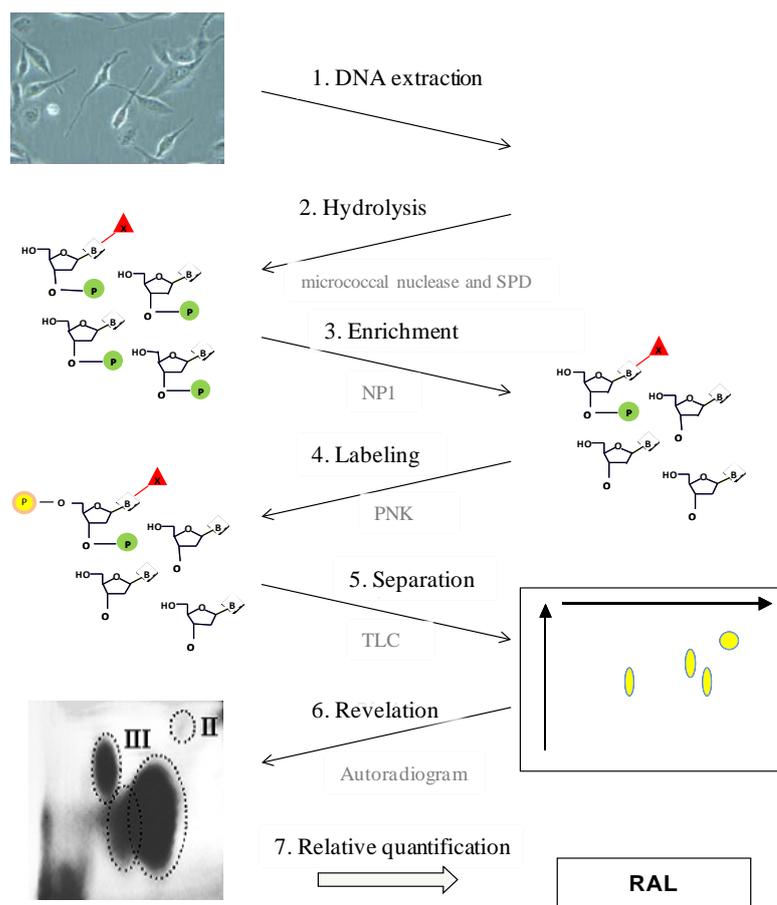


Figure 4.3: Measure of DNA adducts by ^{32}P post-labeling method (Lepers, 2013)

SPD: spleen phosphodiesterase; NP1: Nuclease P1; PNK: T4 polynucleotide kinase; TLC: Thin Layer Chromatography; RAL: Relative Level adduct.

4.1.9 Histone 2AX phosphorylation (γ H2AX) quantification by flow cytometry

4.1.9.1 Principle

The phosphorylation of the histone H2AX, variant of histone H2A, is known to be a marker of double-strand breaks. Specific antibodies for the phosphorylated form of H2AX (γ H2AX) coupled to fluorophores can be used in flow cytometry. It is thus possible to determine the amount of γ H2AX for each cell, and then to divide these results based on the DNA amount in order to obtain an evaluation of double strand breaks according to cell cycle phases. The evaluation of the phosphorylation of histone H2AX in cells exposed to particles, under industrial and rural influences, was carried out by flow cytometry using the Flow collect γ -H2AX detection kit (Merck-Millipore, Darmstadt, Germany). *More details about flow cytometry are present in Appendix 22.*

4.1.9.2 Protocol

Quantification of double-strand breaks was performed on BEAS-2B cells exposed (or not) to PM_{2.5-0.3} for 72h, under industrial and rural influence at 3.75 or 15 $\mu\text{g}/\text{cm}^2$ or B[a]P, fixed and permeabilized directly at the end of the incubation period (*Paragraph 4.1.3.3.d*). A sample of cells exposed to 50, 100, 200 μM of H₂O₂ for 20 min was used as positive control.

Flow collect γ H2AX detection kit (Merck-Millipore, Darmstadt, Germany) was used to quantify phosphorylation of H2AX as a marker of double-strand breaks (Lepers, 2013). For immunostaining, 200 μL of permeabilized cells (about 2×10^5 cells) were placed into two microtubes and centrifuged (300g, 5 min, 4°C). Pelleted cells were resuspended in 95 μL of 1X assay buffer provided in FlowCollect™ histone H2AX phosphorylation assay kit and incubated with fluorochrome-conjugated antibody: 5 μL anti- γ H2AX (mouse IgG1-Alexa Fluor®488) or 5 μL 1/100 Isotype control (mouse IgG1-Alexa Fluor®488) for 30 min at room temperature in the dark. Isotype control nonspecific antibody was used to estimate the extent of nonspecific binding of anti- γ H2AX antibody in the cells. Cells were centrifugated, the supernatant was aspirated and pellets were resuspended in 500 μL PBS. 0.5 μL of DNA dye (FxCycle™ Violet DNA stain (4', 6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen®, France) was added and cells were incubated for 30 min in the dark and immediately analyzed by flow cytometer (Attune® Acoustic Focusing Cytometer; Life Technologies, France) in high sensitivity mode. A fluorescent dye specifically recognizes the double-stranded DNA in the fixed cells. It is used to quantify DNA in the cells and help in the analysis of the cell cycle phase (phase G0/G1, S and G2/M).

4.1.9.3 Analysis

At least 15,000 individual cells were taken into account for analysis. After analyzing the samples by flow cytometry, the quantification of the results was performed using the Flowing Software V.2.4.1 (Turku Centre for Biotechnology, Finland). γ H2AX was quantified as the

differences in Median Fluorescence Intensity (ΔMFI) between antibody-labeled cells and isotype control-labeled cells for each sample in each phase of the cell cycle ($\Delta\text{MFI sample} = \text{MFI}_{\gamma\text{H2AX}} - \text{MFI}_{\text{iso}}$). The fluorescence is normalized compared to the amount of DNA in each cell. Results are expressed as median [Q1;Q3] of 4 biological replicates. Finally, for each type of exposure, we reported the results of γH2AX to the control, corresponding to a relative normalization to the unexposed cells ($\Delta\Delta\text{MFI} = \Delta\text{MFI}_{\text{exposed}} / \Delta\text{MFI}_{\text{unexposed}}$).

4.1.10 Histone 2AX phosphorylation (γH2AX) by In-Cell Western

4.1.10.1 Principle

The test is based on the quantification of the phosphorylation of the histone H2AX (γH2AX), which reflects a global genotoxic insult. Different genotoxic assays have recently been developed based on the quantification of this biomarker by ELISA, fluorescence-activated cell sorter (FACS) or immunofluorescence microscopy techniques. The In-Cell Western (ICW) Assay is a quantitative immunofluorescence assay performed in microplates (96-well format). It combines the specificity of Western blotting with the reproducibility and throughput of ELISA. In-Cell Western™ assays allows the detection of proteins in fixed cultured cells using target-specific primary antibodies and IRDye® fluorescent secondary antibodies, the quantification of two targets at 700 nm and 800 nm, using spectrally-distinct infrared dyes, the measurement of relative protein levels in many samples, and the detection of proteins *in situ* in a relevant cellular context. In this study, this test is used for quantification of γH2AX and allows the simultaneous examination of the cytotoxicity and genotoxicity of xenobiotics on human cells as in study by Khoury *et al.* (2013).

4.1.10.2 Protocol

Quantification of double-strand breaks was performed on BEAS-2B cells exposed (or) to $\text{PM}_{2.5-0.3}$ for 72h, under industrial and rural influences, at 3.75 or 15 $\mu\text{g}/\text{cm}^2$ or B[a]P, fixed directly at the end of the incubation period (*Paragraph 4.1.3.3.e*).

After fixing the cells with 4% paraformaldehyde and washing them with PBS, paraformaldehyde was neutralized with 200 μL of 20 mM NH_4Cl and the wells were then washed with PBS to be used directly to quantify γH2AX by In-Cell Western. Cells were permeabilized with 0.2% Triton X-100 in PBS and washed with PBS, 2% fetal calf serum, 0.2% Triton X-100 (PST buffer). Cells were incubated with 200 μL of mouse monoclonal anti γH2AX (mouse IgG1-Alexa Fluor®488) for 2 hours in the dark. After three washes with PST, secondary detection was carried out using 200 μL of FxCycle™ Violet DNA stain (4', 6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen®, France). After 30 minutes of incubation in the dark and three washes with PST, γH2AX was visualized using a Fluoroskan Ascent (Thermo Labsystem).

4.1.10.3 Analysis

Raw absorbance data median was calculated from 16 replicas for every sample and corrected for background. The relative fluorescence units from the scanning enabled quantitative analysis. For the determination of genotoxicity, relative fluorescent units for γ H2AX per cell were divided by the respective controls to determine the change in phosphorylation of H2AX levels compared with control. Statistical analysis was performed to assess significant effects of PM_{2.5-0.3} using Kruskal-Wallis and Mann-Whitney tests.

4.1.11 Proteins expression by Western blot

4.1.11.1 Principle

By using western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize. *The principle of western blot is present in Appendix 23.A.*

4.1.11.2 Protocol

Protein expression of PARP1, OGG1 and DNMT1 was studied in BEAS-2B cells exposed (or not) to PM_{2.5-0.3} for 72 h, under industrial and rural influences at 3.75 or 15 $\mu\text{g}/\text{cm}^2$ or B[a]P as described in *Paragraph 4.1.3.3.b. More detailed protocol is present in Appendix 23.B.*

- **Sample preparation**

Proteins were extracted, quantified by BCA method and stored at -80°C as described in *Paragraph 4.1.5.* Proteins samples were thawed on ice and then 15 μg of proteins, prepared in distilled water to a volume of 10 μL , were added to 10 μL of Tris glycine SDS (Novex® Tris-Glycine SDS Sample Buffer (2X)). The mixture was heated for 5 min at 95°C to denature the proteins.

- **Gel electrophoresis**

Protein expression of PARP1 (115 kDa), DNMT1 (185 kDa), OGG1 (39 kDa) were done on prepared Novex™ 4-12 % Tris- glycine gels (Invitrogen). Actin (42 kDa) was used as a reference protein when testing DNMT1 and OGG1. After placing the gel in the electrophorator (XCell SureLock Mini (Invitrogen)), the running buffer (1X) was poured until it covered the gel completely. A volume of 5 μL of the protein ladder (molecular weight marker: PageRuler Prestained™ Protein Ladder, 10 to 250 kDa) and 20 μL of the prepared samples were loaded into the wells.

- **Electrotransfer**

On completion of the separation of proteins by polyacrylamide gel electrophoresis (PAGE), the proteins were transferred from the gel to a solid support membrane, usually made of a chemically inert substance, such as nitrocellulose or polyvinylidene difluoride (PVDF). The gel, membrane, and

electrodes are assembled in a sandwich so that proteins move from gel to membrane, where they are captured, in a pattern that perfectly mirrors their migration positions in the gel. A wet transfer was applied for 90 minutes at 800 mA and a constant voltage (30 V). The glass plates containing the gel were then separated and the gel was removed and equilibrated in transfer buffer (1X) (40 mL transfer buffer (25X), 200 mL methanol and 760 mL distilled H₂O). The PVDF membrane, the filters and the sponges were equilibrated in transfer buffer.

- Confirmation of protein transfer to the membrane

After electrotransfer, it may be necessary to confirm that all the proteins in the gel have been completely eluted. This can be achieved by staining the gel using a total protein stain after electrotransfer. In addition, in order to ensure that all the proteins are on the membrane (and have not passed through or migrated in the opposite direction), the membrane can also be treated with a total protein stain. For gel staining, Coomassie Brilliant Blue was used. The membrane was stained with Pierce™ kit Reversible Protein Stain Kit or with Ponceau S.

- Blocking and antibody incubation:

The membrane was blocked with a solution of PBS-Tween (0.05%) (100 mL PBS 10X, 900 mL H₂O and 0.5 mL Tween 20) with 5% milk powder for 90 minutes. After blocking, the membrane was rinsed with PBS-Tween 0.05% (3 times for 5min, with agitation).

Following the blocking step, the protein of interest can be detected using antibodies. The primary antibody was prepared at a concentration of 1/1000 in blocking solution, added to the membrane in a way to cover it and incubated overnight at 4°C with agitation. In case of determining the protein expression of PARP1, only anti-PARP1 (rabbit monoclonal IgG, Abcam ab191217) was added to the membrane. While in the case of both DNMT1 and OGG1, anti-β-Actin (a housekeeping protein, R and D systems) was tested in the same membrane as anti-DNMT1 (polyclonal IgG, Abcam ab19905) or anti-OGG1 (Abcam), and therefore the membrane was cut according to the ladder and the target membrane's size in a way to add on each fragment the specific antibody. The next day, the membrane was rinsed with PBS-Tween 0.05% (3 times for 5min, with agitation). The secondary antibody was prepared at 1/1000 in blocking solution, added to the membrane in a way to cover it and incubated for one hour with shaking. Anti-mouse secondary antibody was used for β-Actin while anti-rabbit second antibody was used for PARP1, DNMT1 and OGG1. The membrane was rinsed with PBS-Tween 0.05% (3 times for 5 min, with agitation).

- Detection and imaging:

In this study, chemiluminescence detection was used. Pierce® ECL 2 Western Blotting Substrate kit (Thermo Scientific) was used to prepare the ECL mixture by adding 2 mL of the solution A and 50 µL of the solution B provided in the kit. Excess reagent was drained from the membrane by tapping the membrane's edge with forceps against an absorbent tissue. The membrane was incubated with ECL and the light signal was detected by digital imaging with a charge-coupled device (CCD)

camera-based imager: Gbox (SynGene) and Snap Gene software was used. Another type of detection, CN/DAB Substrate Kit (Thermo Scientific) was also used. It is for chromogenic detection of horseradish peroxidase in immunohistochemistry and immunoblotting applications. 125 μL of the (10X) CN/DAB Concentrate were combined with 1.125 mL of the Stable Peroxide Substrate Buffer. The substrate solution was applied to the membrane and incubated for 2 to 5 minutes until significant color develops. To stop the reaction, the membrane was washed with ultrapure water.

4.2 Results and Discussions

4.2.1 $\text{PM}_{2.5-0.3}$ mutagenicity by the Ames fluctuation test

4.2.1.1 Results

- Ames fluctuation test with TA98:

Industrial (FP-I) and rural (FP-R) $\text{PM}_{2.5-0.3}$ samples were tested at different concentrations using the Ames fluctuation Test with TA98 strain with and without metabolic activation. MRs were calculated for each treatment plate. If the MR is high enough and significant by *Chi*-squared test, a sample is considered genotoxic with significances of $a < 0.05$, $b < 0.01$, or $c < 0.001$ (Figure 4.4).

FP-I was mutagenic starting with a concentration of 5 $\mu\text{g}/\text{mL}$ before metabolic activation and at 1.25 $\mu\text{g}/\text{mL}$ after metabolic activation. FP-R did not show any significant mutagenicity with the tested concentrations on the *Salmonella* TA98 before activation. After activation, a slight mutagenic activity was observed at 5 and 10 $\mu\text{g}/\text{mL}$. When comparing FP-I and FP-R, a higher response with the industrial sample is observed with and without metabolic activation (Figure 4.4).

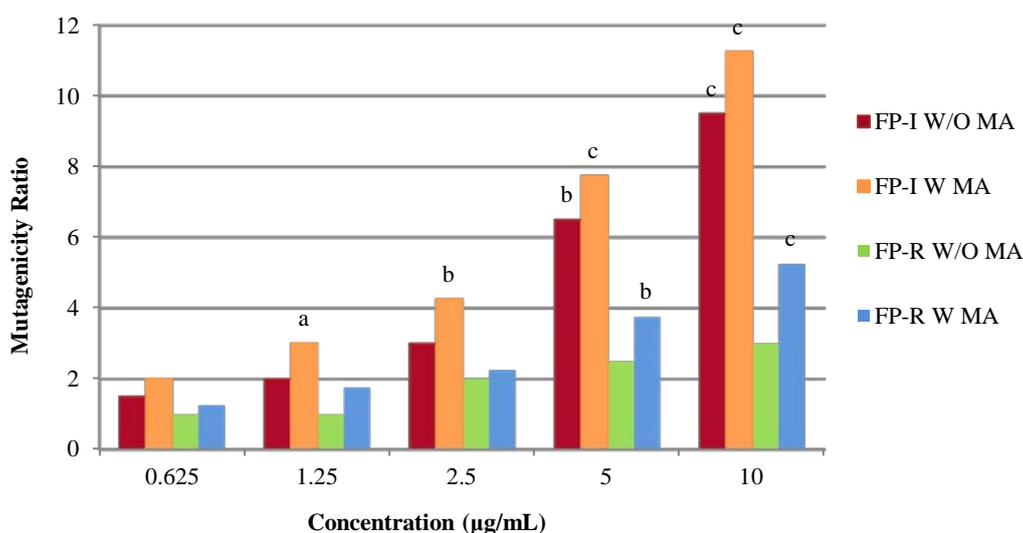


Figure 4.4: Mutagenicity of the industrial and rural $\text{PM}_{2.5-0.3}$ on *Salmonella* TA98

Mutagenicity ratios were calculated and significance was evaluated by Chi-square analysis (χ^2) (^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$).

W/O MA: without metabolic activation, W MA: with metabolic activation.

- Ames fluctuation test with YG1041:

FP-I and FP-R were tested at different concentrations with *Salmonella* YG1041 strain with and without metabolic activation (Figure 4.5). FP-I was mutagenic starting with a concentration of 0.625 µg/mL with and without metabolic activation. MRs were dose-dependent. FP-R had a mutagenic activity starting from 2.5 µg/mL without metabolic activation. After activation, FP-R showed a higher mutagenic activity starting from a concentration of 1.25 µg/mL. When comparing FP-I and FP-R, the industrial sample showed a higher response with and without metabolic activation (Figure 4.5).

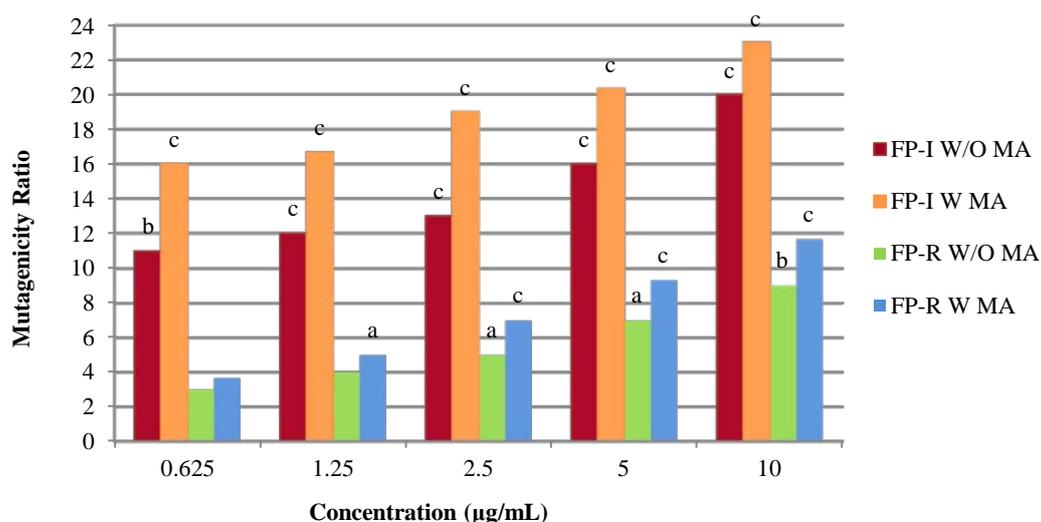


Figure 4.5: Mutagenicity of the industrial and rural PM_{2.5-0.3} on *Salmonella* YG1041

Mutagenicity ratios were calculated and significance was evaluated by Chi-square analysis (χ^2) (^ap < 0,05; ^bp < 0,01; ^cp < 0,001).

W/O MA: without metabolic activation, W MA: with metabolic activation.

- Ames fluctuation test with TA102:

Ames Fluctuation tests were performed on FP-R and FP-I using the *Salmonella* TA102 strain with and without metabolic activation (Figure 4.6). FP-I was mutagenic starting with a concentration of 1.25 µg/mL with and without metabolic activation. MRs were dose-dependent. The FP-R showed a dose-dependent mutagenic activity starting from a concentration of 2.5 µg/mL with the *Salmonella* TA102 with and without metabolic activation. When comparing FP-I and FP-R, the industrial sample showed a higher response with and without activation (Figure 4.6).

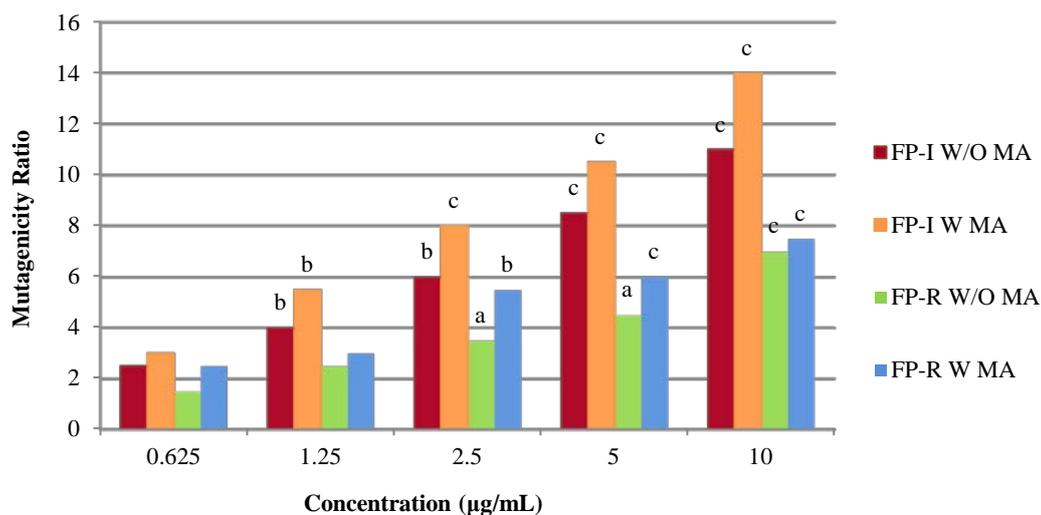


Figure 4.6: Mutagenicity of the industrial and rural PM_{2.5-0.3} on *Salmonella* TA102

Mutagenicity ratios were calculated and significance was evaluated by Chi-square analysis (χ^2) (^a $p < 0,05$; ^b $p < 0,01$; ^c $p < 0,001$).

W/O MA: without metabolic activation, W MA: with metabolic activation.

4.2.1.2 Discussion

In air pollutant-related studies, the *Salmonella* assay is overwhelmingly the most-used bioassay (Claxton *et al.*, 2004). Following the lead of Pitts (Pitts, 1977), a number of investigators examined PM and source emission using the *Salmonella* assay (Lewis *et al.*, 1988; Talcott and Wei, 1977; Waters *et al.*, 1980). Studies were undertaken with different objectives: risk assessment, site-to-site comparisons, source identification, and method development. Latin American studies (Adonis and Gil, 1993; Villalobos-Pietrini *et al.*, 1995), Asian studies (Houk *et al.*, 1992; Tokiwa *et al.*, 1977, 1980), European studies (Alfheim *et al.*, 1983; Pagano *et al.*, 1996), American studies (Barale *et al.*, 1989; Claxton *et al.*, 2001; Talcott and Wei, 1977; Walker *et al.*, 1982) provide estimates of the airborne extracts' mutagenicity. Most of these studies used the *Salmonella* strain TA98, this was the strain of choice because it had a low spontaneous background while being responsive to PAHs (Bronzetti *et al.*, 1997; Matsumoto *et al.*, 1998; Sato *et al.*, 1995; Sutou *et al.*, 1980). Most of the studies that have used TA98 and TA100, showed an increase in mutagenicity of PM upon addition of S9 (Cerná *et al.*, 2000; Martinis *et al.*, 1999; Squadrito *et al.*, 2001). In studies using the YG1041 strain, S9 reduced the mutagenicity of PM (Cerná *et al.*, 1999). As a result of the relatively high activity of nitroreductase and O-acetyltransferase in this specific strain, the highest direct mutagenicity in this strain was indeed found in the polar subfractions, which are most likely to contain nitro-PAH and nitro-PAH derivatives (de Kok *et al.*, 2006). Studies showed that the S9-mediated mutagenicity was higher in PM samples taken during winter than during summer episodes (Binková *et al.*, 2003; Du Four *et al.*, 2004). The majority of these studies tend to correlate the PM mutagenicity with PAHs or nitro-PAHs concentrations (Delgado-Rodríguez *et al.*, 1999; Kuo *et al.*, 1998; Massolo *et al.*, 2002; Müller *et al.*, 2001; De Martinis *et al.*, 1999). They observed a positive relationship

between mutagenicity and PAH concentration, as well as nitro-PAH concentration (Vargas, 2003). It has been demonstrated that different extraction procedures or solvents applied to PM samples and particle size result in differences in mutagenic activities (De Flora *et al.*, 1989; Hayakawa *et al.*, 1995; Kado *et al.*, 1986). In some studies, the samples were chemically fractionated in order to identify the compound classes and compounds responsible for the genotoxicity. The more mutagenic fractions typically contained three to seven ring compounds and included PAHs, oxy-PAHs, nitroarenes, and aromatic anhydrides. Among the mutagens identified were 2-nitropyrene, cyclopenta[c,d]pyrene, benz[a]anthracene, benzo[a]pyrene, and benzo[g,h,i]perylene (de Raat and de Meijere, 1988). Other studies studied the sensibility of different *Salmonella* strains (Ducatti and Vargas, 2003), and the two types of Ames test, plate incorporation (Ames *et al.*, 1973) and microsuspension test (Kado *et al.*, 1986), by testing PM extracts (Cerná *et al.*, 1999; Claxton *et al.*, 2001; Claxton *et al.*, 1991; Houk *et al.*, 1992). Several conclusions can be drawn from these studies and related studies: i) liquid preincubation and spiral assay procedures tend to be more sensitive than plate incorporation procedures for ambient air extractable organics; however, the plate incorporation test provides more reproducible results (Umbuzeiro *et al.*, 2010); ii) when mutagenic nitroaromatic compounds are present in a sample, the YG series of strains and the liquid suspension assays are more sensitive indicators of mutagens than the TA strains and the plate assay (Cerná *et al.*, 2000; de Kok *et al.*, 2006). Several studies that involved determining the mutagenicity of actual airborne organic matter gave some indication that long-range transport and/or atmospheric transformation processes affect the level and type of mutagens associated with airborne gases and particles (Crebelli *et al.*, 1995). Other more recent studies are summarized in Table 4.3.

When the studies are assessed as a group, the following observations can be made: i) although PAHs contribute significantly to the mutagenicity of airborne organics, PAHs are not the predominate class of mutagens found; ii) much of the PM mutagenicity is associated with moderately and highly polar compounds such as nitroaromatics, aromatic amines, and aromatic ketones; iii) when measured, specifically measured PAHs accounted for only a very small portion (<5%) of the total mutagenicity detected; iv) S9 exogenous activation systems increased the mutagenicity of organics and decreased the mutagenicity with some ambient air samples; v) NO_x concentrations correlated with the level of mutagenic activity.

Therefore mutagenicity of the PM_{2.5} is quite well studied in the literature, but most studies were performed on the extractable organic matter (EOM). This raises several issues: i) the very high concentration of organic compounds in the EOM; ii) the representativeness of the sample compared to the original sample; iii) the inability to take into account the core of the PM_{2.5}; iv) the effect of extraction solvents and methods used (Belcik *et al.*, 2014; Claxton *et al.*, 2004; Claxton and Woodall, 2007; de Kok *et al.*, 2006). Few studies tested the mutagenic activity of native PM_{2.5}, by its entirety, by Ames test. According to our knowledge, this is the first study to test the mutagenic effect of native PM_{2.5} by its entirety using the Ames fluctuation test.

The Ames fluctuation test was used to assess the intrinsic mutagenic potential of the collected PM_{2.5}. Three strains of *Salmonella typhimurium* with different sensitivity were assayed with and without metabolic activation.

The TA98 strain is useful in detection of deletions or additions of base pair (frameshifts). This strain is sensitive to frameshift mutagens without activation, and sensitive to PAHs with metabolic activation. The obtained results showed that FP-I was mutagenic starting with a concentration of 5 µg/mL before metabolic activation and 1.25 µg/mL after metabolic activation. For FP-R, although no significant response was observed without activation, MR increased after activation at 5 µg/mL (Figure 4.5). The mutagenic activity of PM_{2.5} on this strain is suggested to be mainly associated with the PAH concentrations (Gilli *et al.*, 2007). It has been shown that PAH content is higher in FP-I than that in FP-R, and consequently our results appear in agreement with previous studies (Bełcik *et al.*, 2014; Lepers *et al.*, 2014).

The YG1041 strain is a derivative of the TA98 strain and shows increased sensitivity to nitro-, amino- and hydroxylamino-PAH derivatives due to an overproduction of the nitroreductase and O-acetyltransferase (Hagiwara *et al.*, 1993). FP-I was mutagenic starting with the lowest tested concentration, 0.625 µg/mL, with and without metabolic activation. FP-R had a mutagenic activity starting from 2.5 µg/mL without metabolic activation and it showed a higher mutagenic activity starting from a concentration of 1.25 µg/mL after metabolic activation (Figures 4.5). Similar to other studies, higher MR values were obtained from FP-I and FP-R with YG1041 strain (Bełcik *et al.*, 2014; Binková *et al.*, 2003; Claxton and Woodall, 2007). The mutagenic activity was higher in FP-I than in FP-R especially after metabolic activation. Usually in more polar fractions the concentrations of PAH nitro derivatives are higher than the concentrations of non-substituted PAHs (Bamford *et al.*, 2003; Cecinato *et al.*, 2001; Purohit and Basu, 2000). Therefore, by including the YG series strains, it is possible to detect the effects of nitro-PAH derivatives (Maeda *et al.*, 2007). The strong correlation between the concentrations of PAHs and the response of the YG1041 strain does not contradict this hypothesis, because the aromatic nitro compounds are formed in the atmosphere in the presence of PAHs and NO₂ (Jariyasopit, 2013). The increase of the mutagenicity after metabolic activation, that leads to the degradation of the nitro-PAHs to PAHs, suggest that the initial PAHs are an important factor in the mutagenic activity of the samples (Laskin, 1984). Furthermore, the principal activation pathway of the nitro-HAP involves reduction of the nitro group to the N-hydroxyamino-PAH intermediates that are capable of binding to DNA leading DNA adduct formation (Yu, 2002).

The strain TA102 was used to investigate the involvement of oxidative stress generated by PM, possibly related: i) to ROS production on the surface of the particles or ii) to the presence of transition metals in the PM, such as iron, copper, manganese, aluminum or chromium, that may catalyze the Fenton reaction and iii) to PAHs whose metabolism can generate ROS. FP-I was mutagenic starting with a concentration of 1.25 µg/mL with and without metabolic activation. Whereas, FP-R showed a dose-dependent mutagenic activity starting at 2.5 µg/mL with the

Salmonella TA102, with and without metabolic activation (Figure 4.6). Therefore, the mutagenic activity was higher in FP-I than in FP-R and with metabolic activation, in agreement with the hypothesis (iii) discussed above.

The results obtained by Ames assay on the native particles allow us to draw main conclusions: i) particles collected under industrial influence showed dose-dependent higher mutagenicity compared to the rural influenced sample; ii) the high MRs obtained with and without metabolic activation confirm the presence of both promutagens and direct mutagens; iii) the YG1041 strain, sensitive to PAHs, is probably the most likely to reveal a mutagenic potential of the studied PM, iv) the PAHs present in higher concentrations in FP-I, seem to play a leading role in the global mutagenicity of the PM even if the nitroaromatics compounds could be also involved in the mutagenicity of these particles; iv) the inorganic and organic composition of PM could result in oxidative capabilities that lead to the mutagenicity. However, other components than PAHs may also lead to similar effects; this hypothesis requires further validation.

It must be underlined that the Ames test is generally used in liquid sample, and in this work, we prove that it also allows studying mutagenicity of particulate samples suspended in a liquid. Our original approach was to consider total particulates and not only PM organic extracts, which maximizes the bioavailability of the PM organic fraction. Due to the PM dispersion procedure, the organic concentration in the culture media is probably poor. In our study, bacteria are in contact with the particles and the positive responses obtained in the Ames test suggest that chemical compounds (such as PAHs or PAHs derivatives) adsorbed onto particles surface can interact with bacteria and that such compounds are bioavailable. Furthermore, the effect of whole particles on mutagenicity/genotoxicity is shown. Finally, literature data showed that complementary information on PM mutagenicity/genotoxicity was obtained from tests on mammalian cell lines and permit a comparison with bacterial assays. In Lepers *et al.* (2014), mutagenicity was investigated using Ames test on the same bacterial strains as in our work and clastogenicity was evaluated using comet and micronucleus assays and γ H2AX phosphorylation on human bronchial epithelial BEAS-2B cells exposed to PM. Ames responses were found in agreement with clastogenicity parameters, suggesting the potential of Ames test to predict mutagenicity effects of PM on mammalian cells.

Table 4.3: Recent studies that determine the mutagenicity of PM at different sites and samples used

Study	Sample	Ames Type	Sites	Chemistry	Strains (-S9/+S9)	Results
(Brits <i>et al.</i> , 2004) Flanders, Belgium	PM ₁₀ extracts	Plate-incorporation assay	3 sites: urban, industrial and rural	17 PAHs, 7 cPAHs	TA98 -/+	- Mutagenicity for all sites and due to PAHs - For particles, PAHs, cPAHs, urban > others - mutagenicity +S9 > -S9
(Cassoni <i>et al.</i> , 2004) Italy	PM _{2.5} extracts	Plate-incorporation assay	8 cities: 2000–2002	CO, NO ₂	TA98 -/+	- mutagenicity +S9 < -S9 - CO, NO, and PM: good indicators of vehicle emissions (reflect trends in mutagenicity)
(Du Four <i>et al.</i> , 2004) Flanders, Belgium	PM ₁₀ extracts, Semivolatiles	Plate-incorporation assay	3 sites: urban, industrial and rural	16 PAHs, 6 cPAHs	TA98 -/+	- PM levels at all sites winter and summer - PAHs higher in winter than summer - PAH distribution (particles vs. semivolatiles) different for winter and summer - Total mutagenicity higher in winter
(Erdinger <i>et al.</i> , 2004) Mannheim, Germany	PM extracts	Plate-incorporation assay	5 consecutive days once a month for 1 year in industrialized town		TA7000, TA7001, TA7003, TA100	- Highest responses in cold months - G- T base substitutions: primary class of mutation induced by the samples and primary class of mutations produced by PAHs and nitroarenes.
(Hansen <i>et al.</i> , 2004) Denmark	PM extracts	Plate-incorporation assay	One city	1-hydroxypyrene, and the N-acetyltransferase	YG1021 -/+	- Most potent airborne mutagens: dinitropyrenes (e.g., from diesel exhaust), not PAHs.
(Kameda <i>et al.</i> , 2004a) Osaka, Japan	PM extracts	Plate-incorporation assay	One site	1-nitropyrene (1-NP), 2-nitrofluoranthene (2-NF), NO _x	YG1024 -/+	- Mutagenicity correlated with 1-NP, CO, and NO - Lack of change in 2-NF levels > lack of atmospheric transformation
(Kameda <i>et al.</i> , 2004b) Osaka, Japan	PM extracts	Plate-incorporation assay	2 sites: high-traffic and suburban	1-NP, 2-NF, NO _x	YG1024 -/+	- Major sources: combustion sources - Automotive emissions major factor at both sites - Atmospheric transformation may not have played a major role
(Kawanaka <i>et al.</i> , 2004) Japan	12 fractions of different sizes of PM: PM _{11-0.11}	Plate-incorporation assay	One site: Saitama	PAHs, 2-NF	TA98, YG1024 -/+	- 88-100% of mutagenicity in fine particles and 5.1-5.9% in ultrafine particles - The highest contents of PAHs, 2-NF and mutagenicity per unit mass of PM in the fraction of <0.11 μm or 0.11-0.19 μm
(Oda <i>et al.</i> , 2004) Japan	PM extracts	Microsuspension assay	4 sites in Osaka		NM3009, NM2009, TA1535/pSK1002 -/+	- NM strains much more sensitive (up to 75-fold) than the parental strain. - Mutagenicity due to nitrated polycyclic aromatic hydrocarbons
(Pastorková <i>et al.</i> , 2004) Czech Republic	PM ₁₀ extracts	Plate-incorporation assay	4 urban cities: 1999–2003	12 PAHs	YG1041, TA98 -/+	- YG1041 > TA98: nitroarenes - Mutagenicity correlated with PAHs
(de Kok <i>et al.</i> , 2005) Maastricht, The Netherlands	TSP, PM ₁₀ , and PM _{2.5} extracts	Plate-incorporation assay	6 urban sites	16 PAHs, 6 cPAHs, metals, Oxygen-radical production	TA98 -/+	- No indicator correlated with traffic volume intensity so other source of pollution - PM _{2.5} had more total and carcinogenic PAHs - PM _{2.5} -associated PAHs only correlated with mutagenicity when +S9 conditions were used
(Du Four <i>et al.</i> , 2005) Flanders, Belgium	PM ₁₀ extracts, Semivolatiles	Plate-incorporation assay	7 sites in winter and in summer: 1 background, 6 industrial, 6 residential; 2 mobile source	16 PAHs, 10 mPAHs	TA98 -/+	- S9 increased mutagenicity - Highest mutagenicity values at industrial sites - Monitored PAHs account for only 3% of mutagenicity activity
(Erdinger <i>et al.</i> , 2005) Southwest Germany	PM extracts	Plate-incorporation assay	8 sites	NO, NO ₂ , SO ₂	TA100, TA98, TA98NR -/+	- No difference between -/+S9 - Higher mutagenicity values in congested areas probably due to nitroarenes - TA98 mutagenicity correlated with NO and NO ₂
(Seagrave <i>et al.</i> , 2005) Texas	PM extracts, Semivolatiles	Plate-incorporation assay	3 buses fueled by compressed natural gas		TA98, TA100 -/+	- Metabolic activation reduced mutagenicity in strain TA100, but not TA98. - Mutagenic potencies for the CNG emission samples were similar to the range observed in the gasoline and diesel emission samples
(ElAssouli <i>et al.</i> , 2007) Saudi Arabia	PM ₁₀ extracts	Plate-incorporation assay	11 urban sites in Jeddah	16 PAHs	TA98 -/+	- Polluted sites showed indirect mutagenic response
(Gilli <i>et al.</i> , 2007b) Turin	PM _{2.5} extracts	Plate-incorporation assay	2002-2004	PAHs	TA98, TA100 -/+	- Mutagenicity statistically correlated to PM _{2.5} and PAHs levels
(A. K. Sharma <i>et al.</i> , 2007) Copenhagen	PM _{2.5} extracts	Plate-incorporation assay	1–2 weeks: incineration energy plant, and from urban air in a heavy-traffic street and from	Inorganic elements and 8 PAHs	TA98, YG1041, YG5161	- PM _{2.5} concentration was about two-fold greater in the oven hall than in the receiving hall - Chemically, the samples from the oven hall were highly different from the other

			background air			samples. - PM extracts from the receiving hall, street and background air were more mutagenic than the PM extracts from the oven hall.
(Umbuzeiro <i>et al.</i> , 2008) Brazil	PM extracts (PAH, nitro- and oxy-PAH)	Plate-incorporation assay	One site Sao Paulo		TA98, YG104 -/+	- The highest mutagenic activity was found for the nitro-PAH fraction using YG1041 without metabolic activation
(Traversi <i>et al.</i> , 2009) Italy	PM _{2.5} extracts	Plate-incorporation assay	Daily monitored, during 2006: 3 cities		TA98, TA98NR, YG1021	- Greater mutagenic response of the YG1021 - Relevant role played by the nitro compounds in the mutagenic properties of the urban PM _{2.5}
(Piekarska, 2010) Poland	PM _{2.5} extract	Plate-incorporation assay	Summer and autumn: urban agglomeration	PAHs, Nitro-PAHs	TA98, TA100, YG1041, YG1042 -/+	- The highest PAH content and nitro-PAH content were found in the autumn season (heating season). - The highest mutagenicity of PM _{2.5} in autumn. - The high MRs were obtained -/+S9 > presence of promutagens and direct mutagens. - YG1041, the most effective in detection of mutagenicity of the suspended dust extracts (high sensitivity to nitro-aromatic compounds).
(Umbuzeiro <i>et al.</i> , 2010)	TSP extracts	Microsuspension assay, Microplate fluctuation protocol			TA98 -/+	- Both protocols produced similar results and similar sensitivities based on the lowest dose that resulted in a positive response were obtained - MPF procedure is a promising tool to test environmental samples for mutagenic activity
(André <i>et al.</i> , 2011) France	PM _{2.5} , Thermally desorbed PM _{2.5}	Plate-incorporation assay	Urbano-industrial site: Dunkerque		TA98, TA102, YG1041 -/+	- No significant effect on strain TA98, after exposure to PM or to dPM, -/+ S9 - Significant effect in YG1041 after exposure to PM at the highest dose (50 mg per plate), and ↓ +S9 - No significant effect on strain TA102
(ElAssouli, 2011) Makkah, Saudi Arabia	PM ₁₀ extracts	Plate-incorporation assay	Arafat and Muzdalifa	16 PAHs	TA98, TA100 -/+	- Samples were mutagenic with TA98
(Piekarska <i>et al.</i> , 2011) Poland	PM ₁₀ extracts	Plate-incorporation assay	Winter and summer: 2 locations of Wroclaw	PAHs, Nitro-PAHs	TA98, YG1041 -/+	- Evidence for occurrence of PAHs and their nitro-, amino- and hydroxylamino derivatives - Similar MR values -/+S9 - Moderately and highly polar classes of compounds are responsible for the mutagenic effect.
(Lemos <i>et al.</i> , 2012) Brazil	TSP and PM _{2.5} organic and aqueous extracts	Microsuspension assay	Urban and industrial areas		TA98, YG1021, YG1024 -/+	- No mutagenic effect of aqueous extracts - All organic extracts presented mutagens with a higher potential associated with PM _{2.5}
(Singla <i>et al.</i> , 2012) India	PM _{2.5} and PM ₁₀ extracts	Plate-incorporation assay	July- December 2010	PAHs	TA98	- PM and PAH concentrations were higher in the winter and were negatively correlated with temperature and wind speed - Presence of mutagens capable of causing base pair and frame shift mutagenicity
(Brito <i>et al.</i> , 2013)	PM _{2.5} extracts	Microsuspension assay	Urban and urban-industrial sites		TA98, YG1021, YG1024 -/+	- Presence of nitro-PAHs and hydroxylamines in PM _{2.5} was shown by positive mutagenic responses with YG1021 and YG1024
(de Rainho <i>et al.</i> , 2013) Brazil	PM _{2.5} extracts	Plate-incorporation assay	Urban Areas of Rio de Janeiro: 1 low traffic and 2 heavy traffic	6PAHs	TA98, YG1021, YG1024	- High mutagenic frameshift responses -/+ in site with heavy traffic - The participation of nitroarenes and dinitroarenes was detected in the total mutagenicity of the extracts
(Jariyasopit, 2013) Western North America	PM _{2.5} extracts	Plate-incorporation assay		PAH, NPAH and OPAH (nitro- and oxy-PAH)	TA98 -/+	- PAHs were associated with both local and regional emissions, while the NPAH and OPAH concentrations were only correlated with the NO concentrations, indicating that the NPAH and OPAH were primarily associated with local emissions - NO ₂ and NO ₃ /N ₂ O ₅ were effective oxidizing agents in transforming PAHs deposited on filters to NPAH - The direct-acting mutagenicity increased the most after NO ₃ /N ₂ O ₅ exposure, particularly for benzo[k]fluoranthene-d ₁₂ in which dinitro PAHs were observed.
(Belcik <i>et al.</i> , 2014) Poland	PM ₁₀ extracts	Plate-incorporation assay, Microsuspension assay	Winter and summer: Wroclaw	PAHs, Nitro-PAHs	A98, YG1041 -/+	- Highest content of total PAH and nitro-PAH content were found in winter extracts. - Higher MR values were observed -S9 - The YG1041 strain is the most sensitive > large amounts of nitro-aromatic compounds.

(Lepers <i>et al.</i> , 2014) France	PM _{2.5}	Plate-incorporation assay	spring–summer or autumn–winter: industrial, urban or rural area	PAHs, ions, metals	YG1041, TA98, TA102 -/+	- Slight mutagenic effect of PM samples on TA102 tester strain, without variation depending on PM origin and season, nor upon +/-S9 - PAHs are not responsible of mutagenicity increase in TA98 tester strain. - Very high inductions factors with YG1041
(Rainho <i>et al.</i> , 2014) Rio de Janeiro	10-50 µg/ plate PM _{2.5} organic extracts	Plate-incorporation assay	August and October 2010 3 sites: 1 with light traffic and 2 with heavy traffic		TA98NR, TA98/1,8-DNP6, YG1021, YG1024 -/+	- The YG1021 and YG1024 strains showed the highest rev/m3 values, confirming their enhanced sensitivity. - YG1024 also demonstrated sensitivity to nitro and amino compounds
(Coulibaly <i>et al.</i> , 2015) Japan	TSP extract	Plate-incorporation assay	June 2012- May 2013: Yurihama	Inorganic composition and PAHs	YG1024 -/+	- High levels of PAHs were detected in highly mutagenic TSP collected from January to March - Ratios of fluoranthene to fluoranthene and pyrene > the main source of PAHs in TSP collected in winter and spring was coal and biomass combustion - High levels of mutagens were transported from mainland East Asia to western Japan.
(Dubey <i>et al.</i> , 2015) India	PM _{2.5}	Plate-incorporation assay	May 2010- December 2012: semi-urban site	16 PAHs	TA98, TA100 -/+	- 3-ring and 4-ring compounds were dominant presence of both base pair and frame shift mutagens - Enhanced mutagenic response was observed +S9
(Traversi, 2015)	PM _{2.5} organic extracts	Plate-incorporation assay	Urban site		TA98 -/+	- Lower Mutagenicity with S9
(X. Li <i>et al.</i> , 2016)	PM samples ranging from 10 nm to 10 µm	Plate-incorporation assay	Idestream cigarette smoke		TA98 -/+	- Mutagenicity of PM larger than 1 µm was significantly lower than PM sized 10 nm-1 µm
(Alves <i>et al.</i> , 2016) Brazil	TSP	Microsuspension assay	6 samples Limeira, a small urban center influenced by heavy traffic and sugar cane biomass burning		TA98, YG1041,TA1538 -/+	- YG1041 was the most sensitive strain - Potency for TA1538 was higher than TA98 - The increased response to YG1041 relative to TA98, and the decreased response with S9, suggests that nitroaromatics are the major contributors - Increase in the formation of nitro-PAHs may result from longer contact time between the aromatic compounds and high NOx and ozone concentration

4.2.2 *PM_{2.5-0.3} genotoxicity by the SOS chromotest*

4.2.2.1 Results

The SOS chromotest was assayed to study the genotoxicity of fine PM under industrial and rural influence (FP-I and FP-R), by testing their ability to induce an SOS response using *Escherichia coli* PQ37. *E. coli* PQ37 was exposed to different concentrations of PM_{2.5} samples ranging from 2000 µg/mL till 0.122 µg/mL to assay their genotoxicity with and without metabolic activation.

Compounds are considered non genotoxic if the IF remains < 1.5, as marginally genotoxic if the IF ranges between 1.5 and 2, and as genotoxic if the IF > 2 (Kevekordes *et al.*, 1999). The samples were tested in triplicates.

- FP-I with and without metabolic activation:

PM_{2.5-0.3} under industrial influence (FP-I) genotoxic activities were studied using the SOS chromotest with and without metabolic activation at concentrations ranging from 1.9 to 2000 µg/mL. Without activation, FP-I sample was genotoxic starting 1.9 µg/mL with an IF between 1.6 and 4.3. Similar results were obtained when FP-I samples were tested with metabolic activation, FP-I were highly genotoxic starting 1.9 µg/mL with an IF between 1.6 and 4.9 (Appendix 24). β-galactosidase activity (β-gal) (Appendix 25A) and induction factor were dose dependant (Figure 4.7). Phosphatase alkaline (PAL) activity was almost stable at different concentrations of FP-I tested (with a value around 11), which indicates stable enzyme activity (Appendix 25B). A slight increase in the genotoxic activity of FP-I was shown after metabolic activation (Figure 4.7).

- FP-R with and without metabolic activation:

E. coli PQ37 was exposed to different concentrations of rural PM to assay their genotoxicity with and without activation. The different concentrations with two-fold dilutions between 0 µg/mL and 2000 µg/mL did not show genotoxicity (IF < 1.5). Therefore, this sample was not genotoxic with or without metabolic activation, at conditions tested (Appendix 26). β-galactosidase activity (Appendix 27A) and induction factor increased with increasing concentration of FP-R sample between 0 and 2000 µg/mL (Figure 4.7). Phosphatase alkaline activity is almost stable at different concentrations of FP-R (with a value around 12), which indicates stable enzyme activity (Appendix 27B). IF values of FP-R samples did not vary with metabolic activation (Figure 4.7).

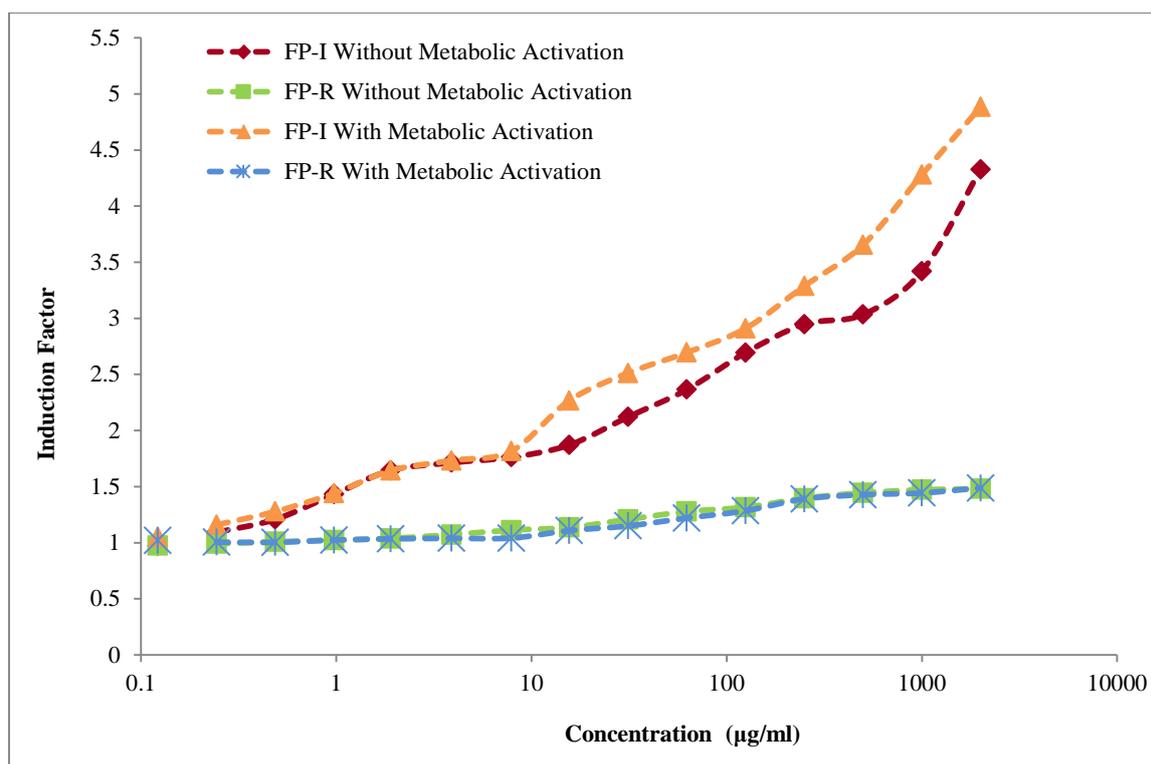


Figure 4.7: Genotoxicity of PM_{2.5-0.3} under industrial and rural influence, using the SOS chromotest

4.2.2.2 Discussion

Genotoxicity corresponds to harmful effect on genetic material, but it is not limited to the mutagenic effect, as detected in the Ames assay. It may lead to DNA damage even if there is no immediate evidence for mutation formation. Thus, using the SOS chromotest can highlight other genetic effects induced by organic air pollutants.

The genotoxicity of FP-I and FP-R was studied by testing the ability of these particles to induce an SOS response using *Escherichia coli* PQ37. SOS chromotest is based on the activation of DNA repair process. Activation of the process indicates that DNA has been modified (Piekarska *et al.*, 2011). β -galactosidase activity and induction factor were dose dependant. Phosphatase alkaline activity which is related to general protein synthesis bacteria was almost stable at different concentrations of FP-I and FP-R. Even without activation, FP-I particles sample were genotoxic starting 1.9 $\mu\text{g}/\text{mL}$ with an IF value between 1.6 and 4.3. Slightly higher results were obtained when FP-I samples were tested with metabolic activation: FP-I were genotoxic starting 1.9 $\mu\text{g}/\text{mL}$ with an IF between 1.6 and 4.9. On the other hand, FP-R was not genotoxic with and without metabolic activation, at the conditions tested till a concentration of 2000 $\mu\text{g}/\text{mL}$ (Figure 4.7). Total mass of PAHs were 13 $\mu\text{g}/\text{g}$ and 9 $\mu\text{g}/\text{g}$ for FP-I and FP-R respectively (*more details in Table 3.12*). Although CPAH/TPAH ratio are similar, we found that concentrations of several PAHs as chrysene, benzo[b]fluoranthene, indeno [1,2,3-c,d] pyrene, dibenz[a,h]anthracene are higher in FP-I than in FP-R. This difference could partly account for explaining the higher response of SOS chromotest for

bacteria exposed to FP-I, even if the contribution of other components of PM, such as PAHs derivatives, has to be also put forward.

Many studies proved that the SOS chromotest is very sensitive to a large spectrum of genotoxic compounds, which includes known organic air pollutants such as PAHs and various PAH-derivatives (Mersch-Sundermann *et al.*, 1992). The obtained results for the FP-I are comparable with the Ames test. Even if there are mechanistic differences between the SOS chromotest and the Ames test, and the assays respond to different stimuli, for most of the tested compounds a good quantitative correlation has already been observed (Rosenkranz *et al.*, 1999). The concordance between the two tests was about 60-100% based on a group of tested compounds. Some authors showed that the SOS chromotest is more sensitive to direct-acting genotoxic compounds (without metabolic activation) than the indirect-acting ones (McDaniels *et al.*, 1990).

For the evaluation of the impact of air pollution samples, SOS chromotest was used in a limited number of studies on PM extracts (Courtois *et al.*, 1988; Kubátová *et al.*, 2004; Malachová, 1998; Nylund *et al.*, 1992; White *et al.*, 1995), but not on native PM. Kubátová *et al.* (2004) showed that fractions of different polarity PM exhibited difference in genotoxicity, suggesting the different constituents in those PM samples were responsible for genotoxicity. This study confirmed the toxicological importance of typically neglected polar PM fractions. Skarek *et al.* (2007) used the SOS chromotest in the examination of TSP and PM_{2.5} organic extracts showing that a significant portion of the direct genotoxic agents was bound to particles larger than 2.5 µm. Whereas, the indirect genotoxic activity was bound predominantly to PM_{2.5}. While the direct genotoxicity may be related to the presence of PAH-derivatives (nitro-, oxy-, and hydroxy-derivatives) as well as some polar organic pollutants, the pro-genotoxicity is related to the detected carcinogenic PAHs (Brits *et al.*, 2004; Claxton *et al.*, 2004; Piekarska *et al.*, 2011; Skarek *et al.*, 2007). Studies done on PM₁₀ and PM_{2.5} organic extracts showed a high degree of similarity between results obtained with SOS chromotest and those of the *Salmonella* assay. Piekarska *et al.* (2011) showed that higher genotoxicity was found in tests carried out without metabolic activation, in relation with a higher toxicity of nitro-, amino- and oxy-PAHs derivatives compared to non-substituted PAHs. In this work, the genotoxicity of FP-I was a bit higher after metabolic activation. Contrary to the study previously discussed, the obtained results indicate a more important prevalence of pro-genotoxic compounds that require metabolic activation than direct ones. This difference could be related to the composition and the nature of the tested PM samples. Therefore, testing native PM or PM extracts could have an effect on the obtained results as the organic compounds are present in a more concentrated and accessible status. In this study, the genotoxic effect of PAHs may be expressed more than various PAH-derivatives.

4.2.3 PM_{2.5-0.3} cytotoxicity

After revealing the mutagenic potential and the bacterial genotoxic effect of PM_{2.5-0.3}, different genotoxic and epigenetic parameters were assessed on human bronchial cells BEAS-2B. The

first step of the quest is the preliminary evaluation of the $PM_{2.5-0.3}$ cytotoxicity in order to determine relevant exposure concentrations.

5-bromo-2-deoxyuridine incorporation (BrdU) and mitochondrial dehydrogenase (MDH) activity (normalized relative to the control, % of proliferation) in BEAS-2B cells and released lactate dehydrogenase (LDH) activity (normalized relative to the control, % of cytotoxicity) were assayed in cell-free culture supernatants of BEAS-2B cells at 24, 48 or 72h after their incubation with increasing concentrations of $PM_{2.5-0.3}$ (3.75, 7.5, 15, 30, 60, 120, 180 and 240 $\mu\text{g}/\text{cm}^2$) as shown in Figure 4.8. Non-exposed cells were used as negative controls. These values are depicted as mean values and standard deviations of 16 replicas for negative controls and 8 replicas for every PM concentration. The experimental results were modeled by logistic regression with 4-parameter Hill model, typically used to assess the dose-response relationships. In this study, IC_{10} values were calculated by concentration-response curve fitting using Hill equation model (Khinkis *et al.*, 2003). Results were expressed by concentration generating 10% of cytotoxicity or 10 % of inhibition of proliferation and their 95% confidence interval. Concentrations between 0 and 120 $\mu\text{g}/\text{cm}^2$ were assayed. Statistical analysis was performed to assess significant effects of $PM_{2.5-0.3}$ using the Mann-Whitney test (* $p < 0.05$).

There was no significant increase in LDH activity following 24h, 48h or 72h exposure to FP-I and FP-R. Thus, FP-I and FP-R toxicity does not result from a direct disruption of cellular membranes; BEAS-2B cells seem to be able to maintain the integrity of their plasma membrane until they reach the late phase of the death pathway. It has been previously demonstrated that the LDH assay had significantly less sensitivity compared to the WST-1 reduction test (MDH activity) (Pohjala *et al.*, 2007). According to this work, industrial $PM_{2.5-0.3}$ affects cell metabolism as shown by a decrease in MDH activity after 24h and 48h exposure to FP-I, IC_{10} : 117.11 $\mu\text{g}/\text{cm}^2$ (95% CI: 78.16-120.00) and 25.40 $\mu\text{g}/\text{cm}^2$ (95% CI: 1.83-67.63) respectively. No significant increases in MDH activity were observed following 24h, 48h or 72h exposure to FP-R. Concentration dependent decreases in the incorporation of BrdU (proliferation) in FP-I-exposed cells suggest the alteration of cellular division after 24 and 48h exposure, IC_{10} : 83.22 $\mu\text{g}/\text{cm}^2$ (95% CI: 9.41-181.69) and 86.91 $\mu\text{g}/\text{cm}^2$ (CI: 40.61-120) respectively. On the other hand, a slight but significant decrease in BrdU incorporation was observed in BEAS-2B cells exposed at 72h to FP-R (i.e., IC_{10} : 66.98 $\mu\text{g}/\text{cm}^2$ (95% CI: 39.24-95.83)), as compared to negative controls.

Furthermore, in order to determine relevant exposition concentrations, we referred to other studies done on BEAS-2B (LC_{10} : 12 and 28 $\mu\text{g}/\text{cm}^2$ in summer and winter respectively) (Borgie, 2013; Lepers, 2013) and other lung cell lines, A549 (LC_{10} : 6.33 $\mu\text{g}/\text{cm}^2$) (Billet *et al.*, 2007a) and L132 (LC_{10} : 5.04 $\mu\text{g}/\text{cm}^2$) (Garçon *et al.*, 2006). Subsequently, for the rest of the study, BEAS-2B cells were exposed for 24, 48 and 72h to industrial and rural influenced $PM_{2.5-0.3}$ at two concentrations that were not lethal, but having an effect on the BEAS-2B cells (3.75 $\mu\text{g}/\text{cm}^2$ and 15 $\mu\text{g}/\text{cm}^2$) in order to study the underlying mechanisms involved in their pulmonary toxicity.

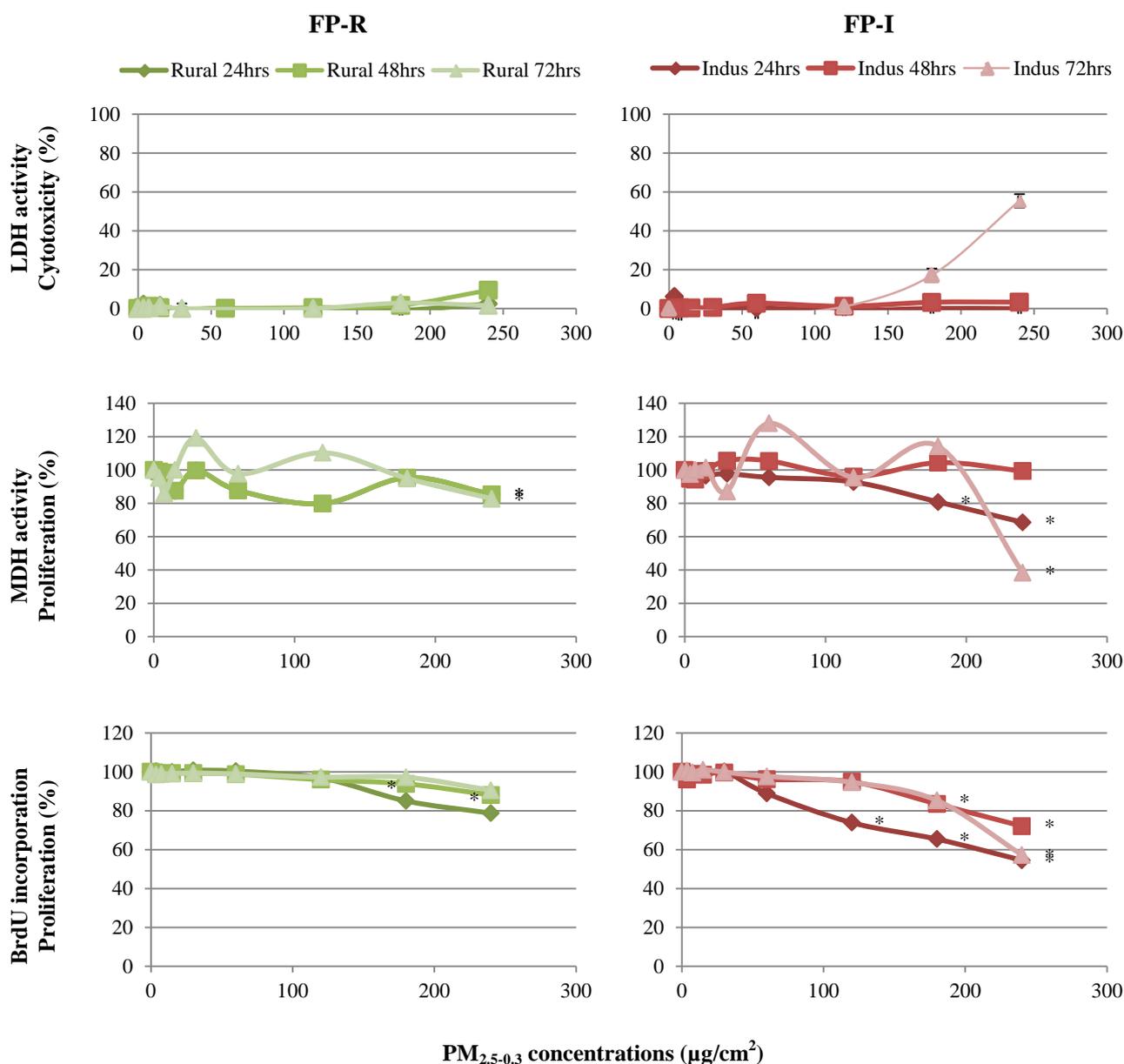


Figure 4.8: 5-bromo-2-deoxyuridine incorporation (BrdU) and mitochondrial dehydrogenase (MDH) activity (% of proliferation) in BEAS-2B cells, and released lactate dehydrogenase (LDH) activity (% of cytotoxicity) in cell-free culture supernatants of BEAS-2B cells 24, 48 or 72 h after their incubation in the continuous presence of increasing concentrations of particulate matter (PM_{2.5-0.3}: 3.75, 7.5, 15, 30, 60, 120, 180 and 240 µg/cm²)

Non-exposed cells were used as negative controls. These values are depicted as mean values and standard deviations of 16 replicates for negative controls and 8 replicates for every PM concentrations. The 4-parameter Hill model was used to calculate IC₁₀ values. Key to statistical significance: * p < 0.05 versus untreated cells (Mann–Whitney U test).

4.2.4 PM_{2.5-0.3}-induced gene expression of xenobiotic-metabolizing enzyme, CYP1A1

One of the most important determinants of xenobiotic persistence in the body and subsequent toxicity to the organism is the extent to which they can be metabolized and excreted. The

bioactivation of pro-toxicants is the biological process through which some chemicals are metabolized into reactive metabolites.

The physicochemical analysis revealed the presence of high concentrations of organic compounds such as PAHs (see Figure 3.15), PCDDs, PCDFs, PCBs (Table 3.14) and paraffins (see Figure 3.16) adsorbed on the $PM_{2.5-0.3}$ surface particularly in the industrial PM. Many of these compounds induce the expression of metabolic enzymes of phase I and phase II such as CYP450. CYP1A1 metabolizes a number of pro-carcinogens, such as benzo[*a*]pyrene (B[*a*]P), to intermediates such as B[*a*]P-7,8-diol-epoxide (BPDE) that can react with DNA and lead to damage by DNA-adduct formation or by oxidative stress. This phenomenon is called metabolic activation.

The *CYP1A1* gene expression was analyzed by RT-PCR in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. Non-exposed cells were used as negative controls and B[*a*]P (1 μM)-exposed cells as positive control.

The relative quantitation (RQ) of target mRNA, which corresponds to the differences in target mRNA levels in exposed cells (i.e., $PM_{2.5-0.3}$ -exposed cells, or B[*a*]P-exposed cells) *versus* in non-exposed cells, corrected by the level of endogenous ribosomal 18S RNA, used as the reference gene, was calculated using the following equation: $RQ = 2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_{t(\text{target gene})} - C_{t(18S \text{ RNA})}$, and $\Delta(\Delta C_t) = \Delta C_{t(\text{exposed cells})} - \Delta C_{t(\text{non-exposed cells})}$. The results are expressed as median form [Q1, Q3] of four biological replicates. The results of mRNA expression of *CYP1A1*, relative to controls, are shown in Figure 4.9. Genes with $RQ > 2$ are considered as overexpressed and with a $RQ < 0.5$ as repressed (Livak and Schmittgen, 2001).

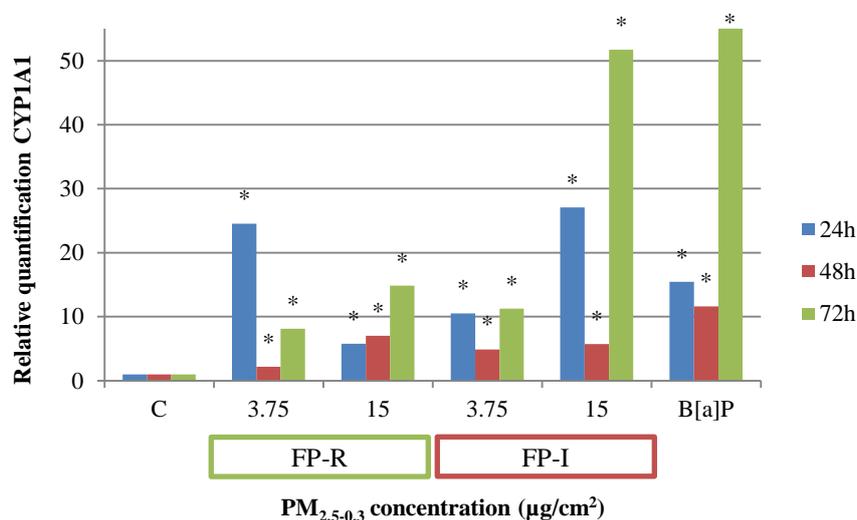


Figure 4.9: mRNA expressions of *CYP1A1*, xenobiotic-metabolizing enzyme, in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$

Key to statistical significance: *: $RQ > 2$, gene overexpressed and **: $RQ < 0.5$, gene repressed.

As shown in Figure 4.9, a significant induction of PAH-metabolizing enzyme, the *CYP1A1* was noted in BEAS-2B cells after 24, 48 and 72 h of exposure to FP-I and FP-R versus non-exposed cells. Whatever the exposure time, industrial influenced $PM_{2.5-0.3}$ induced higher *CYP1A1* mRNA

expression than the rural influenced PM at the same concentration except the exposition to a PM concentration of $3.75 \mu\text{g}/\text{m}^3$ for 24h. Both PM samples induced mRNA expression in a site- and dose-dependent manner and higher expression was noticed at the highest PM concentration. Moreover, following BEAS-2B cells exposure to PAHs positive control (B[a]P, $1 \mu\text{M}$), a significant induction of *CYP1A1* gene expression was observed after 24, 48 and 72 h of exposure.

The amount of mRNA measured in FP-I exposed cells are 5 times ($3.15 \mu\text{g}/\text{m}^3$, 48h) to 52 times ($15 \mu\text{g}/\text{m}^3$, 72h) higher than in the non-exposed cells. Whereas the amount of mRNA measured in FP-R exposed cells are 2 times ($3.15 \mu\text{g}/\text{m}^3$, 48h) to 15 times ($15 \mu\text{g}/\text{m}^3$, 72h) higher than in the non-exposed cells. When monitoring the kinetic response of BEAS-2B to $\text{PM}_{2.5-0.3}$ and B[a]P for *CYP1A1* to control the time scale of gene expression, lower induction was found at 48 hours than in 24 hours and it peaks at 72h. Only after exposition to $15 \mu\text{g}/\text{m}^3$ of FP-R, higher induction was found at 48 hours than in 24 hours.

Our results agree with those of Lepers, Borgie and Gualtieri *et al.*, who showed significant inductions of *CYP1A1* gene expression after exposure of BEAS-2B cells at different concentrations of $\text{PM}_{2.5}$ (Lepers: 3.75 and $15 \mu\text{g}/\text{cm}^2$ of $\text{PM}_{2.5-0.3}$ collected in France, Borgie: 3 and 12 of $\text{PM}_{2.5-0.3}$ collected in Beirut, Lebanon, Gualtieri: 0.5, 2.5 and $5 \mu\text{g}/\text{cm}^2$ of $\text{PM}_{2.5}$ collected in Italy) (Borgie, 2013; Gualtieri *et al.*, 2011; Lepers, 2013). These results are in agreement with the chemical analysis of FP-I and FP-R which showed higher concentrations of organic compounds especially PAHs in FP-I than in FP-R. Different studies showed that PAH-coated onto PM induced significant increases in *CYP1A1* (Billet *et al.*, 2007b, 2008; Gualtieri *et al.*, 2012). In addition, the biotransformation of PCBs is carried out by CYP (1A1, 1A2, 2B1, 2B2, 3A) and leads to the formation of epoxides which can react with nucleic acids or proteins and form adducts (Pereg *et al.*, 2002) and PCBs concentrations were much higher at the industrial influenced site.

Studies of inducible genes have shown that the period required to attain new levels of individual mRNAs and proteins is related to their unique half-lives. The basis for this is a physical principle that predicts rates of accumulation of particles in compartmental systems. The minimum period required to achieve a new level is directly proportional to product half-lives because rates of decay control the ratio between the rate of synthesis and the concentration of gene products at steady state. This kinetic model suggests that sensitivity of gene products to degradation by ribonucleases and proteinases is an important determinant of the time scale of gene expression (Hargrove *et al.*, 1991). Note that these organic compounds are ligands for the AhR cellular receptor; their attachment to the receptor induces the expression of genes such as *CYP1A1*, *CYP1B1* and *AhRR*, which explains the increased induction of gene expression in the presence of the particules. *AhRR* overexpression are usually induced by AhR ligands (Hung *et al.*, 2013) and AhRR-dependent repression of AhR signaling suggest that AhRR is implicated in a negative feedback loop (Evans *et al.*, 2008), thus inhibiting AhR-regulated genes, notably *AhRR*, *CYP1A1*, *CYP1B1* and *NQO1* in BEAS-2B cells (Brauze *et al.*, 2006; Iba and Caccavale, 2013). For example, Rivera *et al.* showed that the regulation

was CYP2S1 involve answers to hypoxia contained in XRE promoter (Rivera *et al.*, 2007). In addition, an autoregulatory loop leads to the fine tuning of the *CYP1A1* gene expression through the down-regulation of nuclear factor I (NFI) activity by CYP1A1-based H₂O₂ production (Morel *et al.*, 1999). This mechanism allows a limitation of the potentially toxic CYP1A1 activity within the cell. This could explain why *CYP1A1* gene expression declined at 48 hours of exposure to our collected PM after an expression increase at 24h and re-increase at 72 hours.

Thus, PAHs-adsorbed PM_{2.5-0.3} induced AhR-regulated genes expression lead to the formation of PAHs-active metabolites which are partly responsible for PM_{2.5-0.3}-induced genotoxicity (Oya *et al.*, 2011). Over-expression of *CYP1A1* could promote the production of reactive intermediates, such as quinones and BPDE which can lead to DNA damage such as DNA adducts and oxidative damage (Castell *et al.*, 2005; Nebert and Dalton, 2006; Shimada, 2006).

4.2.5 Effects of PM_{2.5-0.3} on DNA adduct formation

After showing the induction of CYP1A1, it was legitimate to test the consequences of this activation on the formation of bulky DNA adducts. Formation of DNA adducts were measured by ³²P post-labeling in BEAS-2B cells exposed for 72h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 and 15 µg/cm².

In these tested conditions, no bulky adducts were present on the autoradiograms of TLC sheets. It was quite surprising, knowing the relative high doses of PAH-coated onto air pollution PM_{2.5-0.3}, notably on industrial ones. Several co-contaminants of PAHs, like PCDD/F, DLPCB, and PCB are often detected on PM. While these chemicals are powerful AhR agonists and *CYP1A1* gene expression inducers, they do not form bulky-DNA adducts (Wu *et al.*, 2008). Wu *et al.* findings showed that TCDD treatment causes strain-specific alterations in gene expression and decrease in B[a]P-induced DNA adduct formation in the liver of female mice of the same *AhR^{b2}* genotype. Furthermore, it suggests that TCDD-treated female mice of the BALB strain may have genes whose products modify the toxicity of B[a]P as evidenced by TCDD-induced alterations in B[a]P-DNA adduct formation. Another study done by Shiizaki *et al.* showed that dioxin suppresses benzo[a]pyrene-induced mutations and DNA adduct formation through cytochrome P450 1A1 induction and (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide inactivation in human hepatoma cells (Shiizaki *et al.*, 2013). In agreement with results reported by Wu *et al.* and Shiizaki *et al.*, the high levels of PAH together with the presence of other related compounds (PCDD, PCDF, and PCB)-coated onto FP-I and FP-R could contribute to explain the absence of PAH–DNA adducts in PM-exposed BEAS-2B cells.

In conclusion, the high doses of PAH-coated onto air pollution PM_{2.5-0.3} were able to significantly induce gene expression of *CYP1A1*, but, in the experimental conditions chosen in this work, did not generate PAH–DNA adducts detectable in BEAS-2B cells after a 72 h of exposure.

4.2.6 $PM_{2.5-0.3}$ -induced H2AX phosphorylation

Double strand breaks can be caused by a variety of factors: direct interaction with an agent, ROS, DNA adducts of PAHs, deficient repair, telomere erosion and programmed biological processes (Bonner *et al.*, 2008). Histone H2AX phosphorylation in response to DNA damage on serine 139 within its SQEY motif at the site of DSBs allows the DNA damage signal to spread along the chromatin covering large regions of the chromosome surrounding each DSB lesion, thus providing a platform for recruitment of other proteins that participate in the DNA damage response (DDR) (Kinner *et al.*, 2008; Mariotti *et al.*, 2013). γ H2AX levels were measured by flow cytometry and in-cell western in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$.

By flow cytometry, γ H2AX was quantified as the differences in Median Fluorescence Intensity (ΔMFI) between antibody-labeled cells and isotype control-labeled cells for each sample in each phase of the cell cycle ($\Delta\text{MFI}_{\text{sample}} = \text{MFI}_{\gamma\text{H2AX}} - \text{MFI}_{\text{iso}}$). The fluorescence is normalized compared to the amount of DNA in each cell. Results are expressed as median [Q1;Q3] of 4 biological replicates. Finally, for each type of exposure, we reported the results of γ H2AX to the control, corresponding to a relative normalization to the unexposed cells ($\Delta\Delta\text{MFI} = \Delta\text{MFI}_{\text{exposed}} / \Delta\text{MFI}_{\text{unexposed}}$). An evaluation of H2AX phosphorylation depending on G0/G1, S or G2/M cell cycle's phase was done and $\Delta\Delta\text{MFI}$ is shown in Figure 4.10. Our results showed an increase of γ H2AX in cells exposed to industrial and rural influenced $PM_{2.5-0.3}$. In addition, we noticed higher γ H2AX content in cells exposed to FP-I than to FP-R at the same concentrations. γ H2AX increase was stable during different cell cycle phases but a bit stronger in cells treated with PM in G2/M, compared to G0/G1 and S cells. A positive dose- response relationship was observed for the PM samples and the positive control (H_2O_2).

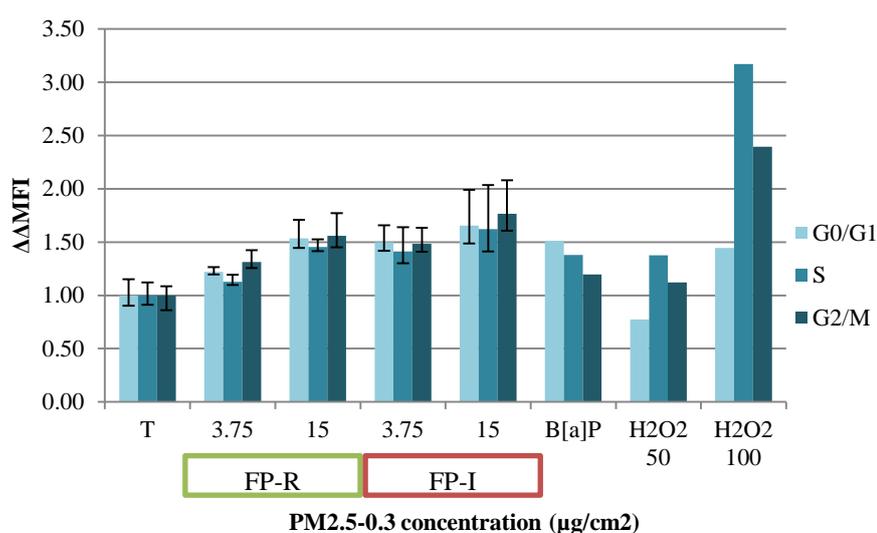


Figure 4.10: Phosphorylated H2AX (γ H2AX) levels measured by flow cytometry in BEAS-2B cells treated for 72 h with industrial and rural influenced $PM_{2.5-0.3}$

By in-cell western, raw absorbance data median was calculated from 16 replicas for every sample, corrected for background; the relative fluorescence units from the scanning enabled quantitative analysis. For the determination of genotoxicity, relative fluorescent units for γ H2AX per cell were divided by the respective controls to determine the change in phosphorylation of H2AX levels compared with control. Statistical analysis was performed to assess significant effects of PM_{2.5-0.3} using Kruskal-Wallis ($p < 0.05$) and Mann-Whitney tests ($*p < 0.05$). Kruskal-Wallis test was used to compare different samples and Mann-Whitney test was used to compare the samples to the control sample. Results were shown in Figure 4.11. This test showed an increase in γ H2AX levels in BEAS-2B exposed to both FP-I and FP-R but this increase was only significant after the exposition to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. In addition, this increase was dose-dependent for both samples. A significant increase of γ H2AX level was observed for the positive control (B[a]P). Kruskal-Wallis test showed that there was a significant difference between the two tested concentrations of FP-I (3.75 and 15 $\mu\text{g}/\text{cm}^2$) ($p = 0.046$) and no significant difference between the two tested concentrations of FP-R (3.75 and 15 $\mu\text{g}/\text{cm}^2$) ($p = 0.380$).

The results we obtained are consistent with others studies which showed, following the appearance of DNA strand breaks, the phosphorylation of H2AX in BEAS-2B cells exposed to PM_{2.5} collected in Milan (Italy) (Gualtieri *et al.*, 2011) and in A549 cells exposed to PM₁₀ (Sánchez-Pérez *et al.*, 2009).

This increase suggest that PAHs, or even their derivatives such as nitro-PAHs, could be involved in DNA double-strand breaks. Indeed, several studies demonstrated the ability of B[a]P and other PAHs to induce H2AX phosphorylation (Audebert *et al.*, 2012; Toyooka *et al.*, 2008). Longhin *et al.* showed that DNA double-strand breaks (DSBs), assessed by measuring the levels of γ H2AX, were increased in cells exposed for 3 hours to PM_{2.5} and organic extract showing the correlation between the organic composition and DSBs (Longhin *et al.*, 2013). In *in vitro* studies, increased levels of γ H2AX was observed after exposure of cells to DNA-damaging compounds such as heavy metals (Bailey *et al.*, 2015; DeLoughery *et al.*, 2015; Peterson-Roth *et al.*, 2005), the dinitrobenzo[e]pyrene (Kawanishi *et al.*, 2009), PAHs and nitro-PAHs (Mattsson *et al.*, 2009; Oya *et al.*, 2011). Lepers *et al.* and Borgie *et al.* showed higher γ H2AX levels observed following cells exposure to PM samples suggesting that PAHs, or even their derivatives such as nitro-PAHs, could be involved in DNA double-strand breaks (Borgie *et al.*, 2015; Lepers *et al.*, 2014). However, the complex mixture of chemical elements present at varying doses in PM, makes difficult to attribute a toxicity to a precise fraction rather another one. It has been described that interactions exist in such mixture of compounds, particularly between inorganic and organic fractions, which can lead to synergistic effects (Garçon *et al.*, 2003; Vakharia *et al.*, 2001).

γ H2AX was also found in pre-cancerous lesions in humans (Gorgoulis *et al.*, 2005) and in different types of tumours such as cervical cancer and melanoma (Banáth *et al.*, 2004; Sedelnikova and Bonner, 2006; Warters *et al.*, 2005). Therefore if this type of DNA damage is not repaired, it may

lead to the initiation of genomic instability and the development of cancer (Jeggo and Löbrich, 2007; McKinnon and Caldecott, 2007). To continue our study, we were interested in testing the effect of the industrial and rural influenced $PM_{2.5-0.3}$ on the DNA repair.

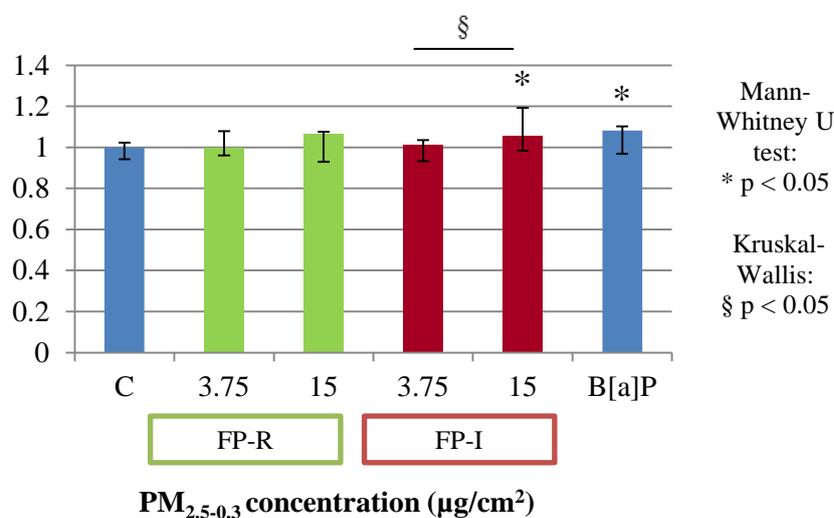


Figure 4.11: γ H2AX levels measured by in-cell western in BEAS-2B cells treated for 72 h with industrial and rural influenced $PM_{2.5-0.3}$

Key to statistical significance: * $p < 0.05$ versus untreated cells (Mann–Whitney U test) and § $p < 0.05$ to compare different samples (Kruskal-Wallis test).

4.2.7 $PM_{2.5-0.3}$ and DNA repair

4.2.7.1 Bulky DNA adducts repair

PAHs can be converted to electrophilic metabolites capable of forming bulky DNA adducts, which can cause mutations which distort the double helix structure. This type of damage is repaired by the nucleotide excision repair (NER) and XPA is a protein involved in this repair mechanism. Gene expression profiles of XPA involved in the NER was measured by RT-PCR in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. The results of mRNA expressions of XPA, relative to controls, are shown in Figure 4.12.

A significant induction of XPA was noted only in BEAS-2B cells after 72h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I and 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R versus non-exposed cells. In addition, significant suppression of XPA was noted in BEAS-2B after 24 h and 48 h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and to 15 $\mu\text{g}/\text{cm}^2$ or FP-I. Moreover, following BEAS-2B cells exposure to B[a]P (1 μM), a significant induction of XPA was observed after 72 h of exposure and a significant repression at 48h.

The expression of XPA was not stable before 72 hours of exposure to PM. It seems that there is a malfunction of this type of repair before 72 hours and this could be related to the ability of metals to interact with zinc ions or magnesium content in certain proteins (Mateuca *et al.*, 2006). Recent studies showed that transition metals (nickel and chromium) and oxidative stress induce lipid

peroxidation (LPO) metabolites such as malondialdehyde (MDA) and trans-4-hydroxy-2-nonenal (4-HNE) and this can inhibit NER and enhance carcinogen-induced mutations (Feng *et al.*, 2006). In addition, our results were consistent with the study done by Mehta *et al.* which showed that in A549 cells exposed to PM_{2.5} for 24 h, the NER repair capacity decreased (Mehta *et al.*, 2008). Rossner *et al.* tested the response of normal human embryonic lung fibroblasts (HEL12469 cells) to treatment with B[a]P and extractable organic matter (EOM) from PM_{2.5} collected in two Czech cities differing in levels and sources of air pollution, by analyzing the levels of bulky DNA adducts and the NER response (expression of *XPE*, *XPC* and *XPA* genes on the level of mRNA and proteins, unscheduled DNA synthesis, UDS). This study indicates that the tested compounds induced low levels of DNA damage and affected the expression of NER genes; however, nucleotide excision repair was not induced (Rossner *et al.*, 2013).

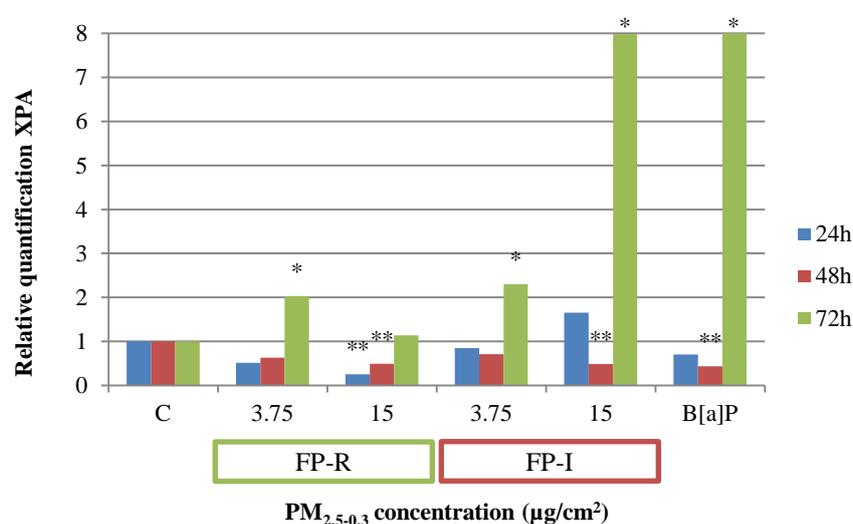


Figure 4.12: mRNA expressions of XPA, involved in DNA adducts repair, in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 µg/cm²

Key to statistical significance: *: RQ > 2, gene overexpressed and **: RQ < 0.5, gene repressed.

We noticed a significant increase of *XPA* expression at 72 hours of exposure to 3.75 and 15 µg/cm² of FP-I and this increase was proportional to the exposed dose. This showed that the industrial influenced particles increased the expression *XPA* gene only after 72 hours of exposure leading to activation of the NER mechanism. Therefore, this increase is caused by an increase in the DNA damage, bulky DNA adducts possibly caused by the high concentration of PAHs and other organic compounds, such as nitro-PAHs (Châtel *et al.*, 2014), in the industrial influenced particles compared to the background rural particles. These results showing no activation of bulky DNA adducts repair at 24 and 48 h of exposure, but only a late activation at 72 h, could be related to delay between the activation of PAHs, the formation of DNA adducts, their detection by the NER system, and the induction of *XPA* expression. These results could correspond to the reparation of DNA

modifications. This could explain why we could not observe any PAH DNA adducts after 72 h of exposure. It could be of interest to measure PAH DNA adducts at shorter times (e.g. 48h).

4.2.7.2 Cellular oxidative stress repair

If cellular antioxidant defence systems are overwhelmed, one consequence of oxidative stress is the oxidation of DNA. As mentioned in chapter 1, different studies showed that the exposure of the lung cells to PM_{2.5} led to the production of ROS leading to oxidative stress that may play an important role in particle toxicity and carcinogenicity. The relationship between oxidative stress and PM is characterized by ROS production, induction of 8-OHdG, DNA strand breaks, MDA, CAT, SOD and decrease of the cellular level of GSH. Different factors can be involved in the ROS formation such as the PM size, the transition metals, the organic compounds, the altered mitochondrial function, or the activation of inflammatory cells capable of producing ROS. Therefore oxidative stress may lead to DNA damage and the repair of such lesion is essential to the integrity of the genome and is ensured by the base excision repair system (BER). Studies demonstrated alterations in the BER system, an excess of iron or zinc in nervous tissue could disrupt the BER, by altering the activity of APE1 or FEN1 and DNA polymerase β , and the ligation step (Li *et al.*, 2009).

Gene expression profiles of different enzymes involved in the BER (*OGG1*, *NTH1*, *APE1*, and *NUDT1*) was measured by RT-PCR in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. Non-exposed cells were used as negative controls and B[a]P (1 μM)-exposed cells as positive control. The results of mRNA expressions of *OGG1* (DNA glycosylase), *NTH1* (DNA glycosylase), *APE1* (endonuclease), and *NUDT1* (8-oxodGTPase), involved in the repair of 8-OHdG, relative to controls, are shown in Figure 4.13. Concerning the study of cellular oxidative stress repair, the results corresponding to the exposure of BEAS-2B cells to 15 $\mu\text{g}/\text{cm}^2$ of PM-R did not show any dose-response relationship in contrary to the exposure to PM-I. These quite surprising results are not fully understood, and further analyses should be performed to validate and/or explain this absence of dose-response relationship.

Protein expression of *OGG1* (39 kDa) was measured by western blot in BEAS-2B cells exposed for 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. Non-exposed cells were used as negative controls and B[a]P (1 μM)-exposed cells as positive control. Actine (42 kDa) was used as a reference protein when testing *DNMT1* and *OGG1*. Western blot analysis of OGG1 protein expression is shown in Figure 4.14.

The results showed an increase in *OGG1* gene expression only after 72 h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^3$ of FP-I and to B[a]P (Figure 4.13). And the test of OGG1 protein expression at 72 h of exposure was in agreement with these results showing the increase of OGG1 protein expression after exposure to PM_{2.5-0.3} especially the ones under industrial influence (Figure 4.14). Moreover, an increase of gene and protein expression was noticed after exposure to higher concentration (from 3.75 to 15 $\mu\text{g}/\text{cm}^3$) of FP-I, therefore there is a dose-response relationship. The results correspond well

with the chemical composition of the particles. As previously mentioned, the inorganic (transition metals) and organic (mainly PAHs) compounds of the particles play an important role in the generation of ROS leading to oxidative stress and these chemicals are present in higher concentrations in the industrial influenced PM_{2.5-0.3}. This increase led to the activation of *OGG1*. Since *OGG1* is a DNA glycosylase involved in 8-oxoG repair or oxidative repair in the lung, FP-I probably causes an oxidation of DNA in our cellular model (Hegde *et al.*, 2008). Similar results were found in studies that showed that PM are related with the frequency of *CYP1A1* and *CYP1B1* in the human body due to their role in PAH metabolism which will lead to increased ROS that contributes to PM-induced toxicity and inflammation (Arlt *et al.*, 2015; Boubilil *et al.*, 2013; den Hartigh *et al.*, 2010; Dumax-Vorzet, 2010; Shang *et al.*, 2013; Totlandsdal *et al.*, 2010). Moreover, the increase in *OGG1* is consistent with other studies' results. Lan *et al.* showed that *OGG1* cause differences in DNA damage after short and long term exposures to particles, where *OGG1* may be upregulated (Lan *et al.*, 2004). Jin *et al.* showed an increased expression of *OGG1* associated with the oxidative DNA damage that occurred in lung epithelial cells (Jin *et al.*, 2015). Yang *et al.* demonstrated that PM_{2.5} leads to mitochondrial dysfunction and the up-regulation of *IL-1β* could be reversed by overexpression of *OGG1* in BEAS-2B cells (Yang *et al.*, 2015). Bind *et al.* showed that PM_{2.5} decreases *OGG1* methylation that may lead to *OGG1* expression (Bind *et al.*, 2016).

At 24h and 48h, the expressions of *APE1*, *NUDT1* and *NTH1* were not stable and dose-dependent. *APE1* was repressed after 24h of exposure to 3.75 and 15 μg/cm² of FP-R and 48h exposure to B[a]P. *APE1* expression significantly increased after 72h of exposure to 3.75 μg/cm² of FP-R and 3.75 and 15 μg/cm² of FP-I and to B[a]P. *NUDT1* was repressed after 24h of exposure to 3.75 and 15 μg/cm² of FP-R and 48h exposure to 15 μg/cm² of FP-I and to B[a]P. Our results showed a significant increase of *NUDT1* and *NTH1* after 72h of exposure to 15 μg/cm² of FP-I and to B[a]P (Figure 4.13).

These mechanisms were probably inhibited due to the oxidative stress related to the composition of the particles in metals, and their induction could be related to the occurrence of DNA damage at 72 hours of exposure.

NTH1 is DNA glycosylase, associated apurinic/aprimidinic (AP) lyase function that catalyzes the first step in base excision repair (BER), the primary repair pathway for the repair of oxidative DNA damage. The DNA N-glycosylase activity releases the damaged DNA base from DNA by cleaving the N-glycosidic bond, leaving an AP site. The AP-lyase activity cleaves the phosphodiester bond 3 to the AP site by a beta-elimination. *NTH1* primarily recognizes and repairs oxidative base damage of pyrimidines but it has also 8-oxo-7,8-dihydroguanine (8-oxoG) DNA glycosylase activity. Different studies showed the alteration of *NTH1* expression in cancer. Couto *et al.* showed that *NTH1* polymorphisms may be associated with non-small-cell lung cancer (NSCLC) pathogenesis (Couto *et al.*, 2015). Goto *et al.* showed the altered expression of the human base excision repair gene *NTH1* in gastric cancer, where *NTH1* expression was downregulated (Goto *et al.*,

2009). PM could affect NTH1 gene expression. Dumax-Vorzet showed indeed that NTH1 and OGG1 protect mammalian cells against PM-induced cell death (Dumax-Vorzet, 2010).

NUDT1 (also called MTH1) the major mammalian 8-oxodGTPase, has generated much interest as its genetic (Patel *et al.*, 2015) and pharmacologic (Gad *et al.*, 2014; Huber *et al.*, 2014) targeting was recently shown to inhibit xenograft tumors by a number of different tumor cell lines. It is a 18 kDa nudix pyrophosphorylase enzyme that eliminates 8-oxodGTP from the nucleotide pool by hydrolyzing it into the monophosphate form which cannot be used by DNA polymerases for genomic incorporation (Nakabeppu, 2001). Our results were in agreement with other studies' results. Giribaldi *et al.* showed that *MTH1* was overexpressed in BEAS-2B immortalized epithelial cells due to the presence of ROS (Giribaldi *et al.*, 2015). Choi *et al.* showed that PM_{2.5} induced the overexpression of *MTH1* (Choi *et al.*, 2004).

Finally, APE1 is an essential enzyme in the base excision repair pathway, which is the primary mechanism for the repair of DNA damage caused by oxidation and alkylation. Several findings demonstrate that the change in expression and activity as well as genetic variability of APE1 caused by environmental chemical (heavy metals and cigarette smoke) and physical carcinogens (ultraviolet and ionizing radiation) is likely associated with various cancers. APE1 overexpression is often observed in tumor cells (Wang *et al.*, 2009; Zaky *et al.*, 2008). Polymorphisms of the *APE1* gene are possibly affected by smoking and may contribute to the development of lung cancer (Lo *et al.*, 2009). Traversi *et al.* showed a marked and significant increase in *APE1* expression levels in the exposed cells to PM_{2.5} (Traversi *et al.*, 2015). Different studies showed the effect of arsenic, lead and cadmium on the expression of *APE1* and therefore on the DNA repair system (Giaginis *et al.*, 2006; Hamadeh *et al.*, 2002; Hu *et al.*, 2002; McNeill *et al.*, 2007).

The DNA damage induced by PM were mainly related to the metal content such as Cd, Cu, Fe, Ni, V, Zn and Pb and to PAHs (Binková *et al.*, 2003; Sánchez-Pérez *et al.*, 2009). These organic compounds can be metabolized in electrophilic reactive metabolites which bind covalently to DNA (Billet *et al.*, 2008). In addition, both groups of compounds adsorbed on the PM can increase the formation of ROS in the exposed cells which can directly interact with DNA and produce different types of damage, including oxidative DNA damage. Therefore, our results suggest that industrial influenced PM_{2.5-0.3} led to the formation of ROS and therefore to an oxidative DNA damage and that *OGG1*, *NTH1*, *NUDT1* and *APE1* have a leading role in repairing such lesions.

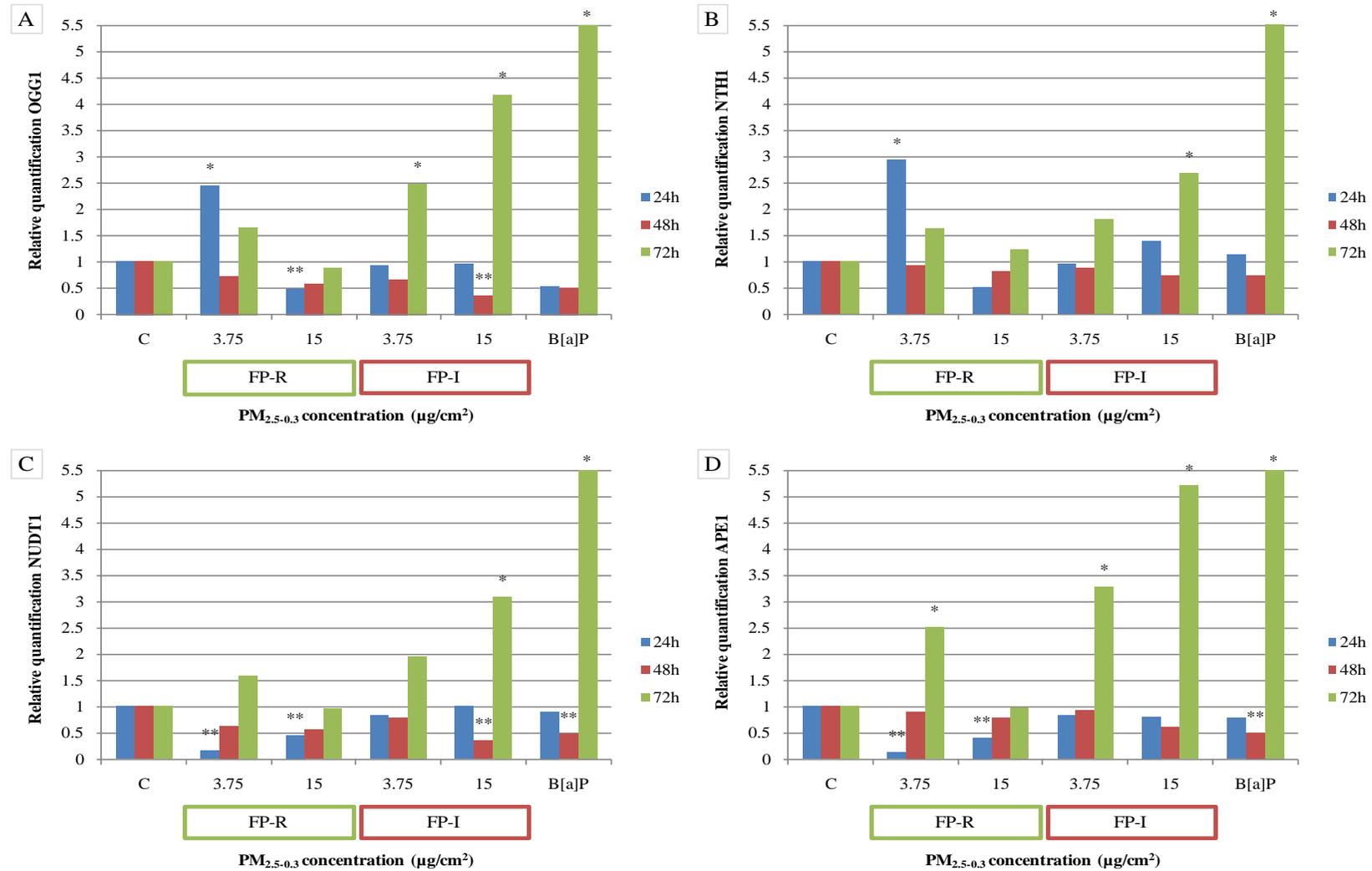


Figure 4.13: mRNA expressions of *OGG1* (A), *NTH1* (B), *NUDT1* (C), *APE1* (D), involved in the base excision repair system, in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu g/cm^2$

Key to statistical significance: *: $RQ > 2$, gene overexpressed and **: $RQ < 0.5$, gene repressed.

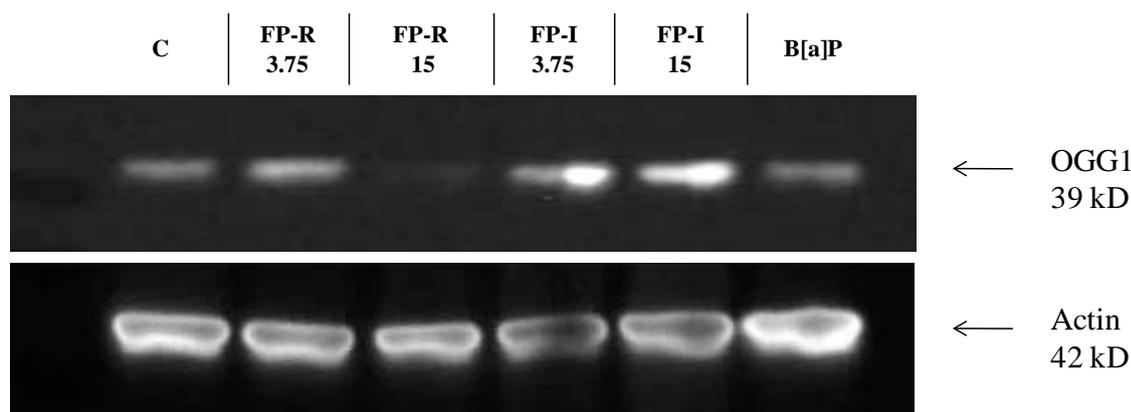


Figure 4.14: Western blotting with affinity-purified anti-OGG1 polyclonal antibody and anti- β -Actin antibody (housekeeping protein), detected by chemiluminescence detection using ECL substrate, in BEAS-2B cells exposed for 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$

4.2.7.3 DNA strand breaks repair

Same as the lesions formed by ROS, damage caused by PAH adducts interfere with repair mechanisms or DNA replication, potentially leading to the formation of double-strand breaks. DSBs can be caused by a variety of factors: direct interaction with an agent, ROS, metabolic processes, deficient repair, telomere erosion and programmed biological processes (Bonner *et al.*, 2008). XRCC1 and PARP1 serve as SSBs and DSBs sensor proteins. Therefore, gene expression of *XRCC1* was measured by RT-PCR and protein expression of PARP1 was measured by western blot in BEAS-2B cells exposed for 24, 48 or 72h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. Non-exposed cells were used as negative controls and B[a]P (1 μM)-exposed cells as positive control. For the RT-PCR, results are presented as relative quantitation (RQ), genes with $RQ > 2$ are considered as overexpressed and with a $RQ < 0.5$ as repressed (Livak and Schmittgen, 2001).

4.2.7.3.a XRCC1 gene expression

The expression of *XRCC1* at 24h and 48h was not stable and it was suppressed after 24h of exposure to FP-R (Figure 4.15). In a Cd-exposed group, 3 DNA repair genes (*XRCC1*, *OGG1*, and *ERCC1*) significantly decreased in the rat liver, kidney, heart, and lung according to the β -actin internal standard (Lei *et al.*, 2015). Whereas, Traversi *et al.* showed no effect of $PM_{2.5}$ exposition (24 hours) on *XRCC1* expression (Traversi *et al.*, 2015). Many DNA repair genes carry genetic polymorphisms, with the potential to modulate gene function and alter DNA repair capacity (Xi *et al.*, 2004).

XRCC1 gene expression increased only after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R, 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I and to B[a]P. This gene expression was inhibited after 24h of exposure to 3.75

and 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I and to B[a]P. The XRCC1 protein acts as a facilitator or coordinator through its interaction with PARP, DNA polymerase h, and DNA ligase III in BER and single-strand break repair (Huang *et al.*, 2013; Leng *et al.*, 2005). Different studies showed the importance of the XRCC1 in the repair and the association between the alteration of its expression with cancer risk (Surdu *et al.*, 2014). Chandirasekar *et al.* showed that XRCC1 variants are associated with cancer risk and linked the increase of XRCC1 to tobacco consumption and the polymorphism of XRCC1 gene influenced the frequencies of chromosomal aberrations and micronuclei (Chandirasekar *et al.*, 2011, 2014). Stern *et al.* data supported an XRCC1 gene expression alteration and smoking interaction ($P = 0.048$) (Stern *et al.*, 2006). Grlickova-Duzevik *et al.* showed that XRCC1 protects against particulate chromate-induced chromosome damage and cytotoxicity in Chinese hamster ovary cells (Grlickova-Duzevik *et al.*, 2006).

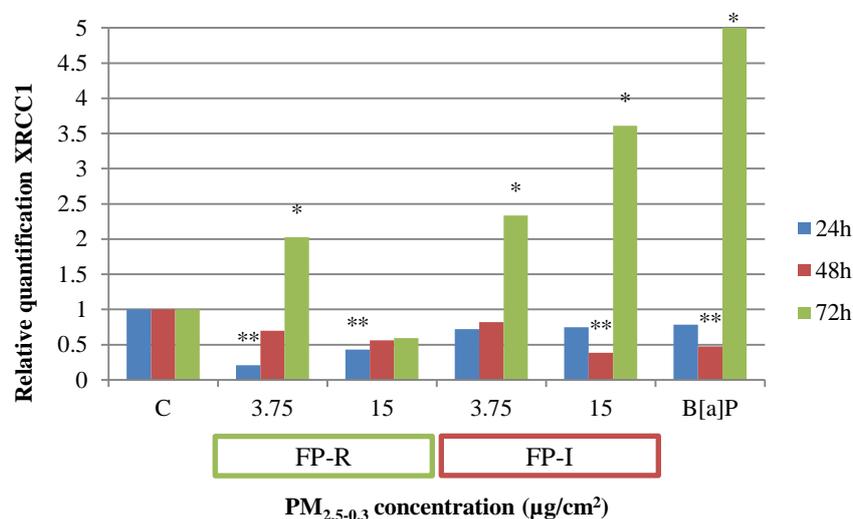


Figure 4.15: mRNA expressions of XRCC1, involved in DSBs repair, in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$

Key to statistical significance: *: RQ > 2, gene overexpressed and **: RQ < 0.5, gene repressed.

4.2.7.3.b Protein expression of PARP-1

Despite its normal function in the repair of DNA damage and its participation in diverse physiological and pathological functions, PARP1 is also a preferred substrate for several ‘suicidal’ proteases. The proteolytic action of suicidal proteases (caspases, calpains, cathepsins, granzymes and matrix metalloproteinases (MMPs)) on PARP-1 produces several specific proteolytic cleavage fragments with different molecular weights. Its cleavage has become a useful hallmark of apoptosis. These PARP-1 signature fragments are recognized biomarkers for specific patterns of protease activity in unique cell death programs.

Protein expression of PARP1, presented in Figure 4.16, reported the cleavage of PARP-1 into 3 fragments of 98, 50 and 24 kDa. According to different studies, PARP-1, a 113 kDa nuclear

enzyme, is cleaved into fragments of 98 and 24 kDa during apoptosis. This cleavage is well studied and is generated by the caspases 3 and 7, proteases activated during apoptosis (Gobeil *et al.*, 2001).

The presence of the 50 kDa fragment is related to necrosis and it is generated by lysosomal proteases released into the cytosol during necrosis that might allow the proteases liberated to access to cytosolic and nuclear proteins like PARP-1 and process them (Casiano *et al.*, 1998; Shah *et al.*, 1996). This explanation was further developed by Gobeil *et al.*, (2001), where it was showed that the necrotic signature of PARP-1 is not inhibited by zVAD-fmk, a broad spectrum caspases inhibitor, suggesting that the known apoptotic proteases may not be implicated in this type of cell death and that the necrotic cleavage of PARP-1 is caused in part or in totality by lysosomal proteases released into the cytosol during necrosis.

Therefore, these cleavages showed the presence of apoptosis in some exposed cells and necrosis in others.

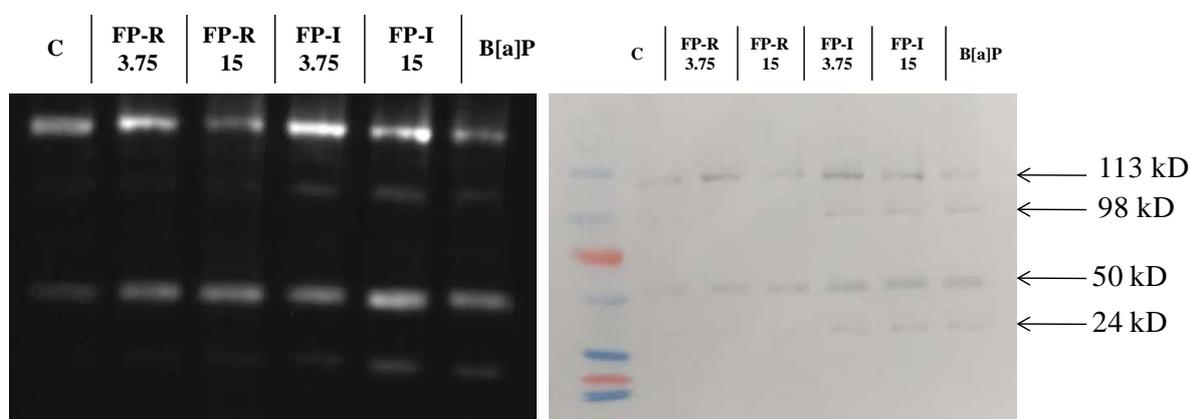


Figure 4.16: Western blotting with affinity-purified anti-PARP1 I antibody, detected by chemiluminescence detection using ECL substrate and by chromogenic detection using CN/DAB substrate, in BEAS-2B cells exposed for 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 µg/cm²

4.2.7.4 Methylation stability

DNA methylation is the most frequently studied epigenetic modification. It provides a stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in association with histone modifications and other chromatin associated proteins. PM can exhibit a strong interference on DNA methylation, evidenced not only by modulated global DNA methylation but also by regulated DNA methyltransferase (DNMT) activities (Liu *et al.*, 2010; Song *et al.*, 2011). DNMT1 has a role in the establishment and regulation of tissue-specific patterns of methylated residues. DNMT1 is thought to play a major role in the changes in DNA methylation observed in human cancer cells and an increase in DNMT1 abundance has been linked to cigarette smoke exposure induced lung carcinogenesis in mice and humans (Lin *et al.*, 2010). Some of the

methyl groups can be removed by a protein encoded by *MGMT* that transfers the methyl group from DNA by covalently coupling it to an internal cysteine residue of the MGMT protein (Yi and He, 2013).

DNMT1 and *MGMT* gene expression was measured in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced $PM_{2.5-0.3}$ at a concentration of 3.75 or 15 $\mu\text{g}/\text{cm}^2$. Non-exposed cells were used as negative controls and B[a]P (1 μM)-exposed cells as positive control. DNMT1 protein expression was measured by western blot and detected by chemiluminescence detection using ECL substrate.

DNMT1 and *MGMT* gene expressions were not stable at 24h and 48 of exposure to $PM_{2.5-0.3}$. *DNMT1* gene expression, presented in Figure 4.17, was repressed after 24h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I and to B[a]P. *DNMT1* gene expression increased after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R, 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I, and to B[a]P. DNMT1 protein expression, shown in Figure 4.18, was increased after exposition to particles especially to FP-I. According to many studies, heavy metals and PAHs adsorbed on $PM_{2.5}$ can upregulate *DNMT1* expression, by increasing ROS production (Bind *et al.*, 2016; Soberanes *et al.*, 2012). Other studies suggest that PM composition (Reichard and Puga, 2010; Wang *et al.*, 2013) and PAHs (Chen *et al.*, 1998; Subach *et al.*, 2007; Weisenberger and Romano, 1999) can inhibit *DNMT1* gene expression or reduce binding and activity of DNMT1 protein senic. High PAHs concentrations especially in the industrial influenced PM can explain DNMT1 decrease after 48h of exposure to FP-I at 15 $\mu\text{g}/\text{cm}^2$. Findings provide a potential mechanism by which PM exposure inhibits *DNMT1* expression and increases the risk of lung cancer. PM-induced up-regulation of the catechol-O-methyltransferase (COMT) depletes the SAM intracellular pool, therefore indirectly inhibiting DNMT (Fiskin *et al.*, 2006). Georgia *et al.* explained that DNMT1 is bound to the p53 regulatory region and that loss of DNMT1 results in derepression of the p53 locus (Georgia *et al.*, 2013). In alveolar epithelial cells, PM exposure increased ROS production, expression of the DNMT1 and increased transcription of *DNMT1* and methylation of the p16 promoter were inhibited by a mitochondrially targeted antioxidant and a c-jun-n-terminal protein kinase (JNK) inhibitor (Soberanes *et al.*, 2012).

MGMT gene expression, presented in Figure 4.19, decreased after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I and to B[a]P and was increased after 24h of exposure to FP-R at 3.75 $\mu\text{g}/\text{cm}^2$ and after 72h of exposure to B[a]P. *MGMT* gene expression is known to be repressed in lung cancer (Agarwal *et al.*, 2013). Duan *et al.* demonstrated hypomethylation of *MGMT* after exposure to PAHs (Duan *et al.*, 2013). In addition, expression of MGMT protein varies in normal and tumoral tissue (Sharma *et al.*, 2009). Miousse *et al.* showed the effect of PM on DNMT1 mRNA and protein expression, suggesting that epigenetic alterations, in concert with cytotoxicity, oxidative stress, and inflammation, might contribute to the pathogenesis of PM-associated respiratory diseases (Miousse and Chalbot, 2014).

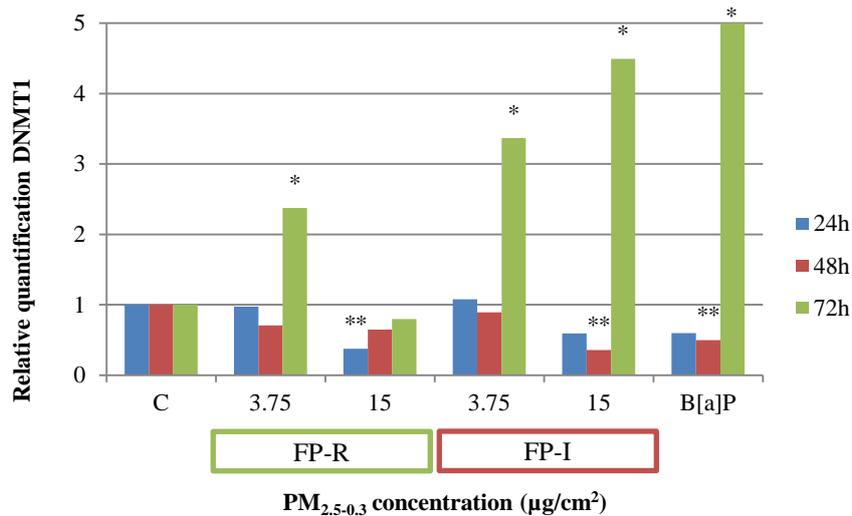


Figure 4.17: mRNA expressions of *DNMT1* in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 µg/cm²

Key to statistical significance: *: RQ > 2, gene overexpressed and **: RQ < 0.5, gene repressed.

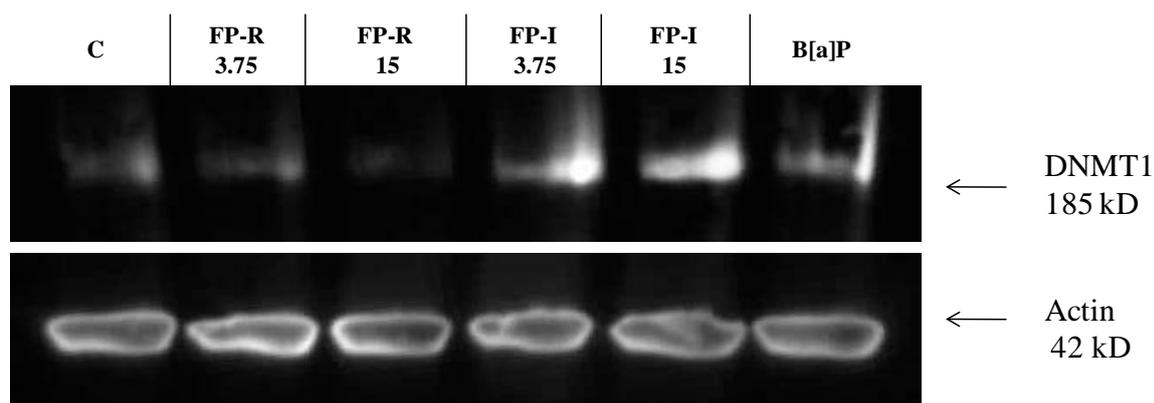


Figure 4.18: Western blotting with affinity-purified anti-DNMT1 antibody, detected by chemiluminescence detection using ECL substrate and by chromogenic detection using CN/DAB substrate, in BEAS-2B cells exposed for 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 µg/cm²

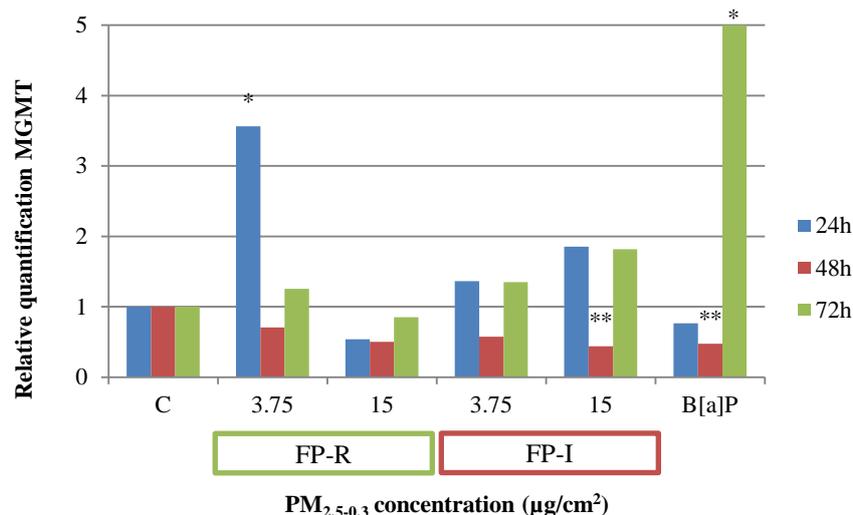


Figure 4.19: mRNA expressions of *MGMT* in BEAS-2B cells exposed for 24, 48 or 72 h to industrial and rural influenced PM_{2.5-0.3} at a concentration of 3.75 or 15 µg/cm²

Key to statistical significance: *: RQ > 2, gene overexpressed and **: RQ < 0.5, gene repressed.

4.3 Conclusion

Environmental air pollution is a serious public health concern throughout the world. Human exposure to PM_{2.5} is associated with increased mortality, particularly due to cardiovascular and respiratory diseases, including cancer. The aim of this work was thus to assess the mutagenicity and genotoxicity of fine (PM_{2.5-0.3}) particles collected in two sites under industrial and rural influence in Northern Lebanon that is affected by several sources of pollution and to study genotoxicity mechanisms involved in the pathogenicity of PM.

Mutagenicity and genotoxicity effects were studied, using the Ames fluctuation test and the SOS chromotest. In order to identify PM and the effects associated with early events in carcinogenesis, human bronchial epithelial cell line (BEAS-2B) were exposed to sampled PM_{2.5-0.3}. The cytotoxicity of the PM_{2.5-0.3} was determined by measuring of membrane integrity (testing the activity of extracellular LDH), assessing cell viability and testing the extent of cell proliferation (measuring the activity of the MDH and the incorporation of 5-bromo-2-deoxyuridine). Particles toxicity was then examined through the study of PAH metabolic activation (CYP1A1), DNA adducts (³²P-postlabelling), DNA strands breaks (γH2AX quantification by flow cytometry analysis and in-cell western assay), and effects on DNA repair (genes and proteins expressions by RT-qPCR and Western Blot).

The first step of the present study demonstrates that the Ames fluctuation test and SOS chromotest can be applied for studying genotoxicity and mutagenicity of whole airborne particulate matter. For the first time using the Ames fluctuation test, the ability of native PM to induce such effects was observed. The toxicological results obtained allow us to draw main conclusions: i)

particles collected under industrial influence showed dose-response higher mutagenicity and genotoxicity, compared to the rural one confirming the higher presence of promutagens and direct mutagens; ii) FP-I was highly mutagenic compared to FP-R with three *Salmonella typhimurium* strains, TA98, YG1041 and TA102; iii) the use of TA98 and YG1041 suggest that PAHs seem to play a leading role in the global mutagenicity and genotoxicity of the FP-I and that the PAHs derivatives could also be involved; iv) the use of TA102 showed that the inorganic composition of FP-I could result in oxidative capabilities that lead to its toxicity; v) the rural influenced sample was not genotoxic even at high concentrations.

After showing the mutagenic potential and the bacterial genotoxic effect of $PM_{2.5-0.3}$, we were interested to assess different genotoxic parameters on human bronchial cells BEAS-2B. The first step of the quest was the preliminary evaluation of the $PM_{2.5-0.3}$ cytotoxicity. No significant increase in extracellular LDH activity was observed following 24h, 48h or 72h of exposure to FP-I and FP-R. Only industrial $PM_{2.5-0.3}$ affected cell metabolism, which was shown by a decrease in MDH activity after 24h and 48h of exposure to FP-I (IC_{10} : 117.11 $\mu\text{g}/\text{cm}^2$ and 25.40 $\mu\text{g}/\text{cm}^2$). Concentration dependent decreases in the incorporation of BrdU in FP-I-exposed cells suggested the alteration of cellular division after 24 and 48h of exposure (IC_{10} : 83.22 $\mu\text{g}/\text{cm}^2$ and 86.91 $\mu\text{g}/\text{cm}^2$ respectively). On the other hand, a slight but significant decrease in BrdU incorporation was observed in BEAS-2B cells exposed 72h to FP-R (IC_{10} : 66.98 $\mu\text{g}/\text{cm}^2$). The determination of these IC_{10} allowed us to expose BEAS-2B cells at concentrations lower than IC_{10} .

In order to study the underlying mechanisms involved in their pulmonary toxicity, BEAS-2B cells were exposed during 24, 48 and 72h to industrial and rural influenced $PM_{2.5-0.3}$ at two concentrations that are not lethal, but had an effect on the BEAS-2B cells (3.75 and 15 $\mu\text{g}/\text{cm}^2$).

The bioactivation of pro-toxicants is the biological process through which some chemicals are metabolized into reactive metabolites which can lead to DNA damage. CYP1A1 metabolizes a number of pro-carcinogens to intermediates that can react with DNA and lead to damage by DNA-adduct formation or by oxidative stress. When testing the expression of CYP1A1, a significant induction was noted in BEAS-2B cells after 24, 48 and 72 h of exposure to FP-I and FP-R versus non-exposed cells, with higher expression when exposed to industrial influenced $PM_{2.5-0.3}$. This increase is related to high concentration of PAHs adsorbed on the tested PM especially the FP-I as shown by the chemical characterization. After showing the induction of CYP1A1, it was legitimate to test the consequences of this activation on the formation of bulky DNA adducts. In the tested conditions, quite surprisingly, no bulky adducts were shown on the autoradiograms of TLC sheets. This could be explained by several hypothesis: i) the presence of PCDD, PCDF and PCB, adsorbed in high concentration on the tested PM, can inhibit the formation of bulky-DNA adducts by inducing CYP1A1 gene expression and inhibiting AhR; ii) the XPA gene expression results showing an activation of bulky DNA adducts repair at 72h could be related to reparation of DNA modifications. Therefore the

absence of PAH DNA adducts after 72 h of exposure maybe detectable at shorter times of exposure (e.g. 48h).

Reactive oxygen species (ROS) and PAHs metabolic processes can lead to the formation of double strand breaks. Histone H2AX phosphorylation in response to DNA damage was measured by both flow cytometry and in-cell western. Results showed the appearance of H2AX phosphorylation in BEAS-2B cells exposed to PM_{2.5} collected especially to FP-I, suggesting that PAHs, or even their derivatives such as nitro-PAHS, could be involved in DNA double-strand breaks and therefore the formation of γ H2AX.

These types of DNA damage, if not repaired, may lead to the initiation of genomic instability and the development of cancer. Therefore, we were interested in studying the effect of the industrial and rural influenced PM_{2.5-0.3} on the DNA repair mechanisms.

- The first step was to determine the expression of gene involved in the repair of bulky DNA adducts. *XPA*, involved in the nucleotide excision repair, was induced in BEAS-2B cells mostly after 72h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I, related to its organic composition. It was suppressed after 24h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R, 48 h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-R or FP-I, maybe related to the ability of metals to interact with zinc ions or magnesium content in certain proteins.

- Studies showed that the exposure of the lung cells to PM_{2.5} let to the production of ROS leading to oxidative stress that may play an important role in particle toxicity and carcinogenicity. Thus, we were interested in testing the expression of genes and proteins involved in the base excision repair, responsible of the repair of such damage. The results of mRNA expressions of *OGG1* (DNA glycosylase), *NTH1* (DNA glycosylase), *APE1* (endonuclease), and *NUDT1* (8-oxodGTPase) showed: i) an increase in *OGG1* gene and protein expressions only after 72 hours of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I; ii) increase of *NTH1* and *NUDT1* genes expressions after 72h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I; iii) increase of *APE1* gene expression after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R and 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I.

These alterations are mainly related to FP-I contents in metals such as Cd, Cu, Fe, Ni, V, Zn and Pb, and PAHs. *APE1* was repressed after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R. *NUDT1* was repressed after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R and 48h exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. This latter could be probably related to the metal composition of the particles.

- ROS production and PAHs metabolites potentially lead to the formation of double-strand breaks. XRCC1 serves as SSBs sensor protein, while PARP1 and γ H2AX detect DSBs. *XRCC1* gene expression was inhibited after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. *XRCC1* gene expression increased only after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R and to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I. Protein expression of PARP1 reported the cleavage of PARP-1 into 3 fragments of 98, 50 and 24 kDa, showing the presence of apoptosis and necrosis. These results showed that the genotoxicity of such complex toxics as PM, could be delayed,

due to an inhibition by the oxidative stress, but, after a few days, PM exposure was clearly able to induce serious DNA damages like SSB and DSB.

- DNA methylation is the most frequently studied epigenetic modification. When testing the expression of genes involved in the regulation of the DNA methylation: i) *DNMT1* gene expression was repressed after 24h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I, probably due to the oxidative stress induced by the presence of particles; ii) *DNMT1* gene and protein expressions at 72h of exposure were increased after exposition to particles especially to FP-I; iii) *MGMT* gene expression decreased after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. It should be interesting to look at its promoter's methylation level which could be affected by PM exposure.

These findings provide potential mechanisms that might contribute to the pathogenesis of PM-associated respiratory diseases. Taken together, these results support the leading role of inorganic (metals: Cd, Cu, Fe, Ni, V, Zn and Pb) and organic components (PAHs) in PM oxidative properties and effects on both DNA damage and repair. The comparison between industrial and rural influenced PM showed a significant increase of several markers of genotoxicity after exposure to industrial PM, compared to rural PM. Taking into account the major differences in chemical composition between the PM from two different origins, that is to say higher concentrations in PAHs, and PCDD/Fs, our *in vitro* results highlighted the contribution of high molecular weight organic compounds in the global mutagenicity and genotoxicity, critical endpoints for carcinogenesis.

GENERAL CONCLUSION AND PERSPECTIVES

Air pollution is now the world's largest single environmental health risk (WHO, 2014a). Airborne particulate matter (PM) pollution imparts a tremendous burden to the global public health, ranking it as the 13th leading cause of mortality (3 million premature deaths) (WHO, 2014c). Epidemiological studies, from chamber studies to large-scale epidemiological analyses of acute effects in time series studies and of chronic effects in cohort studies, revealed an association between exposure to PM, and many cardiopulmonary diseases, lung cancer and mortality. Lebanon is a developing country (no laws and regulations for air emissions) where exposure to environmental pollution is a major source of health risk. Northern Lebanon is affected by several sources of pollution: cement factories and quarries, phosphate fertilizer industry, transport section, and other human activities. However, toxicity, and knowledge about air pollutants and their impact on health remains very patchy in the region.

According to our knowledge, in Lebanon no study has examine the perception of air pollution and few epidemiological studies were done to relate air pollution to respiratory diseases, although lung cancer is thought to be of primary importance in the region (Salim *et al.*, 2010) and has a relatively high incidence in Lebanon (Shamseddine *et al.*, 2014). Although PM is defined as carcinogenic to humans by the International Agency for Research on Cancer, toxicity mechanisms and pathways are not fully identified.

The aim of this work is to test the health impact of PM in Northern Lebanon, from a epidemiological perspective to chemical and toxicological perspective. This was achieved by: i) examining the levels of perceived air pollution annoyance and health risk and evaluating the relationship between respiratory diseases and industrial activities; ii) assessing the mutagenic and genotoxic effects of fine particulate matter (PM_{2.5-0.3}) samples collected in background area or an area under industrial influence; iii) relating the PM composition to the observed genotoxic effects.

In order to achieve these objectives, this work was divided into three successive pillars:

- Perception and epidemiological study
- Physicochemical and characteristics of fine PM
- Toxicological analyzes of fine PM.

The survey conducted in two areas in Northern Lebanon, rural and industrial (310 treatable questionnaires/area), showed significant differences between the inhabitants' perceptions and therefore an indicator of the real situation in these areas. This survey confirmed the seriousness of the situation at the industrial area. It reveals the opinion of the inhabitants of the industrial area regarding air pollution from industrial activities in terms of its effect on their quality of life, health and

identifying this attitude of nuisance from industrial emissions. The answers were affected by many factors such as working in the industries, the distance from the industries, seeing and feeling the industrial emissions. People who are accustomed to relatively poor air quality may be more sensitive to questions on air quality perceptions. Moreover, the epidemiological part of this survey confirmed the significant association between living in the proximity of industries and respiratory symptoms and diseases. Preliminary evidence suggested that industrial air pollution may be a risk factor for the increase in breathlessness and other respiratory problems at the industrial site. Besides air pollution, smoking, and advanced age were also reported to increase the risk of respiratory diseases. In addition, children were vulnerable to ambient air pollution as they spend more time outdoors, are more physically active, and have higher ventilation rates than adults. Our findings showed that people's perception about air quality is conditioned not only by the surrounding environmental parameters but also by their health state and their past illnesses. Therefore, our results showed the annoyance and health consequences of living in the vicinity of industries. These findings opened the doors for new inquiries about the effects of air pollution and if the nature of air pollution in the industrial area could explain the differences between the two areas.

Epidemiology is valuable because it generally deals with the full spectrum of susceptibility in human populations. This survey provides an early, somewhat speculative, discussion of the contribution of epidemiology to evaluating biologic mechanisms, and evaluates who's at risk or is susceptible to adverse health effects. Though such studies are limited but it confirmed the need to conduct a more detailed study, chemical characterization and toxicological study, in order to identify potential toxicity mechanisms induced by PM associated with early events in carcinogenesis and to relate the composition of PM to the observed effects. Since $PM_{2.5}$ is more closely associated with adverse health effects than larger particles, and the 2006 World Health Organization Air Quality Guidelines recommended $PM_{2.5}$ rather than PM_{10} as the indicator of air particle pollution, we were thus interested in assessing the mutagenicity and genotoxicity of fine particles ($PM_{2.5-0.3}$) collected in two sites under industrial and rural influence in Northern Lebanon.

Sampling strategy consisted of a simultaneous sampling of fine particles ($PM_{2.5-0.3}$) and quasi-ultrafine particles ($PM_{0.3}$) by cascade impaction using a TFIA-2 high volume air sampler at two sites in Northern Lebanon in Koura district: Zakroun, a site affected by industrial influences, and Kaftoun, a background rural site. During the whole sampling period, the mean concentration of $PM_{2.5}$ in Zakroun ($36.5 \mu\text{g}/\text{m}^3$) was higher than in Kaftoun ($22.8 \mu\text{g}/\text{m}^3$). The concentrations in both sites were higher than the annual standard set by the WHO ($10 \mu\text{g}/\text{m}^3$). Zakroun is influenced by numerous direct and indirect sources of fine particles such as industrial activities (cement factories, phosphate fertilizer industries...) and burning fuels (dense traffic, generators, and industrial power plants). All these factors favor the formation of fine particles and explain their higher concentrations encountered in the industrial influenced site compared to the rural background site.

Hence, to better understand what component of PM_{2.5} could have adverse health effects, some physicochemical characteristics were studied. The physicochemical analyses showed that for major species, both samples had similar distribution between species. Nevertheless, for these species and for metals, higher concentrations were very often observed for particles collected in the industrial influenced site. The PAHs concentrations were slightly higher in particles collected under industrial influence, especially for InPy, B[b]Fl, B[a]An, Chr and B[ghi]Pe. The detected PAHs are emitted by numerous combustion sources, including motor vehicles (mainly gasoline vehicles), biomass burning, coal combustion and industrial processes. In particular, the PAH characteristic ratios evidenced the contribution of diesel and gasoline combustion sources at both site. The highest difference between industrial and rural site PAH concentrations was observed for InPy. This PAH is notably emitted during tires combustion, and such attribution need to be investigated as metal industrial impact is also compatible with such assumption. Finally, the major difference in the composition concerns PCDDs and PCDFs that were considerably higher (125 times) in particles collected under industrial influence. Some industrial processes and in particular waste combustion, can lead to the formation of dioxins. In the frame of this study, the origin of such high dioxin level has not been clarified.

The lung constitutes the most important interface between the environment and the body, which gives it a particular interest in toxicology. Lung cancer is a major cause of cancer-related death worldwide and the overall survival rate is still extremely poor. DNA damage and alterations of DNA repair processes are considered as an essential feature in the initiation of cancer. Moreover, a number of studies have shown that PM_{2.5} poses a nontrivial risk to public health even at very low levels, but till present, the mechanisms underlying these effects have not been fully understood. We assessed and studied the mutagenic activity, genotoxic effect and genotoxicity mechanisms involved in the pathogenesis of PM-associated respiratory diseases. For a start, mutagenicity and genotoxicity were studied, using the Ames fluctuation test and the SOS chromotest with and without activation. According to our knowledge, this study is the first to perform the Ames fluctuation test and the SOS chromotest on the native PM_{2.5-0.3} to assess their genotoxicity. The results of these two bacterial tests reveal that: i) particles collected under industrial influence showed dose-response higher mutagenicity and genotoxicity, compared to the rural one confirming the higher presence of promutagens and direct mutagens; ii) FP-I was highly mutagenic compared to FP-R with three *Salmonella typhimurium* strains, TA98, YG1041 and TA102; iii) the use of TA98 and YG1041 suggest that PAHs seem to play a leading role in the global mutagenicity and genotoxicity of the FP-I and that the PAHs derivatives could also be involved; iv) the use of TA102 showed that oxidative stress could participate to the mutagenicity of FP-I; v) the rural influenced sample was not genotoxic even at high concentrations.

Subsequently, we were interested in assessing different genotoxic parameters on human bronchial cells BEAS-2B exposed to collected PM. The first step was the evaluation of the PM_{2.5-0.3} cytotoxicity. No significant increase in the percentage of cell death was observed following 24h, 48h or 72h of exposure to FP-I and FP-R. Only industrial PM_{2.5-0.3} affected cell metabolism, which was

shown by a decrease in mitochondrial activity after 24h and 48h of exposure to FP-I (IC₁₀: 117.11 µg/cm² and 25.40 µg/cm²). Concentration dependent decreases in the incorporation of BrdU in FP-I-exposed cells suggested the alteration of cellular division after 24h and 48h of exposure (IC₁₀: 83.22 µg/cm² and 86.91 µg/cm² respectively).

This study was oriented in a way to study the DNA damages caused by PM_{2.5-0.3} and the DNA repair specific of every type of damage. Thus, we tested: i) the metabolic activation of organic compounds in response to PM_{2.5-0.3}; ii) consecutive DNA damages such as DNA adducts and DNA strands breaks; iii) the effects of PM_{2.5-0.3} on DNA repair responsible of the repair of bulky DNA adducts, oxidative stress, DNA strands breaks, and the regulation of methylation. With this intention, in order to study the underlying mechanisms involved in PM_{2.5-0.3} pulmonary toxicity, BEAS-2B cells were exposed for 24h, 48h and 72h to industrial and rural influenced PM_{2.5-0.3} at two concentrations that are not lethal, but having an effect on the BEAS-2B cells (3.75 µg/cm² and 15 µg/cm²). Genes' expression was tested after exposure during 24h, 48h and 72h and DNA damage and proteins expressions testing were conducted after 72h of exposure in order to detect preferentially more stable lesions.

One of the most important determinants of xenobiotic persistence in the body and subsequent toxicity to the organism is the extent to which they can be metabolized and excreted. CYP1A1 metabolizes a number of pro-carcinogens to intermediates that can react with DNA and lead to damage by DNA-adduct formation or by oxidative stress. When testing the expression of *CYP1A1*, a significant induction was noted in BEAS-2B cells after 24h, 48h and 72h of exposure to FP-I and FP-R versus non-exposed cells, with higher expression when exposed to industrial influenced PM_{2.5-0.3}. This increase may be related to high concentration of PAHs adsorbed on the tested PM especially the FP-I as shown by the chemical characterization.

Hence, it was legitimate to test the consequences of this activation on the formation of bulky DNA adducts. In the tested conditions, quite surprisingly, no bulky adducts were shown after ³²P postlabeling. This could be explained by two hypotheses: i) the presence of PCDD, PCDF and PCB, adsorbed in high concentration on the tested PM, can inhibit the formation of bulky-DNA adducts by inducing *CYP1A1* gene expression and inhibiting AhR; ii) the *XPA* gene expression (involved in the nucleotide excision repair and the repair of bulky DNA adducts) results showing an activation of bulky DNA adducts repair at 72h could be related to reparation of DNA modifications. The *XPA* gene was suppressed after 24h of exposure to 3.75 µg/cm² of FP-R, 48 h of exposure to 15 µg/cm² of FP-R or FP-I, which may be related to the ability of metals to interact with zinc ions or magnesium content in certain proteins involved in this type of repair. It was induced in BEAS-2B cells mostly after 72h of exposure to 15 µg/cm² of FP-I, which may be related to its organic composition, metabolized after 72h of exposure.

Therefore the absence of PAH DNA adducts after 72 h of exposure maybe detectable at shorter times of exposure (e.g. 48h). ROS and PAHs metabolic processes can lead to the formation of double

strand breaks. Histone H2AX phosphorylation in response to DNA damage was measured by both flow cytometry and in-cell western. Results showed the phosphorylation of H2AX in BEAS-2B cells exposed to collected PM_{2.5-0.3} especially to FP-I, suggesting that PAHs, or even their derivatives such as nitro-PAHs, could be involved in DNA double-strand breaks and therefore the formation of γ H2AX. γ H2AX increase was stable during different cell cycle phases but a bit stronger in cells in G2/M, compared to G0/G1 and S cells. These types of DNA damage, if not repaired, may lead to the initiation of genomic instability and the development of cancer. We were thus next interested in testing the effect of the industrial and rural influenced PM_{2.5-0.3} on the DNA repair mechanisms:

- When testing the expression of genes and proteins involved in the base excision repair, responsible for the repair of damage caused by the production of ROS and oxidative stress, the results showed: i) an increase in *OGG1* (DNA glycosylase) gene and protein expressions only after 72 hours of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I; ii) increase of *NTH1* (DNA glycosylase) and *NUDT1* (8-oxodGTPase) genes expressions after 72h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I; iii) increase of *APE1* (endonuclease) gene expression after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R and 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I. These alterations are mainly related to FP-I contents in metals such as Cd, Cu, Fe, Ni, V, Zn and Pb, and PAHs that could lead to oxidative stress and therefore to the activation of this type of repair only at 72h of exposure. *APE1* was repressed after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R. *NUDT1* was repressed after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R and 48h exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. This repression is probably related to the metal composition of the particles and the ability of metals to modify the structure of certain proteins after 24h and 48h of exposure. Therefore, the base excision repair was activated only after 72h of exposure to PM.

- ROS production and PAHs metabolites potentially lead to the formation of double-strand breaks. XRCC1 serves as SSBs sensor protein, while PARP1 and γ H2AX detect DSBs. *XRCC1* gene expression was inhibited after 24h of exposure to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-R, and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. *XRCC1* gene expression increased only after 72h of exposure to 3.75 $\mu\text{g}/\text{cm}^2$ of FP-R and to 3.75 and 15 $\mu\text{g}/\text{cm}^2$ of FP-I. Protein expression of PARP1 reported the cleavage of PARP-1 into 3 fragments of 98, 50 and 24 kDa, showing the presence of apoptosis and necrosis in different exposed cells, even at low doses. This is related to the presence of different types of proteases that could be induced by different chemicals. These results showed that the genotoxicity of such complex toxins as PM, could be delayed, due to an early inhibition by the oxidative stress, but, after a few days, PM exposure was clearly able to induce serious DNA damages like SSB and DSB.

- When testing the expression of genes involved in the regulation of the DNA methylation: i) *DNMT1* gene expression was repressed after 24h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-R and after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. This is probably due to the oxidative stress induced by the presence of PM and the metabolization of organic compounds; ii) DNMT1 gene and protein expressions at 72h of

exposure were increased after exposition to particles especially to FP-I; iii) *MGMT* gene expression decreased after 48h of exposure to 15 $\mu\text{g}/\text{cm}^2$ of FP-I. It could be interesting to look at its promoter's methylation level which could be affected by PM exposure.

Therefore, a number of compounds found in PM can interact with DNA either directly or after enzymatic transformation to induce DNA modifications. The complex mixture of chemical elements present at varying doses in PM makes it difficult to attribute a toxicity to a precise fraction rather. Thus there are interactions in such mixture of compounds, particularly between inorganic and organic fractions leading to synergistic effects. In addition, we should take into consideration the chemical speciation of the metals adsorbed into the PM. PM are complex entities and their nature, the chemical form of their components, including the metal fraction should be considered during the evaluation of their toxicological impact. Chemical speciation modulates pathophysiological consequences related to the presence of an element in the body. This concept also takes into consideration the evolution of the properties of the xenobiotic in the environment and biological matrices.

The comparison between industrial and rural influenced PM showed a significant increase of several markers of genotoxicity after exposure to industrial PM, compared to rural PM. Taking into account the major differences in chemical composition between the PM from two different origins, such as higher concentrations in some PAHs, and PCDD/Fs, our *in vitro* results highlighted the contribution of high molecular weight organic compounds in the global mutagenicity and genotoxicity, critical endpoints for carcinogenesis.

The toxicological findings provide potential mechanisms that might contribute to the pathogenesis of PM-associated respiratory diseases. Taken together, these results support the leading role of inorganic (metals: Cd, Cu, Fe, Ni, V, Zn and Pb) and organic components (PAHs) in PM oxidative properties and effects on both DNA damage and repair. The repression of the DNA repair genes at 24h and 48h of exposure could be related to the composition of the particles in metals and their ability to interact with zinc ions or magnesium content in certain proteins. The late activation of the repair genes after 72h of exposure could be related to the bio-activation of the PAHs that took time to induce the expressions of these genes. Therefore this can also explain why we did not observe any PAH DNA adducts after 72h of exposure. It could be of interest to measure PAH DNA adducts at shorter time of exposure (e.g. 48h). Besides bulky DNA adducts of carcinogenic PAHs, such as BPDE-DNA adduct, a wide variety of non-bulky base damages and single-strand breaks formed by free radicals during metabolic activation of PAHs could be also involved in the PAH carcinogenesis.

Future work may include an epidemiological study in the North of Lebanon to assess the respiratory diseases and lung cancer cases found in the hospitals present in the vicinity of the industrial areas. Although toxicological studies have limitations, and it is impossible to mirror the complexity of the human environment or the complexity of the respiratory system with the defense mechanisms involved, they are the essential data base that show the real risk assessment and help to understand the potential mechanisms contributing to the pathogenesis of xenobiotics. This work should be further pursued along different axes:

- i) better understanding of the toxicity mechanisms by: testing the global DNA methylation and the oxidative damage (8-oxodG)
- ii) consolidating the obtained results by: looking for MGMT promoter's methylation level, studying the kinetic response of the toxicity mechanisms, and measuring the PAHs DNA adducts at shorter time of exposure (48h)
- iii) searching for toxicity mechanisms of the ultrafine particles
- iv) seeking for chemical parameters that can be used as tracers for different sources' contributions.

As a conclusion, this study showed that particles collected at the vicinity of industries have a more important genotoxic and mutagenic potential than rural ones. Proximity to industries is a parameter affecting the PM concentrations and composition. The mean concentrations of PM_{2.5} in Zakroun (industrial influenced site) and Kaftoun (background rural site) are 36.5 and 22.8 µg/m³ respectively. The effects of the collected particles are undoubtedly related to their composition and concentrations mostly the organic fraction. In particular, we have shown slightly higher levels of PAHs and up to 100 times' higher dioxins concentrations at the vicinity of industries. Moreover our results proved the annoyance and health consequences of living in the industrial influenced areas. Comprehensive evaluation of these evidences is needed in order to formulate effective pollution reduction strategies and national and international policies for reducing health risks due to pollution. This also showed the necessity of controlling pollutants' emissions and combustion processes. It is essential that tight operating procedures be in place and that close attention be paid to constant pre-treatment to industrial emissions.

At the national level, this study aims to contribute to the awareness of the government on the harmful effects of air pollution related to industrial releases, help to articulate policy objectives in risk minimization, and contribute to the design of programs for the evaluation of risk management and organizational structures to identify, monitor and control risks. In Northern Lebanon, this study aims to articulate design and implementation of measures to improve the air quality and to enunciate the need of pollution prevention and abatement for industries.

Finally, reducing car use and upgrading to more energy efficient appliances are the most common individual actions to reduce emissions. Providing higher financial incentives for low

emitting products is perceived as a good measure to tackle air pollution. More public information on the health and environmental effects of air pollution is also seen as a measure to reduce pollution, probably by increasing awareness and motivation. Stricter pollution controls on industrial activity and energy production are seen as the most effective way to tackle air-related problems. Harnessing appropriate technology for use in the cement industry and phosphate fertilizer industry could go a long way towards minimizing on-site wastes and pollution.

The priority in the cement industry is to minimize the increase in ambient particulate levels by reducing the mass load emitted from the stacks, from fugitive emissions, and from other sources. Collection and recycling of dust in the kiln gases is required to improve the efficiency of the operation and to reduce atmospheric emissions. Units that are well designed, operated, and maintained can normally achieve generation of less than 0.2 kilograms of dust per metric ton (kg/t) of clinker, and include dust recovery systems. For control of fugitive particulate emissions, ventilation systems should be used in conjunction with hoods and enclosures covering transfer points and conveyors. Material handling should be conducted in enclosed systems where negative pressure is maintained by exhaust fans and ventilation air and dust is removed using cyclones and bag filters. Drop distances should be minimized by the use of adjustable conveyors (Burange and Yamini, 2009). Dusty areas such as roads should be wetted to reduce dust generation. Appropriate storm water and runoff control systems should be provided to minimize the quantities of suspended material carried off site. For PM emissions which are associated with the procedure of kiln systems, clinker coolers, and mills, including clinker and limestone burning, different pollution prevention and control techniques are recommended: i) capturing using filters (sticky or dry filters): kiln and cooler dusts can be captured and recovered particulates can be recycled into the kiln feed and into the clinker (Boudaghpour and Jadidi, 2009); ii) mechanical systems such as cyclones trap the larger particulates in kiln gases and act as preconditioners for downstream collection devices; iii) electrostatic precipitators (ESPs) and fabric filter systems (baghouses) are the principal options for collection and control (achieving over 99% removal efficiency) of fine particulates (Chikowore *et al.*, 2012; Williams, 2010); iv) industries should stop the non-conventional combustible use (domestic waste, used tires) (Greer, 2003). For the fertilizer plants, scrubbers should be used to remove fluorides and acid from air emissions (WorldBank, 2007).

We have the right to breathe fresh air, so we should think globally and act locally to reduce air pollution.

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APPENDICES

APPENDIX 1 : Questionnaire

Section 1: Personal Information

1. What is your city of residence?

2. How long have you lived in this particular address?

< 2 years

2-5 years

> 5 years

3. Are you present in this particular address in a:

Temporary way Second city of residence: _____ Permanent way

4. Have you ever lived in Beirut or Tripoli:

Yes How long? _____ No

5. Sex: Male

Female

6. Please select your age range:

18-25

26-35

36-45

46-55

Above 55

7. Marital status:

Single

Married

Widowed

Separated/Divorced

8. Do you have children? Yes

No

9. What is your present occupation and the nature of work performed?

Part-time Full-time

10. Occupational history:

A. Have you ever worked for a year or more in any dusty job? Yes No

Specify job/ industry: _____ Total years worked: _____

Was the exposure: Mild

Moderate

Severe

B. Do you think you have ever been exposed to gas or chemical fumes in your work?

Yes No

Specify job/ industry: _____ Total years worked: _____

Was the exposure: Mild

Moderate

Severe

11. A. Did you ever smoke cigarettes? Yes No

How long? _____ Number of cigarettes per week? _____

B. Do you smoke now? Yes No

C. If yes, how many cigarettes do you smoke per week now? Number of cigarettes: _____

D. How old were you when you first started regular cigarette smoking? Age in years: _____

E. If you have stopped smoking completely, since when? _____

12. A. Have you ever smoked shisha? Yes

No

How long? _____

Number of bowls per week _____

- B. Do you smoke now? Yes No
 C. If yes, how many bowls do you smoke per week now? Number of bowls: _____
 D. How old were you when you first started regular shisha smoking? Age in years: _____
 E. If you have stopped smoking completely, since when? _____

13. Heating home:

by gas by wood by diesel electrically by hot air centrally

Section 2: General environmental concerns

- According to you, what are the three major environmental problems in Lebanon (1 to 3 from most to least important)?
 Air Pollution Water Pollution Soil Pollution Food Contamination
- How would you describe the air quality in Northern Lebanon?
 Very Good Poor Don't know
 Good Very Poor No answer
- How do you perceive air pollution?
 Odour Smoke Don't know
 Dust Poor vegetation condition No answer
- How often do you notice the presence of air pollution in your region?
 Always Seldom Don't know
 Often Never No answer
- Do you think the air quality in your village is better or worse than other villages in the North?
 Much better Worse Similar No answer
 Better Much worse Don't know
- Do you feel the pollution mostly during day time or at night?
 Day Night Both Don't know No answer
- In your opinion, is air pollution affected by the weather conditions?
 More in summer More in winter No Don't know No answer
- In your opinion, the impact of air pollution on your health is:
 Very important Important Low Very low
 No impact Don't know No answer
- In your opinion, has air pollution ever affected the health of any of your family or friends?
 Yes No Don't know
- According to you, what are the three main sources of air pollution in Northern Lebanon (1 to 3 from most to least important)?
 Transport Industries Agriculture Generators Other _____
- In your opinion, the industries in the region:
 are a source of wealth are harmful to health
 are harmful to the environment give a good image of the region
 are a source of jobs are a source of accident

Section 3: Health State

1. A. Do you usually have a **cough**? If no skip to question 1C. Yes No
 B. Do you usually cough as much as 4 to 6 times a day, 4 or more days of the week?
 Yes No
 C. Do you usually cough at all on getting up, or first thing in the morning? Yes No
 D. Do you usually cough at all during the rest of the day or at night? Yes No
 If yes to any of above (1A, B, C or D), answer the following, if no, skip to next question.
 E. Do you usually cough like this on most days for 3 consecutive months or more during the year? Yes No
 F. For how many years have you had this cough? Number of years: _____

2. A. Do you usually bring up **phlegm** of your chest? If no skip to question 2C.
 Yes No
 B. Do you usually bring up phlegm like this as much as twice a day, 4 or more days out of the week? Yes No
 C. Do you usually bring up phlegm at all on getting up, on first thing in the morning?
 Yes No
 D. Do you usually bring up phlegm at all during the rest of the day or at night? Yes No
 If yes to any of above (2A, B, C or D), answer the following, if no, skip to next question.
 E. Do you bring up phlegm like this on most days for 3 consecutive months or more during the year? Yes No
 F. For how many years have you had trouble with phlegm? Number of years: _____

3. A. Have you had periods or episodes of increased cough and phlegm lasting for 3 weeks or more each year? Yes No
 B. For how long have you had at least 1 such episode per year? Number of years: _____

4. A. Does your chest ever sound wheezy or **whistling**:

1. When you have a cold?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
2. Occasionally apart from colds?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
3. Most days?	Yes <input type="checkbox"/>	No <input type="checkbox"/>

 B. For how many years has this been present? (If Yes in 4A: 1, 2, or 3) Number of years: _____

5. A. Have you ever had a wheezing attack that has made you feel short of breath?
 Yes No
 If yes to 5A:
 B. How old were you when you had your first wheezing attack? Age in years: _____
 C. Have you had 2 or more such episodes? Yes No
 D. Have you ever required medicine or treatment for the(se) attack(s)? Yes No

6. If disabled from walking by any condition other than heart or lung disease, please describe and proceed to Question 7. Nature of condition(s): _____
 A. Are you troubled by **shortness of breath** when hurrying or walking up a slight hill?
 Yes No
 B. Do you have to walk slower than people of your age because of breathlessness?
 Yes No
 C. Do you ever have to stop for breath when walking at your own pace?
 Yes No
 D. Do you ever have to stop for breath after walking about 100 meters (or after a few minutes)? Yes No
 E. Are you too breathless to leave the house or breathless while dressing or undressing?
 Yes No

7. If you get a cold, does it usually go to your chest? (Usually means more than half the time.)
 Yes No Don't get colds
8. A. During the past 3 years, have you had any chest illnesses that have kept you off work, at home, or in bed? Yes No If Yes to 8A:
 B. Did you produce phlegm with any of these chest illnesses? Yes No
 C. In the last 3 years, how many such illnesses, with (increased) phlegm, did you have which lasted a week or more? _____

Section 4: Past Illnesses

1. Did you have any lung trouble before the age of 16? Yes No
2. Have you ever had any of the following: (confirmed by a doctor):
- A. Attacks of **bronchitis**? Yes No At what age was your first attack? _____
- B. **Pneumonia**? Yes No At what age did you first have it? _____
- C. **Hay fever**? Yes No At what age did it start? _____
- D. **Chronic bronchitis**? Yes No At what age did it start? _____
 Do you still have it? Yes No
- E. **Emphysema**? Yes No At what age did it start? _____
 Do you still have it? Yes No
- F. **Asthma**? Yes No At what age did it start? _____
 Do you still have it? Yes No If you no longer have it, at what age did it stop?_
3. Have you ever had:
- A. Any other chest illness? Yes No If yes, please specify _____
- B. Any chest operations? Yes No If yes, please specify _____
- C. Any chest injuries? Yes No If yes, please specify _____
4. A. Has a doctor ever told you that you had **heart problems**? Yes No
 B. Have you ever had treatment for heart problems in the past 10 years? Yes No
5. A. Has a doctor ever told you that you had **high blood pressure**? Yes No
 B. Have you had any treatment for high blood pressure in the past 10 years? Yes No
6. During which months do all these respiratory illnesses give you the most trouble?
 Month or season: _____

Section 5: Family History and Children health

1. Does or did any of your parents work in an industry? Yes No
2. Were either of your natural parents ever told by a doctor that they had a chronic lung condition such as:
 Chronic bronchitis Asthma Other chest conditions
 Emphysema Lung cancer
3. Did anyone in your family die of lung cancer? Yes No
4. Did your child have any chest illness or chest cold? Yes specify _____ No
5. Was he/she ever hospitalized for a severe chest illness or chest cold before the age of 2 years?
 Yes No

6. Did a physician state that your child had an allergic reaction to pollen or dust?

Yes No

7. To what extent do you think air quality affects your child/ children's respiratory health?

Seriously affects Somewhat affects No impact Don't know

If you have anything to add about the issues raised in this questionnaire or any comments about the questionnaire itself, please write them here:

Thank you for giving up your time to complete this questionnaire. It is very much appreciated.

Much better	9 (5.7)	35 (20.5)	44 (13.4)	8 (5.2)	30 (21.6)	38 (13)	0.66	0.929	0.582
Better	38 (24.2)	90 (52.6)	128 (39)	41 (26.8)	65 (46.8)	106 (36.3)			
Worse	71 (45.2)	21 (12.3)	92 (28)	70 (45.8)	20 (14.4)	90 (30.8)			
Much worse	4 (2.5)	1 (0.6)	5 (1.5)	9 (5.9)	1 (0.7)	10 (3.4)			
Similar	34 (21.7)	21 (12.3)	55 (16.8)	24 (15.7)	21 (15.1)	45 (15.4)			
No answer	1 (0.6)	3 (1.8)	4 (1.2)	1 (0.7)	2 (1.4)	3 (1)			
Do you feel the pollution mostly during day time or at night?									
Day	11 (7)	17 (9.9)	28 (8.5)	17 (11.1)	5 (3.6)	22 (7.5)	0.635	0.096	0.592
Night	54 (34.4)	38 (22.2)	92 (28)	59 (38.6)	32 (23)	91 (31.2)			
Both	67 (42.7)	57 (33.3)	124 (37.8)	56 (36.6)	57 (41)	113 (38.7)			
Don't know	8 (5.1)	18 (10.5)	26 (7.9)	7 (4.6)	8 (5.8)	15 (5.1)			
No answer	17 (10.8)	41 (24)	58 (17.7)	14 (9.2)	37 (26.6)	51 (17.5)			
In your opinion, is air pollution affected by the weather conditions?									
More in summer	57 (36.3)	50 (29.2)	107 (32.6)	54 (35.3)	54 (38.8)	108 (37)	0.727	0.327	0.433
More in winter	30 (19.1)	37 (21.6)	67 (20.4)	36 (23.5)	26 (18.7)	62 (21.2)			
No	16 (10.2)	26 (15.2)	42 (12.8)	21 (13.7)	13 (9.4)	34 (11.6)			
Don't know	40 (25.5)	26 (15.2)	66 (20.1)	27 (17.6)	22 (15.8)	49 (16.8)			
No answer	14 (8.9)	32 (18.7)	46 (14)	15 (9.8)	24 (17.3)	39 (13.4)			
In your opinion, the impact of air pollution on your health is:									
Very important	45 (28.7)	47 (27.5)	92 (28)	42 (27.5)	46 (33.1)	88 (30.1)	0.62	0.771	0.739
Important	82 (52.2)	74 (43.3)	156 (47.6)	88 (57.5)	54 (38.8)	142 (48.6)			
Low	18 (11.5)	10 (5.8)	28 (8.5)	17 (11.1)	11 (7.9)	28 (9.6)			
Very low	1 (0.6)	0	1 (0.3)	0	0	0			
No impact	5 (3.2)	32 (18.7)	37 (11.3)	4 (2.6)	24 (17.3)	28 (9.6)			
Don't know	4 (2.5)	5 (2.9)	9 (2.7)	1 (0.7)	2 (1.4)	3 (1)			
No answer	2 (1.3)	3 (1.8)	5 (1.5)	1 (0.7)	2 (1.4)	3 (1)			
In your opinion, has air pollution ever affected the health of any of your family or friends?									
Yes	72 (45.9)	39 (22.8)	111 (33.8)	81 (52.9)	33 (23.7)	114 (39)	0.4	0.933	0.456
No	75 (47.8)	114 (66.7)	189 (57.6)	64 (41.8)	90 (64.7)	154 (52.7)			
Don't know	10 (6.4)	18 (10.5)	28 (8.5)	8 (5.2)	16 (11.5)	24 (8.2)			
According to you, what are the three main sources of air pollution in Northern Lebanon (1 to 3 from most to least important)?									
First main source									
Transport	44 (28)	35 (20.5)	79 (24.1)	48 (31.4)	44 (31.7)	92 (31.5)	0.116	0.078	0.769
Industries	66 (42)	91 (53.2)	157 (47.9)	59 (38.6)	65 (46.8)	124 (42.5)			
Agriculture	47 (29.9)	45 (26.3)	92 (28)	46 (30.1)	30 (21.6)	76 (26)			
Second main source									
Transport	108 (70.6)	116 (67.8)	224 (69.1)	98 (65.3)	88 (63.3)	186 (64.4)	0.436	0.559	0.323
Industries	28 (18.3)	40 (23.4)	68 (21)	38 (25.3)	34 (24.5)	72 (24.9)			
Agriculture	17 (11.1)	15 (8.8)	32 (9.9)	14 (9.3)	17 (12.2)	31 (10.7)			
Third main source									
Transport	5 (3.4)	19 (11.6)	24 (7.7)	6 (4.2)	7 (5.4)	13 (4.8)	0.317	0.148	0.929
Industries	57 (39)	41 (25)	98 (31.6)	54 (37.8)	39 (30.2)	93 (34.2)			
Agriculture	84 (57.5)	104 (63.4)	188 (60.6)	83 (58)	83 (64.3)	166 (61)			
In your opinion, the industries in the region:									
Source of wealth	13 (8.3)	15 (8.8)	28 (8.5)	10 (6.5)	8 (5.8)	18 (6.2)	0.108	0.238	0.569

Harmful to the environment	36 (22.9)	44 (25.7)	80 (24.4)	45 (29.4)	50 (36)	95 (32.5)
Source of job	15 (9.6)	12 (7)	27 (8.2)	13 (8.5)	11 (7.9)	24 (8.2)
Harmful to health	87 (55.4)	93 (54.4)	180 (54.9)	83 (54.2)	68 (48.9)	151 (51.7)
Good image of the region	5 (3.2)	6 (3.5)	11 (3.4)	2 (1.3)	1 (0.7)	3 (1)
Source of accident	1 (0.6)	1 (0.6)	2 (0.6)	0	1 (0.7)	1 (0.3)

APPENDIX 3: Impact of respondents' age on air pollution perception

Variable	Industrial					Rural					Total					p-value range of age		
	18-25	26-35	36-45	46-55	above 55	18-25	26-35	36-45	46-55	above 55	18-25	26-35	36-45	46-55	above 55	Industrial	Rural	Total
According to you, what are the three major environmental problems in Lebanon (1 to 3 from most to least important)?																		
First major environmental problem in Lebanon																		
Air Pollution	85.3	83.3	75.4	79.7	75.4	63.1	50	55.7	67.7	53.1	75	66.7	65.6	73.4	64	0.555	0.393	0.425
Water Pollution	13.3	13	18	15.3	23	24.6	38.9	37.7	26.2	39.1	18.6	25.9	27.9	21	31.2			
Soil Pollution	1.3	3.7	6.6	5.1	1.6	12.3	11.1	6.6	6.2	7.8	6.4	7.4	6.6	5.6	4.8			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Second major environmental problem in Lebanon																		
Air Pollution	12	9.3	16.4	10.3	11.7	26.2	31.5	33.3	27.7	40.6	18.6	20.4	24.8	19.5	26.6	0.912	0.605	0.697
Water Pollution	61.3	57.4	52.5	56.9	51.7	41.5	31.5	33.3	36.9	37.5	52.1	44.4	43	46.3	44.4			
Soil Pollution	26.7	33.3	31.1	32.8	36.7	32.3	37	33.3	35.4	21.9	29.3	35.2	32.3	34.1	29			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Third major environmental problem in Lebanon																		
Air Pollution	2.7	7.4	8.3	12.1	11.7	10.8	18.5	11.7	4.6	6.3	6.4	13	10	8.1	8.9	0.678	0.158	0.704
Water Pollution	26.7	29.6	28.3	27.6	28.3	33.8	29.6	28.3	40	23.8	30	29.6	28.3	34.1	26			
Soil Pollution	70.7	63	63.3	60.3	60	55.4	51.9	60	55.4	69.8	63.6	57.4	61.7	57.7	65			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
How would you describe the air quality in Northern Lebanon?																		
Very good	1.3	0	1.6	13.6	23	6.2	3.7	9.8	15.4	16.9	3.6	1.9	5.7	14.5	19.8	0	0.046	0
Good	48	35.2	50.8	44.1	39.3	80	59.3	68.9	66.2	66.2	62.9	47.2	59.8	55.6	53.2			
Poor	44	63	44.3	40.7	32.8	12.3	35.2	16.4	15.4	13.8	29.3	49.1	30.3	27.4	23			
Very poor	6.7	1.9	3.3	0	4.9	0	1.9	4.9	3.1	1.5	3.6	1.9	4.1	1.6	3.2			
Don't know	0	0	0	0	0	1.5	0	0	0	0	0.7	0	0	0	0			
No answer	0	0	0	1.7	0	0	0	0	0	1.5	0	0	0	0.8	0.8			
How do you perceive air pollution?																		
Odor	16	11.1	13.1	10.2	18	10.8	14.8	9.8	1.5	7.7	13.6	13	11.5	5.6	12.7	0.048	0.017	0.001
Dust	46.7	37	37.7	40.7	39.3	46.2	53.7	47.5	43.1	46.2	46.4	54.4	42.6	41.9	42.9			
Smoke	34.7	44.4	42.6	30.5	21.3	21.5	20.4	23	35.4	13.8	28.6	32.4	32.8	33.1	17.5			
Poor vegetation condition	1.3	0	1.6	0	0	6.2	0	1.6	0	0	3.6	0	1.6	0	0			
Don't know	0	1.9	1.6	5.1	8.2	3.1	3.7	3.3	3.1	3.1	1.4	2.8	2.5	4	5.6			
No answer	1.3	5.6	3.3	13.6	13.1	12.3	7.4	14.8	16.9	29.2	6.4	6.5	9	15.3	21.4			
How often do you notice the presence of air pollution in your region?																		
Always	22.7	22.2	21.3	13.6	19.7	3.1	5.6	6.6	7.7	4.6	13.6	13.9	13.9	10.5	11.9	0.054	0.45	0.081
Often	54.7	48.1	50.8	45.8	42.6	41.5	53.7	37.7	36.9	23.1	48.6	50.9	44.3	41.1	32.5			
Seldom	21.3	20.4	24.6	30.5	18	40	29.6	37.7	36.9	43.1	30	25	31.1	33.9	31			
Never	0	0	0	0	6.6	9.2	7.4	13.1	10.8	18.5	4.3	3.7	6.6	5.6	12.7			
Don't know	0	1.9	0	1.7	1.6	3.1	1.9	0	1.5	1.5	1.4	1.9	0	1.6	1.6			
No answer	1.3	7.4	3.3	8.5	11.5	3.1	1.9	4.9	6.2	9.2	2.1	4.6	4.1	7.3	10.3			
Do you think the air quality in your village is better or worse than other villages in the North?																		
Much better	0	3.7	3.3	8.5	13.1	9.2	20.4	16.4	21.5	36.9	4.3	12	9.8	15.3	25.4	0.19	0.021	0.001
Better	25.3	22.2	24.6	32.2	23	58.5	37	49.2	56.9	46.2	40.7	29.6	36.9	45.2	34.9			
Worse	46.7	50	47.5	42.4	41	10.8	24.1	16.4	9.2	7.7	30	37	32	25	23.8			

Much worse	5.3	3.7	1.6	1.7	8.2	0	1.9	0	1.5	6.2	2.9	2.8	0.8	1.6	4			
Similar	22.7	20.4	23	13.6	13.1	20	14.8	16.4	10.8	3.1	21.4	17.6	19.7	12.1	9.5			
No answer	0	0	0	1.7	1.6	1.5	1.8	1.6	0	0	0.7	0.9	0.8	0.8	2.4			
Do you feel the pollution mostly during day time or at night?																		
Day	10.7	3.7	8.2	13.6	8.2	6.2	7.4	13.1	6.2	3.1	8.6	5.6	10.7	9.7	5.6			
Night	40	38.9	36.1	28.8	37.7	29.2	25.9	18	26.2	13.8	35	32.4	27	27.4	25.4			
Both	42.7	0	47.5	33.9	26.2	35.4	40.7	39.3	41.5	27.7	39.3	44.4	43.4	37.9	27	0.013	0.031	0.001
Don't know	4	0	4.9	8.5	6.6	10.8	9.3	8.2	4.6	9.2	7.1	4.6	6.6	6.5	7.9			
No answer	2.7	9.3	3.3	15.3	21.3	18.5	16.7	21.3	21.5	46.2	10	13	12.3	18.5	34.1			
In your opinion, is air pollution affected by the weather conditions?																		
More in summer	42.7	37	36.1	30.5	31.1	41.5	35.2	36.1	30.8	24.6	42.1	36.1	36.1	30.6	27.8			
More in winter	17.3	22.2	23	25.4	19.7	12.3	33.3	11.5	29.2	16.9	15	27.8	17.2	27.4	18.3			
No	9.3	7.4	9.8	20.3	13.1	10.8	7.4	16.4	12.3	15.4	10	7.4	13.1	16.1	14.3	0.081	0.011	0
Don't know	24	27.8	26.2	15.3	14.8	24.6	11.1	18	10.8	12.3	24.3	19.4	22.1	12.9	13.5			
No answer	6.7	5.6	4.9	8.5	21.3	10.8	13	18	16.9	30.8	8.6	9.3	11.5	12.9	26.2			
In your opinion, the impact of air pollution on your health is:																		
Very important	28	18.5	26.2	27.1	39.3	20	25.9	39.3	36.9	27.7	24.3	22.2	32.8	32.3	33.3			
Important	52	63	49.2	57.6	54.1	40	50	31.1	46.2	40	46.5	56.5	40.2	51.6	46.8			
Low	18.7	5.6	16.4	10.2	3.3	12.3	9.3	6.6	3.1	3.1	15.7	7.4	11.5	6.5	3.2			
Very low	0	0	0	0	1.6	0	0	0	0	0	0	0	0	0	0.8	0.082	0.147	0.038
No impact	0	5.6	4.9	3.4	1.6	26.2	9.3	16.4	12.3	24.6	12.1	7.4	10.7	8.1	13.5			
Don't know	0	5.6	1.6	1.7	0	1.5	3.7	3.3	0	3.1	0.7	4.6	2.5	0.8	1.6			
No answer	1.3	1.9	1.6	0	0	0	1.9	3.3	1.5	1.5	0.7	1.9	2.5	0.8	0.8			
In your opinion, has air pollution ever affected the health of any of your family or friends?																		
Yes	61.3	50	59	37.3	36.1	32.3	22.2	19.7	24.6	16.9	47.9	36.1	39.3	30.6	26.2			
No	32	42.6	41	57.6	54.1	58.5	63	68.9	67.7	70.8	44.3	52.8	54.9	62.9	62.7	0.014	0.596	0.012
Don't know	6.7	7.4	0	5.1	9.8	9.2	14.8	11.5	7.7	12.3	7.9	11.1	5.7	6.5	11.1			
According to you, what are the three main sources of air pollution in Northern Lebanon (1 to 3 from most to least important)?																		
First main source																		
Transport	24	22.2	31.1	37.3	34.4	20	31.5	26.2	26.2	24.6	22.1	26.9	28.7	31.5	29.4			
Industries	37.3	51.9	37.7	40.7	36.1	63.1	50	45.9	41.5	50.8	49.3	50.9	41.8	41.1	43.7	0.32	0.329	0.664
Agriculture	38.7	25.9	31.1	22	29.5	16.9	18.5	27.9	32.3	24.6	28.6	22.2	29.5	27.4	27			
Second main source																		
Transport	72	73.9	68.9	59.6	64.9	70.8	64.8	70.5	61.5	61.5	71.4	69.2	69.7	60.7	63.1			
Industries	21.3	18.9	19.7	22.8	26.3	21.5	20.4	18	32.3	26.2	21.4	19.6	18.9	27.9	26.2	0.593	0.526	0.555
Agriculture	6.7	7.5	11.5	17.5	8.8	7.7	14.8	11.5	6.2	12.3	7.1	11.2	11.5	11.5	10.7			
Third main source																		
Transport	4.1	4	0	5.5	5.8	9.5	4.2	3.5	11.3	14.3	6.6	4.1	1.7	8.5	10.4			
Industries	42.5	30	42.4	32.7	42.3	15.9	29.2	40.4	29	23.8	30.1	29.6	41.4	30.8	32.2	0.578	0.7	0.108
Agriculture	53.4	66	57.6	61.8	51.9	74.6	66.7	56.1	59.7	61.9	63.2	66.3	56.9	60.7	57.4			
In your opinion, the industries in the region:																		
Source of wealth	5.3	1.9	4.9	11.9	13.1	1.5	1.9	13.1	7.7	12.3	3.6	1.9	9	9.7	12.7			
Harmful to the environment	28	16.7	31.1	25.4	27.9	29.2	48.1	32.8	20	24.6	28.6	32.4	32	22.6	26.2			
Source of job	4	7.4	9.8	11.9	13.1	9.2	5.6	0	6.2	4.6	6.4	6.5	10.7	8.9	8.7			
Harmful to health	61.3	70.4	52.5	47.5	42.6	56.9	38.9	11.5	64.6	53.8	59.3	54.6	47.5	56.5	48.4	0.21	0.007	0.071
Good image of the region	1.3	3.7	1.6	1.7	3.3	3.1	5.6	42.6	1.5	1.5	2.1	4.6	0.8	1.6	2.4			
Source of accident	0	0	0	1.7	0	0	0	0	0	3.1	0	0	0	0.8	1.6			

APPENDIX 4: Occupational Impact on Perception of Air Pollution

Variable	Industrial					Rural					Total					p-value csp		
	Primary	Secondary	Tertiary	Don't work	Student	Primary	Secondary	Tertiary	Don't work	Student	Primary	Secondary	Tertiary	Don't work	Student	Ind.	Rur.	Total
According to you, what are the three major environmental problems in Lebanon (1 to 3 from most to least important)?																		
First major environmental problem in Lebanon																		
Air Pollution	75.7	36.4	86.7	77.4	83	57.1	66.7	59	52.8	64.9	66	42.9	74.1	64.2	73.1	0	0.16	0.005
Water Pollution	20	27.3	13.3	17.7	14.9	37.7	33.3	26	41.7	28.1	29.3	28.6	19.1	30.6	22.1			
Soil Pollution	4.3	36.4	0	4.8	2.1	5.2	0	15	5.6	7	4.8	28.6	6.8	5.2	4.8			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Second major environmental problem in Lebanon																		
Air Pollution	14.3	18.2	8.4	11.5	17	29.9	33.3	28.3	43.1	26.3	22.4	21.4	17.4	28.6	22.1	0.502	0.306	0.413
Water Pollution	52.9	45.5	60.5	49.2	61.7	40.3	66.7	33.3	30.6	42.1	46.3	50	48.2	39.1	50			
Soil Pollution	32.9	36.4	31.1	39.3	21.3	29.9	0	38.4	26.4	31.6	31.3	28.6	34.4	21.3	26.9			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
Third major environmental problem in Lebanon																		
Air Pollution	11.6	36.4	5	11.5	0	11.7	0	14.1	4.2	8.8	11.6	28.6	9.2	7.6	4.8	0.003	0.065	0.107
Water Pollution	27.5	36.4	26.9	32.8	23.4	24.7	0	40.4	28.2	29.8	26	28.6	33	30.3	26.9			
Soil Pollution	60.9	27.3	68.1	55.7	76.6	63.6	100	45.5	67.6	61.4	62.3	42.9	57.8	62.1	68.3			
Food contamination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
How would you describe the air quality in Northern Lebanon?																		
Very good	8.6	27.3	3.3	16.1	2.1	14.1	100	5	18.1	7	11.5	21.4	4.1	17.2	4.8	0	0.153	0
Good	40	54.5	35.8	51.6	57.4	61.5	0	70	65.3	77.2	51.4	64.3	51.4	59	68.3			
Poor	51.4	18.2	55	29	34	23.1	0	20	13.9	14	36.5	14.3	39.1	20.9	23.1			
Very poor	0	0	5.8	1.6	6.4	1.3	0	5	1.4	0	0.7	0	5.5	1.5	2.9			
Don't know	0	0	0	0	0	0	0	0	0	1.8	0	0	0	0	1			
No answer	0	0	0	1.6	0	0	0	0	1.4	0	0	0	0	1.5	0			
How do you perceive air pollution?																		
Odour	17.1	9.1	13.3	9.7	17	14.1	0	6	6.9	8.8	15.5	7.1	10	8.2	12.5	0.002	0.003	0
Dust	32.9	36.4	39.2	43.5	53.2	43.6	0	51	44.4	50.9	38.5	28.6	44.5	44	51.9			
Smoke	37.1	27.3	42.5	24.2	25.5	26.9	33.3	24	16.7	22.8	31.8	28.6	34.1	20.1	24			
Poor vegetation condition	0	0	0	1.6	2.1	0	33.3	2	0	3.5	0	7.1	0.9	0.7	2.9			
Don't know	0	18.2	1.7	9.7	0	2.6	0	5	2.8	1.8	1.4	14.3	3.2	6	1			
No answer	12.9	9.1	3.3	11.3	2.1	12.8	33.3	12	29.2	12.3	12.8	14.3	7.3	20.9	7.7			
How often do you notice the presence of air pollution in your region?																		
Always	15.7	0	25.8	14.5	23.4	10.3	66.7	7	1.4	1.8	12.8	35.7	17.3	7.5	11.5	0	0.562	0.004
Often	42.9	45.5	55.8	43.5	46.8	35.9	33.3	42	31.9	43.9	39.2	35.7	49.5	37.3	45.2			
Seldom	28.6	27.3	15.8	25.8	27.7	35.9	0	36	41.7	36.8	32.4	28.6	25	34.3	32.7			
Never	0	27.3	0	1.6	0	12.8	0	8	15.3	12.3	6.8	0	3.6	9	6.7			
Don't know	1.4	0	0.8	1.6	0	2.6	0	1	1.4	1.8	2	0	0.9	1.5	1			
No answer	11.4	0	1.7	12.9	2.1	2.6	0	1	8.3	3.5	6.8	0	3.6	10.4	2.9			

Transport	0	0	4.4	9.3	2.2	11	0	4.3	11.4	10.5	5.7	0	4.4	10.5	6.9	0.063	0.119	0.01
Industries	37.1	75	34.2	44.4	34.8	21.9	100	30.4	32.9	19.6	30	80	32.5	37.9	26.5			
Agriculture	61.2	25	61.4	40.3	63	67.1	0	65.2	55.7	69.6	64.3	20	63.1	51.6	66.7			
In your opinion, the industries in the region:																		
Source of wealth	2.9	63.6	5	8.1	6.4	11.5	0	7	8.3	1.8	7.4	50	5.9	8.2	3.8	0	0.091	0
Harmful to the environment	27.1	9.1	22.5	32.3	29.8	26.9	33.3	31	30.6	33.3	27	14.3	26.4	31.3	31.7			
Source of job	5.7	18.2	5.8	21	4.3	3.8	66.7	10	5.6	7	4.7	28.6	7.7	12.7	5.8			
Harmful to health	61.4	9.1	64.2	35.5	57.4	52.6	0	49	54.2	56.1	56.8	7.1	57.3	45.5	56.7			
Good image of the region	2.9	0	1.7	3.2	2.1	3.8	0	3	0	1.8	3.4	0	2.3	1.5	1.9			
Source of accident	0	0	0.8	0	0	1.3	0	0	1.4	0	0.7	0	0.5	0.7	0			

APPENDIX 5: Positive answers to questions on health state

	Total % (n)	Industrial % (n)	Rural % (n)	OR
Do you usually have a cough ?				
Yes	56 (9)	33 (10.6)	23 (7.4)	1.49 [0.85-2.6]
No	564 (91)	277 (89.4)	287 (92.6)	
Do you usually cough as much as 4 to 6 times a day, 4 or more days of the week?				
Yes	34 (5.5)	21 (6.8)	13 (4.2)	1.66 [0.82-3.38]
No	586 (94.5)	289 (93.2)	297 (95.8)	
Do you usually cough at all on getting up, or first thing in the morning?				
Yes	26 (4.2)	13 (4.2)	13 (4.2)	1.00 [0.46-2.19]
No	594 (95.8)	297 (95.8)	297 (95.8)	
Do you usually cough at all during the rest of the day or at night?				
Yes	23 (3.7)	11 (3.5)	12 (3.9)	0.91 [0.4-2.09]
No	597 (96.3)	299 (96.5)	298 (96.1)	
Do you usually cough like this on most days for 3 consecutive months or more during the year?				
Yes	19 (3.1)	10 (3.2)	9 (2.9)	1.11 [0.44-2.77]
No	601 (96.9)	300 (96.8)	301 (97.1)	
Do you usually bring up phlegm of your chest?				
Yes	20 (3.2)	13 (4.2)	7 (2.3)	1.89 [0.74-4.8]
No	600 (96.8)	297 (95.8)	303 (97.7)	
Do you usually bring up phlegm like this as much as twice a day, 4 or more days out of the week?				
Yes	7 (1.1)	5 (1.6)	2 (0.6)	2.52 [0.49-13.09]
No	613 (98.9)	305 (98.4)	308 (99.4)	
Do you usually bring up phlegm at all on getting up, on first thing in the morning?				
Yes	6 (1)	4 (1.3)	2 (0.6)	2.01 [0.37-11.06]
No	614 (99)	306 (98.7)	308 (99.4)	
Do you usually bring up phlegm at all during the rest of the day or at night?				
Yes	6 (1)	4 (1.3)	2 (0.6)	2.01 [0.37-11.06]
No	614 (99)	306 (98.7)	308 (99.4)	
Do you bring up phlegm like this on most days for 3 consecutive months or more during the year?				
Yes	4 (0.6)	3 (1)	1 (0.3)	3.02 [0.31-29.19]
No	616 (99.4)	307 (99)	309 (99.7)	
Have you had periods or episodes of increased cough and phlegm lasting for 3 weeks or more each year?				
Yes	2 (0.3)	2 (0.6)	0	ND
No	618 (99.7)	308 (99.4)	320 (100)	
Does your chest ever sound wheezy or whistling when you have a cold?				
Yes	32 (5.2)	20 (6.5)	12 (3.9)	1.71 [0.82-3.56]
No	588 (94.8)	290 (93.5)	298 (96.1)	
Occasionally apart from colds?				
Yes	19 (3.1)	11 (3.5)	8 (2.6)	1.39 [0.55-3.50]
No	601 (96.9)	299 (96.5)	302 (97.4)	
Most days?				
Yes	3 (0.5)	2 (0.6)	1 (0.3)	2.01 [0.18-22.28]
No	617 (99.5)	308 (99.4)	309 (99.7)	
Have you ever had a wheezing attack that has made you feel short of breath?				
Yes	9 (1.5)	7 (2.3)	2 (0.6)	3.56 [0.73-17.27]
No	611 (98.5)	303 (97.7)	308 (99.4)	
Have you had 2 or more such episodes?				
Yes	4 (0.6)	4 (1.3)	0	ND
No	616 (99.4)	306 (98.7)	310 (100)	
Have you ever required medicine or treatment for the(se) attack(s)?				
Yes	5 (0.8)	5 (1.6)	0	ND
No	615 (99.2)	305 (98.4)	310 (100)	
Are you troubled by shortness of breath when hurrying or walking up a slight hill? (Grade 1)				
Yes	103 (16.6)	65 (21)	38 (12.3)	1.90 [1.23-2.14]
No	517 (83.4)	245 (79)	272 (87.7)	
Do you have to walk slower than people of your age because of breathlessness? (Grade 2)				
Yes	62 (10)	40 (12.9)	22 (7.1)	1.94 [1.12-3.35]
No	558 (90)	270 (87.1)	288 (92.9)	
Do you ever have to stop for breath when walking at your own pace? (Grade 3)				
Yes	52 (8.4)	35 (11.3)	17 (5.5)	2.19 [1.20-4.00]
No	568 (91.6)	275 (88.7)	293 (94.5)	
Do you ever have to stop for breath after walking about 100 meters (or after a few minutes)? (Grade 4)				
Yes	59 (9.5)	38 (12.3)	21 (6.8)	1.92 [1.10-3.35]
No	561 (90.5)	272 (87.7)	289 (93.2)	
Are you too breathless to leave the house or breathless while dressing or undressing? (Grade 5)				
Yes	32 (5.2)	19 (6.1)	13 (4.2)	1.49 [0.72-3.07]
No	588 (94.8)	291 (93.9)	297 (95.8)	
If you get a cold, does it usually go to your chest? (Usually means more than half the time.)				
Yes	37 (6)	22 (7.1)	15 (4.8)	ND
No	578 (93.2)	285 (91.9)	293 (94.5)	

Don't get colds	5 (0.8)	3 (1)	2 (0.6)	
During the past 3 years, have you had any chest illnesses that have kept you off work, at home, or in bed?				
Yes	11 (1.8)	11 (3.5)	0	ND
No	609 (98.2)	299 (96.5)	310 (100)	
Did you produce phlegm with any of these chest illnesses?				
Yes	2 (0.3)	2 (0.6)	0	ND
No	618 (99.7)	308 (99.4)	310 (100)	

Yes	1 (0.3)	0	1 (0.6)	1 (0.3)	0	1 (0.7)	ND	ND	0.89
No	327 (99.7)	171 (100)	156 (99.4)	291 (99.7)	139 (100)	152 (99.3)			[0.06-14.29]
Does your chest ever sound wheezy or whistling when you have a cold?									
Yes	20 (6.1)	9 (5.3)	11 (7)	12 (4.1)	3 (2.2)	9 (5.9)	1.36	2.83	1.48
No	308 (93.9)	162 (94.7)	146 (93)	280 (95.9)	136 (97.8)	144 (94.1)	[0.55- 3.37]	[0.75- 10.67]	[0.73-3.17]
Occasionally apart from colds?									
Yes	11 (3.4)	6 (3.5)	5 (3.2)	8 (2.7)	2 (1.4)	6 (3.9)	0.9 [0.27- 3.01]	2.8 [0.56- 14.11]	1.22
No	317 (96.6)	165 (96.5)	152 (96.8)	284 (97.3)	137 (98.6)	147 (96.1)			[0.49-3.1]
Most days?									
Yes	3 (0.9)	1 (0.6)	2 (1.3)	0	0	0	2.19	ND	ND
No	325 (99.1)	170 (99.4)	155 (98.7)	292 (100)	139 (100)	153 (100)	[0.2- 24.39]		
Have you ever had a wheezing attack that has made you feel short of breath									
Yes	5 (1.5)		5 (3.2)	4 (1.4)	2 (1.4)	2 (1.3)	ND	0.91 [0.13-6.55]	1.11
No	323 (98.5)	171 (100)	152 (96.8)	288 (98.6)	137 (98.6)	151 (98.7)			[0.3-4.17]
Have you had 2 or more such episodes									
Yes	1 (0.3)	0	1 (0.6)	3 (1)	0	3 (2)	ND	ND	0.3
No	327 (99.7)	171 (100)	156 (99.4)	289 (99)	139 (100)	150 (98)			[0.03-2.8]
Have you ever required medicine or treatment for the(se) attack(s)?									
Yes	2 (0.6)	0	2 (1.3)	3 (1)	0	3 (2)	ND	ND	0.59
No	326 (99.4)	171 (100)	155 (98.7)	289 (99)	139 (100)	150 (98)			[0.1-3.56]
Are you troubled by shortness of breath when hurrying or walking up a slight hill?									
Yes	61 (18.6)	25 (14.6)	36 (22.9)	42 (14.4)	13 (9.4)	29 (19)	1.74	2.27	1.29
No	267 (81.4)	146 (85.4)	121 (77.1)	250 (85.6)	126 (90.6)	124 (81)	[0.99- 3.06]	[1.13- 4.57]	[0.89-2.09]
Do you have to walk slower than people of your age because of breathlessness?									
Yes	39 (11.9)	18 (10.5)	21 (13.4)	23 (7.9)	4 (2.9)	19 (12.4)	1.31	4.79	1.51
No	289 (88.1)	153 (89.5)	136 (86.6)	269 (92.1)	135 (97.1)	134 (87.6)	[0.67- 2.56]	[1.59- 14.45]	[0.92-2.72]
Do you ever have to stop for breath when walking at your own pace?									
Yes	30 (9.1)	13 (7.6)	17 (10.8)	22 (7.5)	4 (2.9)	18 (11.8)	1.48	4.5 [1.48-13.65]	1.21
No	298 (90.9)	158 (92.4)	140 (89.2)	270 (92.5)	135 (97.1)	135 (88.2)	[0.69- 3.16]		[0.7-2.2]
Do you ever have to stop for breath after walking about 100 meters (or after a few minutes)?									
Yes	37 (11.3)	16 (9.4)	21 (13.4)	22 (7.5)	5 (3.6)	17 (11.1)	1.5	3.35 [1.2- 9.34]	1.5
No	291 (88.7)	155 (90.6)	136 (86.6)	270 (92.5)	134 (96.4)	136 (88.9)	[0.75- 2.99]		[0.9-2.71]
Are you too breathless to leave the house or breathless while dressing or undressing?									
Yes	20 (6.1)	10 (5.8)	10 (6.4)	12 (4.1)	3 (2.2)	9 (5.9)	1.1	2.83	1.48
No	308 (93.9)	161 (94.2)	147 (93.6)	280 (95.9)	136 (97.8)	144 (94.1)	[0.45-2.72]	[0.75- 10.67]	[0.73-3.17]
If you get a cold, does it usually go to your chest? (Usually means more than half the time.)									
Yes	22 (6.7)	10 (5.8)	12 (7.6)	15 (5.1)	5 (3.6)	10 (6.5)			
No	304 (92.7)	161 (94.2)	143 (91.1)	274 (93.8)	132 (95)	142 (92.8)	ND	ND	ND
Don't get colds	2 (0.6)		2 (1.3)	3 (1)	2 (1.4)	1 (0.7)			

During the past 3 years, have you had any chest illnesses that have kept you off work, at home, or in bed?									
Yes	4 (1.2)	0	4 (2.5)	7 (2.4)	0	7 (4.6)	ND	ND	0.51
No	324 (98.8)	171 (100)	153 (97.5)	285 (97.6)	139 (100)	146 (95.4)			[0.14-1.73]
Did you produce phlegm with any of these chest illnesses?									
Yes	1 (0.3)	0	1 (0.6)	1 (0.3)	0	1 (0.7)	ND	ND	0.89
No	327 (99.7)	171 (100)	156 (99.4)	291 (99.7)	139 (100)	152 (99.3)			[0.06-14.29]

APPENDIX 7: Symptoms and smoking habits

	Smoker			Non-smoker			OR		
	Total	Rural	Industrial	Total	Rural	Industrial	Smoker-nonsmoker	Smoker (Industrial-rural)	Non smoker (Industrial-rural)
Do you usually have a cough ?									
Yes	22 (15)	9 (15.3)	13 (14.8)	34 (7.2)	7 (2.8)	20 (9.0)	1.81 [1.02-3.2]	0.96 [0.38- 2.41]	3.45 [1.43- 8.32]
No	125 (85)	50 (84.7)	75 (85.2)	439 (92.8)	244 (97.2)	202 (91.0)			
Do you usually cough as much as 4 to 6 times a day, 4 or more days of the week?									
Yes	15 (10.2)	6 (10.2)	9 (10.2)	19 (4.0)	9 (3.6)	12 (5.4)	2.72 [1.35- 5.5]	1.01 [0.34- 3]	1.54 [0.64- 3.73]
No	132 (89.8)	53 (89.8)	79 (89.8)	454 (96.0)	242 (96.4)	210 (94.6)			
Do you usually cough at all on getting up, or first thing in the morning?									
Yes	7 (4.8)	4 (6.8)	3 (3.4)	19 (4.0)	7 (2.8)	10 (4.5)	1.19 [0.49- 2.89]	0.49 [0.11- 2.27]	1.64 [0.61- 4.38]
No	140 (95.2)	55 (93.2)	85 (96.6)	454 (96.0)	244 (97.2)	212 (95.5)			
Do you usually cough at all during the rest of the day or at night?									
Yes	10 (6.8)	5 (8.5)	5 (5.7)	13 (2.7)	4 (1.6)	6 (2.7)	2.58 [1.11-6.01]	0.65 [0.18- 2.35]	1.72 [0.48- 6.18]
No	137 (93.2)	54 (91.5)	83 (94.3)	460 (97.3)	247 (98.4)	216 (97.3)			
Do you usually cough like this on most days for 3 consecutive months or more during the year?									
Yes	9 (6.1)	5 (8.5)	4 (4.5)	10 (2.1)	4 (1.6)	6 (2.7)	3.02 [1.2- 7.58]	0.51 [0.13- 1.98]	1.72 [0.48- 6.18]
No	138 (93.9)	54 (91.5)	84 (95.5)	463 (97.9)	247 (98.4)	216 (97.3)			
Do you usually bring up phlegm of your chest?									
Yes	10 (6.8)	3 (5.1)	7 (8.0)	10 (2.1)	0	6 (2.7)	1.38 [1.38-8.29]	1.61 [0.4- 6.49]	ND
No	137 (93.2)	56 (94.9)	81 (92.0)	463 (97.9)	251 (100.0)	216 (97.3)			
Do you usually bring up phlegm like this as much as twice a day, 4 or more days out of the week?									
Yes	5 (3.4)	2 (3.4)	3 (3.4)	2 (0.4)	2 (0.8)	2 (0.9)	8.29 [1.59- 43.19]	1.01 [0.16- 6.24]	1.13 [0.16- 8.09]
No	142 (96.6)	57 (96.6)	85 (96.6)	471 (99.6)	249 (99.2)	220 (99.1)			
Do you usually bring up phlegm at all on getting up, on first thing in the morning?									
Yes	1 (7)	0	1 (1.1)	5 (1.1)	1 (0.4)	3 (1.4)	0.64 [0.07- 5.52]	ND	3.42 [0.35- 33.12]
No	146 (99.3)	59 (100)	87 (98.9)	468 (98.9)	250 (99.6)	219 (98.6)			
Do you usually bring up phlegm at all during the rest of the day or at night?									
Yes	4 (2.7)	1 (1.7)	3 (3.4)	2 (0.4)	0	1 (0.5)	6.59 [1.19- 36.35]	2.05 [0.21- 20.2]	ND
No	143 (97.3)	58 (98.3)	85 (96.6)	471 (99.6)	251 (100.0)	221 (99.5)			
Do you bring up phlegm like this on most days for 3 consecutive months or more during the year?									
Yes	3 (2.0)	1 (1.7)	2 (2.3)	1 (0.2)	0	1 (0.5)	9.83 [1.01-95.23]	1.35 [0.12- 15.23]	ND
No	144 (98.0)	58 (98.3)	86 (97.7)	472 (99.8)	251 (100.0)	221 (99.5)			
Have you had periods or episodes of increased cough and phlegm lasting for 3 weeks or more each year?									
Yes	0	0	0	2 (0.4)	0	2 (0.9)	ND	ND	ND
No	147 (100.0)	59 (100)	88 (100.0)	471 (99.6)	251 (100.0)	220 (99.1)			
Does your chest ever sound wheezy or whistling when you have a cold?									
Yes	14 (9.5)	4 (6.8)	10 (11.4)	18 (3.8)	8 (3.2)	10 (4.5)	2.66 [1.29-5.49]	1.76 [0.52- 5.9]	1.43 [0.55- 3.69]
No	133 (90.5)	55 (93.2)	78 (88.6)	455 (96.2)	243 (96.8)	212 (95.5)			
Occasionally apart from colds?									
Yes	6 (4.1)	3 (5.1)	3 (3.4)	13 (2.7)	5 (2.0)	8 (3.6)	1.51 [0.56- 4.05]	0.66 [0.13- 3.39]	1.84 [0.59- 5.71]

No	141 (95.9)	56 (94.9)	85 (96.6)	460 (97.3)	246 (98.0)	214 (96.4)			
Most days?									
Yes	2 (1.4)	1 (1.7)	1 (1.1)	1 (0.2)	0	1 (0.5)	6.51 [0.59-72.32]	0.67 [0.04- 10.93]	ND
No	145 (98.6)	58 (98.3)	87 (98.9)	472 (99.8)	251 (100)	221 (99.5)			
Have you ever had a wheezing attack that has made you feel short of breath									
Yes	3 (2.0)	0	3 (3.4)	6 (1.3)	2 (0.8)	4 (1.8)	1.62 [0.4- 6.56]	ND	2.28 [0.41- 12.57]
No	144 (98.0)	59 (100)	85 (96.6)	467 (98.7)	249 (99.2)	218 (98.2)			
Have you had 2 or more such episodes									
Yes	1 (.7)	0	1 (1.1)	3 (0.6)	0	3 (1.4)	1.07 [0.11-10.37]	ND	ND
No	146 (99.3)	59 (100)	87 (98.9)	470 (99.4)	251 (100)	219 (98.6)			
Have you ever required medicine or treatment for the(se) attack(s)?									
Yes	1 (.7)	0	1 (1.1)	4 (0.8)	0	4 (1.8)	0.8 [0.09- 7.21]	ND	ND
No	146 (99.3)	59 (100)	87 (98.9)	469 (99.2)	251 (100)	218 (98.2)			
Are you troubled by shortness of breath when hurrying or walking up a slight hill?									
Yes	41 (27.9)	12 (20.3)	29 (33.0)	62 (13.1)	26 (10.4)	36 (16.2)	2.56 [1.63- 4.01]	1.93 [0.89- 4.19]	1.67 [0.97- 2.87]
No	106 (72.1)	47 (79.7)	59 (67.0)	411 (86.9)	225 (89.6)	186 (83.8)			
Do you have to walk slower than people of your age because of breathlessness?									
Yes	27 (18.4)	9 (15.3)	18 (20.5)	35 (7.4)	13 (5.2)	22 (9.9)	2.82 [1.64- 4.84]	1.43 [0.59- 3.44]	2.01 [0.99- 4.09]
No	120 (81.6)	50 (84.7)	70 (79.5)	438 (92.6)	238 (94.8)	200 (90.1)			
Do you ever have to stop for breath when walking at your own pace?									
Yes	21 (14.3)	8 (13.6)	13 (14.8)	31 (6.6)	9 (3.6)	22 (9.9)	2.38 [1.32- 4.29]	1.11 [0.43- 2.87]	2.96 [1.33- 6.57]
No	126 (85.7)	51 (86.4)	75 (85.2)	442 (93.4)	242 (96.4)	200 (90.1)			
Do you ever have to stop for breath after walking about 100 meters (or after a few minutes)?									
Yes	24 (16.3)	8 (13.6)	16 (18.2)	35 (7.4)	13 (5.2)	22 (9.9)	2.44 [1.4- 4.26]	1.42 [0.57-3.57]	2.01 [0.99- 4.09]
No	123 (83.7)	51 (86.4)	72 (81.8)	438 (92.6)	238 (94.8)	200 (90.1)			
Are you too breathless to leave the house or breathless while dressing or undressing?									
Yes	14 (9.5)	5 (8.5)	9 (10.2)	18 (3.8)	8 (3.2)	10 (4.5)	2.66 [1.29- 5.49]	1.23 [0.39- 3.87]	1.43 [0.55- 3.69]
No	133 (90.5)	54 (91.5)	79 (89.8)	455 (96.2)	243 (96.8)	212 (95.5)			
If you get a cold, does it usually go to your chest? (Usually means more than half the time.)									
Yes	13 (8.8)	3 (5.1)	10 (11.4)	24 (5.1)	12 (4.8)	12 (5.4)	ND	ND	ND
No	133 (90.5)	56 (94.9)	77 (87.5)	445 (94.1)	237 (94.4)	208 (93.7)			
Don't get colds	1 (0.7)	0	1 (1.1)	4 (0.8)	2 (0.8)	2 (0.9)			
During the past 3 years, have you had any chest illnesses that have kept you off work, at home, or in bed?									
Yes	3 (2.0)	0	3 (3.4)	8 (1.7)	0	8 (3.6)	1.21 [0.32- 4.62]	ND	ND
No	144 (98.0)	59 (100)	85 (96.6)	465 (98.3)	251 (100)	214 (96.4)			
Did you produce phlegm with any of these chest illnesses?									
Yes	2 (1.4)	0	2 (2.3)	0	0	0	ND	ND	ND
No	145 (98.6)	59 (100)	86 (97.7)	473 (100)	251 (100)	222 (100.0)			

APPENDIX 8: Past illnesses in the rural and industrial sites

	Total	Rural	Industrial	OR
Did you have any lung trouble before the age of 16?				
Yes	28 (4.5)	1 (0.3)	27 (8.7)	29.48 [3.98- 218.37]
No	592 (95.5)	309 (99.7)	283 (91.3)	
Have you ever had any of the following: (confirmed by a doctor):				
Attacks of bronchitis ?				
Yes	12 (1.9)	6 (1.9)	6 (1.9)	1.00 [0.32- 3.14]
No	608 (98.1)	304 (98.1)	304 (98.1)	
Pneumonia ?				
Yes	4 (0.6)	2 (0.6)	2 (0.6)	1.00 [0.14- 7.14]
No	616 (99.4)	308 (99.4)	308 (99.4)	
Hay fever ?				
Yes	3 (0.5)	1 (0.3)	2 (0.6)	2.01 [0.18-22.28]
No	617 (99.5)	309 (99.7)	308 (99.4)	
Chronic bronchitis ?				
Yes	1 (0.2)	0	1 (0.3)	ND
No	619 (99.8)	310 (100)	309 (99.7)	
Do you still have it?				
Yes	0	0	0	ND
No	620 (100)	310 (100)	310 (100)	
Emphysema ?				
Yes	1 (0.2)	0	1 (0.3)	ND
No	619 (99.8)	310 (100)	309 (99.7)	
Do you still have it?				
Yes	1 (0.2)	0	1 (0.3)	ND
No	619 (99.8)	310 (100)	309 (99.7)	
Asthma ?				
Yes	24 (3.9)	10 (3.2)	14 (4.5)	1.42 [0.62- 3.25]
No	596 (96.1)	300 (96.8)	296 (95.5)	
Do you still have it?				
Yes	17 (2.7)	6 (1.9)	11(3.5)	1.86 [0.68-5.09]
No	603 (97.3)	304 (98.1)	299 (96.5)	
Have you ever had:				
Any other chest illness?				
Yes	12 (1.9)	3 (1)	9 (2.9)	3.06 [0.82-11.41]
No	608 (98.1)	307 (99)	301 (97.1)	
If yes, please specify : allergy, clotting, heart problems, valve problems				
Any chest operations?				
Yes	5 (0.8)	0	5 (1.6)	ND
No	615 (99.2)	310 (100)	305 (98.4)	
If yes, please specify : ballon, open heart, pacemaker, stent placement				
Any chest injuries?				
Yes	0	0	0	ND
No	620 (100)	310 (100)	310 (100)	
Has a doctor ever told you that you had heart problems ?				
Yes	43 (6.9)	19 (6.1)	24 (7.7)	1.29 [0.69-2.41]
No	577 (93.1)	291 (93.9)	286 (92.3)	
Have you ever had treatment for heart problems in the past 10 years?				
Yes	42 (6.8)	19 (6.1)	23 (7.4)	1.23 [0.66-2.31]
No	578 (93.2)	291 (93.9)	287 (92.6)	
Has a doctor ever told you that you had high blood pressure ?				
Yes	106 (17.1)	44 (14.2)	62 (20)	1.51 [0.99- 2.31]
No	514 (82.9)	266 (85.8)	248 (80)	
Have you had any treatment for high blood pressure in the past 10 years?				
Yes	102 (16.5)	43 (13.9)	59 (19)	1.46 [0.95-2.24]
No	518 (83.5)	267 (86.1)	251 (81)	

APPENDIX 9: Differences in past illnesses prevalence between men and women

	Men			Women			OR		RR
	Total	Rural	Industrial	Total	Rural	Industrial	Men (Industrial- rural)	Women (Industrial- rural)	Total
Did you have any lung trouble before the age of 16?									
Yes	9 (2.7)	0	9 (5.7)	19 (6.5)	1 (0.7)	18 (11.8)	ND	18.4 [2.42- 139.77]	0.42 [0.18-0.92]
No	319 (97.3)	171 (100)	148 (94.3)	273 (93.5)	138 (99.3)	135 (88.2)			
Have you ever had any of the following: (confirmed by a doctor):									
Attacks of bronchitis ?									
Yes	6 (1.8)	2 (1.2)	4 (2.5)	6 (2.1)	4 (2.9)	2 (1.3)	2.21 [0.1- 12.24]	0.45 [0.08- 2.5]	0.89 [0.28-2.79]
No	322 (98.2)	169 (98.8)	153 (97.5)	286 (97.9)	135 (97.1)	151 (98.7)			
Pneumonia ?									
Yes	3 (0.9)	1 (0.6)	2 (1.3)	1 (0.3)	1 (0.7)	0	2.19 [0.2-24.39]	ND	2.67 [0.28-26]
No	325 (99.1)	170 (99.4)	155 (98.7)	291 (99.7)	138 (99.3)	153 (100)			
Hay fever ?									
Yes	0	0	0	3 (1)	1 (0.7)	2 (1.3)	ND	1.83 [0.16- 20.41]	ND
No	328 (100)	171 (100)	157 (100)	289 (99)	138 (99.3)	151 (98.7)			
Chronic bronchitis ?									
Yes	1 (0.3)	0	1 (0.6)	0	0	0	ND	ND	ND
No	327 (99.7)	171 (100)	156 (99.4)	292 (100)	139 (100)	153 (100)			
Do you still have it?									
Yes	0	0	0	0	0	0	ND	ND	ND
No	328 (100)	171 (100)	157 (100)	292 (100)	139 (100)	153 (100)			
Emphysema ?									
Yes	1 (0.3)	0	1 (0.6)	0	0	0	ND	ND	ND
No	327 (99.7)	171 (100)	156 (99.4)	292 (100)	139 (100)	153 (100)			
Do you still have it?									
Yes	1 (0.3)	0	1 (0.6)	0	0	0	ND	ND	ND
No	327 (99.7)	171 (100)	156 (99.4)	292 (100)	139 (100)	153 (100)			
Asthma ?									
Yes	14 (4.3)	6 (3.5)	8 (5.1)	10 (3.4)	4 (2.9)	6 (3.9)	1.48 [0.5- 4.36]	1.38 [0.38- 5]	1.25 [0.55-2.88]
No	314 (95.7)	165 (96.5)	149 (94.9)	282 (96.6)	135 (97.1)	147 (96.1)			
Do you still have it?									
Yes	11 (3.4)	4 (2.3)	7 (4.5)	6 (2.1)	2 (1.4)	4 (2.6)	1.95 [0.5- 4.36]	1.38 [0.38-5]	1.63 [0.6-4.52]
No	317 (96.6)	167 (97.7)	150 (95.5)	286 (97.9)	137 (98.6)	149 (97.4)			
Have you ever had:									
Any other chest illness?									
Yes	6 (1.8)	1 (0.6)	5 (3.2)	6 (2.1)	2 (1.4)	4 (2.6)	5.59 [0.65- 48.38]	1.84 [0.33- 10.21]	0.89 [0.28- 2.79]
No	322 (98.2)	170 (99.4)	152 (96.8)	286 (97.9)	137 (98.6)	149 (97.4)			
Any chest operations?									
Yes	4 (1.2)	0	4 (2.5)	1 (0.3)	0	1 (0.7)	ND	ND	3.56 [0.4-32.3]
No	324 (98.8)	171 (100)	153 (97.5)	291 (99.7)	139 (100)	152 (99.3)			
Any chest injuries?									
Yes	0	0	0	0	0	0	ND	ND	ND

No	328 (100)	171 (100)	157 (100)	292 (100)	139 (100)	153 (100)			
Has a doctor ever told you that you had heart problems ?									
Yes	28 (8.5)	15 (8.8)	13 (8.3)	15 (5.1)	4 (2.9)	11 (7.2)			1.66
No	300 (91.5)	156 (91.2)	144 (91.7)	277 (94.9)	135 (97.1)	142 (92.8)	0.94 [0.43- 2.04]	2.61 [0.81- 8.4]	[0.9-3.29]
Have you ever had treatment for heart problems in the past 10 years?									
Yes	27 (8.2)	15 (8.8)	12 (7.6)	15 (5.1)	4 (2.9)	11 (7.2)			1.6
No	301 (91.8)	156 (91.2)	145 (92.4)	277 (94.9)	135 (97.1)	142 (92.8)	0.86 [0.39- 1.9]	2.61 [0.81-8.4]	[0.86-3.19]
Has a doctor ever told you that you had high blood pressure ?									
Yes	59 (18)	28 (16.4)	31 (19.7)	47 (16.1)	16 (11.5)	31 (20.3)			1.12
No	269 (82)	143 (83.6)	126 (80.3)	245 (83.9)	123 (88.5)	122 (79.7)	1.26 [0.72- 2.22]	1.95 [1.01- 3.75]	[0.75-1.74]
Have you had any treatment for high blood pressure in the past 10 years?									
Yes	57 (17.4)	27 (15.8)	30 (19.1)	45 (15.4)	16 (11.5)	29 (19)			1.13
No	271 (82.6)	144 (84.2)	127 (80.9)	247 (84.6)	123 (88.5)	124 (81)	1.26 [0.71- 2.23]	1.8 [0.93- 3.48]	[0.75-1.76]

Yes	6 (4.1)	0	6 (6.8)	6 (1.3)	3 (1.2)	3 (1.4)	3.31 [1.05- 10.42]	ND	1.13 [0.23- 5.66]
No	141 (95.9)	59 (100)	82 (93.2)	467 (98.7)	248 (98.8)	219 (98.6)			
If yes, please specify : allergy, clotting, heart problems, valve problems									
Any chest operations?							13.2 [1.46- 119.05]	ND	ND
Yes	4 (2.7)	0	4 (4.5)	1 (0.2)	0	1 (0.5)			
No	143 (97.3)	59 (100)	84 (95.5)	472 (99.8)	251 (100.0)	221 (99.5)			
If yes, please specify : ballon, open heart, pacemaker, stent placement									
Any chest injuries?							ND	ND	ND
Yes	0	0	0	0	0	0			
No	147 (100.0)	59 (100)	88 (100.0)	473 (100)	251 (100.0)	222 (100.0)			
Has a doctor ever told you that you had heart problems?							2.02 [1.06- 3.86]	2.17 [0.66- 7.09]	0.9 [0.41- 1.97]
Yes	16 (10.9)	4 (6.8)	12 (13.6)	27 (5.7)	15 (6.0)	12 (5.4)			
No	131 (89.1)	55 (93.2)	76 (86.4)	446 (94.3)	236 (94.0)	210 (94.6)			
Have you ever had treatment for heart problems in the past 10 years?							2.01 [1.09- 4.03]	2.17 [0.66- 7.09]	0.82 [0.37- 1.82]
Yes	16 (10.9)	4 (6.8)	12 (13.6)	26 (5.5)	15 (6.0)	11 (5.0)			
No	131 (89.1)	55 (93.2)	76 (86.4)	447 (94.5)	236 (94.0)	211 (95.0)			
Has a doctor ever told you that you had high blood pressure?							1.5 [0.94- 2.38]	2.39 [0.99- 5.77]	1.23 [0.75- 2.02]
Yes	32 (21.8)	8 (13.6)	24 (27.3)	74 (15.6)	36 (14.3)	38 (17.1)			
No	115 (78.2)	51 (86.4)	64 (72.7)	399 (84.4)	215 (85.7)	184 (82.9)			
Have you had any treatment for high blood pressure in the past 10 years?							1.43 [0.89- 2.3]	2.13 [0.88- 5.18]	1.23 [0.74- 2.03]
Yes	30 (20.4)	8 (13.6)	22 (25.0)	72 (15.2)	35 (13.9)	37 (16.7)			
No	117 (79.6)	51 (86.4)	66 (75.0)	401 (84.8)	216 (86.1)	185 (83.3)			

APPENDIX 11: Family history and children health

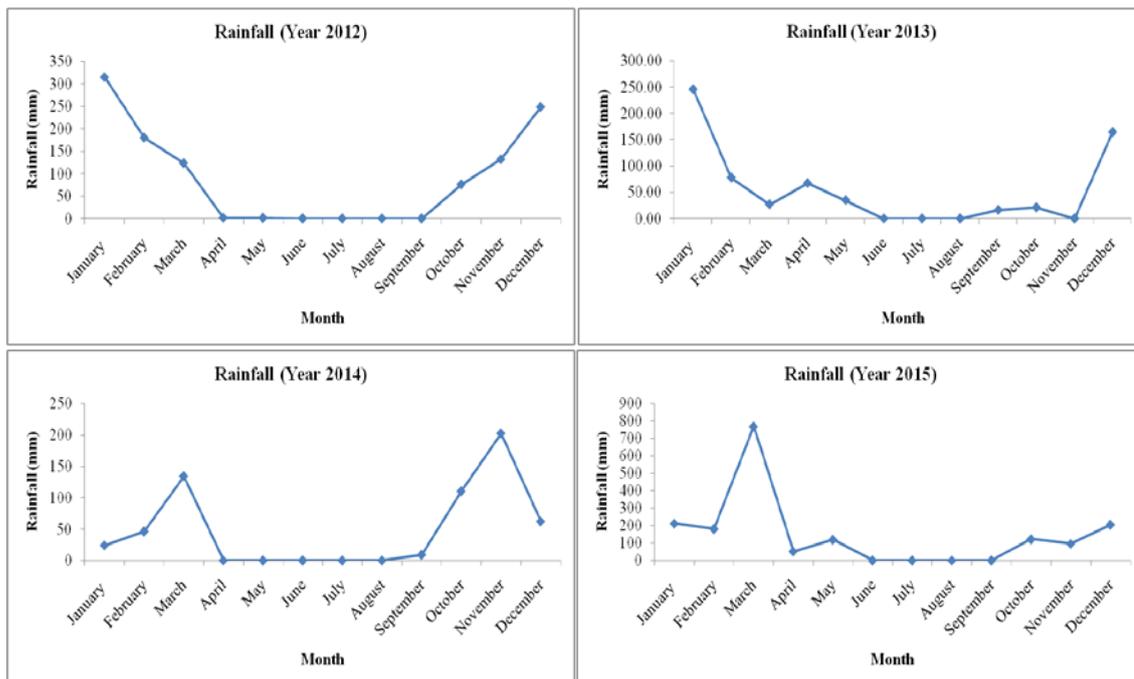
	Total	Rural	Industrial	OR
Does or did any of your parents work in an industry?				
Yes	89 (14.4)	18 (5.8)	71 (22.9)	4.82 [2.8-8.31]
No	531 (85.6)	292 (94.2)	239 (77.1)	
Were either of your natural parents ever told by a doctor that they had a chronic lung condition such as:				ND
Chronic bronchitis	9 (1.5)	1 (0.3)	8 (2.6)	
Emphysema	8 (1.3)	1(0.3)	7 (2.3)	
Asthma	68 (11)	23 (7.4)	45 (14.5)	
Lung cancer	78 (12.6)	24 (7.7)	54 (17.4)	
Lung cancer and other chest conditions	1 (0.2)	0	1 (0.3)	
Other chest conditions: allergy	2 (0.3)	2 (0.6)	8(2.6)	
Other chest conditions	10 (1.6)	2 (0.6)	0	
Did anyone in your family die of lung cancer?				
Yes	66 (10.6)	21 (6.8)	45 (14.5)	2.34 [1.36-4.03]
No	554 (89.4)	289 (93.2)	265 (85.5)	
Did your child have any chest illness or chest cold?				
Yes	13 (4.2)	1 (0.7)	12 (7.7)	12.67 [1.63-98.69]
Specify: abdominal pain, allergy, asthma, bronchitis, cold				
No	296 (95.8)	152 (99.3)	144 (92.3)	
Was he/she ever hospitalized for a severe chest illness or chest cold before the age of 2 years?				
Yes	9 (2.9)	4 (2.6)	5 (3.2)	1.24 [0.33-4.71]
No	299 (97.1)	149 (97.4)	150 (96.8)	
Did a physician state that your child had an allergic reaction to pollen or dust?				
Yes	68 (22.1)	34 (22.2)	34 (22.1)	3.07 [1.69-5.58]
No	239 (77.9)	119 (77.8)	38.7 (77.9)	
To what extent do you think air quality affects your child/children's respiratory health?				
Seriously affects	98 (31.8)	45 (29.4)	53 (34.2)	ND
Somewhat affects	143 (46.4)	70 (45.8)	73 (47.1)	
No impact	63 (20.5)	34 (22.2)	29 (18.7)	
Don't know	4 (1.3)	4 (1.3)	0	

APPENDIX 12: Stratification over gender: Family history and children health

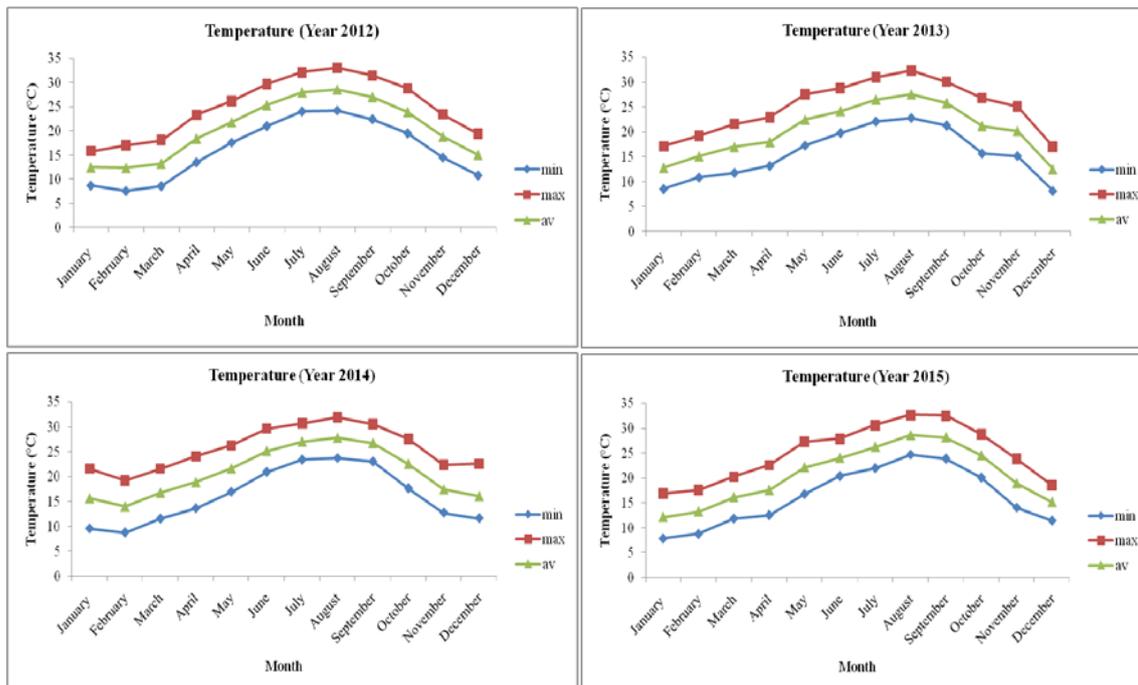
	Men			Women			OR		RR
	Total	Rural	Industrial	Total	Rural	Industrial	Men (Industrial- rural)	Women (Industrial- rural)	Total
Does or did any of your parents work in an industry?									
Yes	43 (13.1)	12 (7)	31 (19.7)	46 (15.8)	6 (4.3)	40 (26.1)	3.26	7.85	0.83
No	285 (86.9)	159 (93)	126 (80.3)	246 (84.2)	133 (95.7)	113 (73.9)	[1.61- 6.61]	[3.21- 19.19]	[0.52-1.27]
Were either of your natural parents ever told by a doctor that they had a chronic lung condition such as:									
Chronic bronchitis	8 (2.4)	1 (0.6)	7 (4.5)	1 (0.3)	0	1 (0.7)			
Emphysema	4 (1.2)	0	4 (2.5)	4 (1.4)	1 (0.7)	3 (2)			
Asthma	34 (10.4)	12 (7)	22 (14)	34 (11.6)	11 (7.9)	23 (15)			
Lung cancer	43 (13.1)	14 (8.2)	29 (18.5)	35 (12)	10 (7.2)	25 (16.3)	ND	ND	ND
Lung cancer and other chest conditions	1 (0.3)	0	0	0	0	0			
Other chest conditions: allergy	0	0	0	2 (0.7)	2 (1.4)	0			
Other chest conditions	4 (1.2)	1 (0.6)	3 (1.9)	6 (2.1)	1 (0.7)	5 (3.3)			
Did anyone in your family die of lung cancer?									
Yes	35 (10.7)	12 (7)	23 (14.6)	31 (10.6)	9 (6.5)	22 (14.4)	2.27	2.43	1.01
No	293 (89.3)	159 (93)	134 (85.4)	261 (89.4)	130 (93.5)	131 (85.6)	[1.09- 4.73]	[1.08- 5.48]	[0.61-1.68]
Did your child have any chest illness or chest cold?									
Yes	4 (2.5)	0	4 (5.3)	9 (6.1)	1 (1.5)	8 (10)	ND	7.44	0.41
No	157 (97.5)	85 (100)	72 (94.7)	139 (93.9)	67 (98.5)	72 (90)		[0.91- 61.08]	[0.12-1.29]
Was he/she ever hospitalized for a severe chest illness or chest cold before the age of 2 years?									
Yes	5 (3.1)	3 (3.5)	2 (2.6)	4 (2.7)	1 (1.5)	2 (3.8)	0.74	2.7	1.14
No	156 (96.9)	82 (96.5)	74 (97.4)	143 (97.3)	67 (98.5)	49.7 (96.2)	[0.12- 4.55]	[0.24- 30.62]	[0.3-4.37]
Did a physician state that your child had an allergic reaction to pollen or dust?									
Yes	30 (18.8)	18 (21.2)	12 (16)	38 (25.9)	16 (23.5)	22 (27.8)	0.71	1.25	0.73
No	130 (81.3)	67 (78.8)	63 (84)	109 (74.1)	52 (76.5)	57 (72.2)	[0.32- 1.59]	[0.59- 2.63]	[0.38-1.14]
To what extent do you think air quality affects your child/ children's respiratory health?									
Seriously affects	48 (29.8)	1 (30.6)	22 (28.9)	50 (34)	19 (27.9)	31 (39.2)			
Somewhat affects	75 (46.6)	37 (43.5)	38 (50)	68 (46.3)	33 (48.5)	35 (44.3)			
No impact	35 (21.7)	19 (22.4)	16 (21.1)	28 (19)	15 (22.1)	13 (16.5)	ND	ND	ND
Don't know	3 (1.9)	3 (3.5)	0	1 (0.7)	1 (1.5)	0			

APPENDIX 13: Variation of precipitation (in mm) (A) and evolution of temperatures (in °C) (B) in Tripoli between 2012 and 2015

A



B



APPENDIX 14: Physicochemical characterization techniques' principles

A. Size distribution

Laser diffraction measures particle size distributions by measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample. The angular scattering intensity data is then analyzed to calculate the size of the particles responsible for creating the scattering pattern. Particles below a few microns in diameter have very similar light scattering patterns that are alike in both shape and intensity. Large particles scatter light strongly at low angles and with readily detectable maxima and minima in the scattering pattern. This means that detectors placed at low angles relative to the optical path and with sufficient angular resolution can detect these maxima and minima. Conversely small particles scatter light weakly and without any discernible maxima and minima until extremely high angles of measurement are reached. This makes the detection and the resolution of the scattering pattern difficult. Polarization Intensity Differential Scattering (PIDS) technique relies upon the transverse nature of light i.e., that it consists of a magnetic vector and an electric vector at ninety degrees to it. Light at three wavelengths (450 nm, 600 nm, and 900 nm) sequentially irradiates the sample, with first vertical and then horizontal polarized light. The scattered light from the samples is measured over a range of angles. By analyzing the differences between the horizontally and the vertically radiated light for each wavelength, we gain information about the particle size distribution of the sample. The intensity vs. scattering angle information from the signals is then incorporated into the standard algorithm from the intensity vs. scattering angle data from the laser light scattering to give a continuous size distribution. Therefore the basis of the method is that a laser light source is used to illuminate particulates, usually contained within a suitable sample cell. The light scattered by the particles is then detected by silicon photo-detectors. The intensity of light on each detector, measured as a function of angle, is then subjected to mathematical analysis using a complex inversion matrix algorithm. The result is a particle size distribution displayed as volume % in discrete size classes.

B. Morphology and single particle analysis

Morphology and single particle analysis were performed using the scanning electron microscope with energy dispersive X-ray analysis (SEM-EDX). SEM provides detailed high resolution images of the sample by rastering a focused electron beam across the surface and detecting secondary or back-scattered electron signal. An Energy Dispersive X-Ray Analyzer (EDX) is also used to provide elemental identification and quantitative compositional information. An electron beam of a few nanometers in diameter scans the surface of the sample. When electrons strike the sample, they cause several phenomena: the sample itself emits secondary electrons, a part of the primary electrons is reflected (back-scattering), electrons are absorbed by the sample, and the sample

emits X-rays. All these phenomena are interrelated with the topography, the atomic number and the chemical state of the sample. Secondary electrons are thus exploited to obtain the topographic images, while the backscattered electrons are used to obtain an image based on the difference in atomic number of sample's elements (chemical contrast image). X rays emitted allow the recognition of the chemical composition of the sample.

C. Identification of crystalline phases

X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda = 2d \sin \theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of 2θ angles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns.

D. ICP-AES

ICP-AES was used to quantify some elements that are usually found in moderate to high concentrations in the atmosphere and hence do not require any additional sensitivity in the detection procedure. Electrons of an atom absorb energy and jump to higher energy levels. When they return to normal states, they emit characteristic photons of energy. By isolating these photon wavelengths, the types and concentrations of the elements present can be determined. The analysis begins with the sample injection (in liquid form) in the analyzer, and then the solution containing the elements passes through a nebulizer which transforms it into fine droplets and injects it in the plasma torch in a flux of argon gas which excites the atomic species in the aerosol. The argon gas is burned with the droplets at 6000- 1000°C which is sufficient to ionize all the atoms in the sample by eliminating one peripheral electron from each. Inside the torch, the sample goes through different phases: liquid, solid, gas, atom then ion. The plasma produces a double action on atoms: it excites making them emit photons (photons that are detected when using ICP-AES) and it passed the state of positive mono-charged ions (these ions are then directed and identified by a mass spectrometer in the case of the ICP-MS). Instantly after the transformation, the excited atom will return to its initial non-excited status and emit a ray that is characteristically linked to the atom. The group of rays emitted from the excited atoms is transferred inside a dispersive system and then diverged to separate each ray by its wavelength. The

diverged rays are redirected to face a plate formed by photomultiplier that increase the wavelengths intensity and transform it into electric signals corresponding to each element. The electric signals are captured and projected as peaks that are proportional to the corresponding elements concentrations. The emitted rays are detected in this case by a charge injection device (CID).

The limitations of this technique are essentially linked to phenomena of spectral interference formed due to the superposition of atomic emission rays of different elements. Thus one atom can emit many rays (having different wavelengths) while going back to non-excited status depending on which high energy level he is found at after excitation. The selection of rays was based on the capacity of these latter to give the highest intensity for each element and on the minimum interferences between them.

E. ICP-MS

Mass spectrometry is an analytical technique based on the separation, identification and quantification of different isotopes of the elements of a sample according to their mass. Each sample is injected and then sprayed with a nebulizer inside a plasma torch where the elements of the sample change phases starting from liquid, passing through solid and gas, and ending in an excited atom form. The ions formed in the plasma are extracted by an interface constituted of two cones (sampler and skimmer) allowing the passage of these ions to the separation system. The ion formed enters a quadrupole unit that separates in a fraction of a second the ions depending on their mass to charge ratio (m/z). Hence, each ion quits the quadrupole at a certain specific time. Once the ions have been separated by their mass-to-charge ratio, they must then be detected or counted by a suitable detector. The fundamental purpose of the detector is to translate the number of ions striking the detector into an electrical signal that can be measured and related to the number of atoms of that element in the sample via the use of calibration standards. Finally, the concentration relative to each element that hits the detector is proportional to its quantity in the sample and is measured by the mass spectrometer in counts per second.

Within the plasma or the ion beam going out from the plasma, recombination can take place between the elements from the plasma (Ar), air (O_2 , N_2 , CO_2), solvent, and even the sample. Many formed polyatomic species such as ArC^+ , ArO^+ , $NaAr^+$, $CaAr^+$, and $MgAr^+$ can disrupt the measurements due to the fact that they have the same m/z ratio than that of other analyzed elements and therefore their presence leads to an overestimation. Other than polyatomic ions, isobaric interferences may occur when two different isotopes of two elements have the same m/z ratio. Polyatomic interferences can be reduced by using the collision gases: helium, hydrogen and air. These gases are injected one at a time in a Collision Reaction Cell (CRC) located between the plasma torch and the mass spectrometer. This section of the instrumentation is also known as Collision Reaction Interface (CRI). The gas collides with polyatomic species, breaks them and continues its path. Collision gases selection is based on their efficiency from a thermodynamic point of view, and also on

the atomic mass. Smaller masses reduce the loss of sensitivity due to atoms dispersion during the collisions.

F. Ion chromatography

This technique separates ions based on their affinity to the ion exchanger. The equilibrated stationary phase consists of an ionizable functional group where the targeted molecules of a mixture to be separated and quantified can bind while passing through the column. This method applies the idea of the interaction between molecules and the stationary phase which are charged oppositely to each other. Cation exchange chromatography is used when the desired molecules to separate are cations; and anion exchange chromatography is used to separate anions, meaning that the beads in the column contain positively charged functional groups to attract the anions. The bound molecules then can be eluted and collected using an eluent which contains anions and cations by running higher concentration of ions through the column or changing pH of the column. The concentration of the exchangeable counter ions, which competes with the molecules for binding, can be increased or the pH can be changed. The greater the charge density of a solute, the more the ion is retained by the stationary phase. A change in pH changes the charge on the particular molecules and, therefore, alters binding. An electrochemical suppressor is used to decrease the mobile phase signal.

G. Elemental microanalysis by the CHNS-O Analyzer

This analyzer is equipped with a quartz reactive column and two chromatographic columns: one for CHNS analysis and another column for O. The results are analyzed by the EagerXperience software. The quartz reactive column is composed of a combustion area at the top, which is a few centimeters of empty space that is heated up to 900°C to allow the combustion of the introduced sample. Afterwards, a 10 mm layer of quartz wool separates between the combustion zone and a few centimeters layer of copper oxide granules. This latter is separated from copper metallic bars (up to 10 cm) by another 10 mm layer of quartz wool. At the end of the copper bars, another 25 mm of quartz wool are laying on the bottom of the column. A constant helium flux passes through the column and plays the role of carrier gas.

The samples are dropped into the vertical quartz combustion tube, through which a constant flow of helium is run. When the sample is introduced, the helium stream is temporarily enriched with pure oxygen and tin rapidly atomize, thus increasing the temperature around the sample up to 1800°C approximately. Flash combustion takes place, primed by the oxidation of the tin container. Quantitative combustion is then achieved by passing the mixture of gases over tungstic oxide. At this high temperature, all carbon content of the sample in the capsules is atomized. The mixture of gases is then passed over copper to remove the excess of oxygen and reduce oxides of nitrogen to nitrogen. Total released carbon is then oxidized into CO₂ after passing through the oxidation section formed by

cooper oxide granules. It then passes through a chromatographic column to be separated and then quantified by a thermal conductivity detector (TCD) (also called a katharometer). The gases eluted are N_2 , CO_2 , H_2O , and SO_2 (in the latter case, the addition of a vanadium pentoxide catalyst is required).

For the total carbon measurement, the detection at this level is measured by the difference in the thermal conductivity between the carrier gas and the formed CO_2 .

H. GC-MS

GC-MS is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. It is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions as well as the phase properties. The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The compounds are brought to the gaseous state at the injection system and are transported by an inert carrier gas (helium in our case) in the chromatography column where they are retained by the stationary phase. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio. A chromatogram representing all the ions according to their retention time is obtained at the end of the analysis and desired compounds can be identified according to their m/z ratios and their retention time.

APPENDIX 15: Concentrations of inorganic ions, major elements, trace elements and total carbon in individual samples of fine and ultrafine particles collected in Zakroun (Industrial site)

ng/m ³	Industrial fine particulate (FP-I)					Industrial quasi-ultrafine particulate (UFP-I)				
	FP-I1	FP-I2	FP-I3	FP-I4	FP-I5	UFP-I1	UFP-I2	UFP-I3	UFP-I4	UFP-I5
F ⁻	9.7	34.2	4.9	25.4	10.7	22.8	52.3	162	43.6	18.7
Cl ⁻	427	1850	170	1067	708	164	120	541	361	276
NO ₃ ⁻	1915	1732	541	3740	2146	1612	581	2486	1762	1838
PO ₄ ³⁻	9.0	5.8	15.3	22.5	3.6	14.0	11.5	39.4	27.6	40.0
SO ₄ ²⁻	1065	1570	900	2686	1559	3500	4948	7456	6500	8418
Na ⁺	437	1371	210	1293	733	670	450	1500	1162	1071
NH ₄ ⁺	117	14.6	27.5	143	11.1	650	1134	1000	850	1260
K ⁺	46.8	83	16.2	114	55	84	26.4	145	118	244
Mg ²⁺	63	187	29.4	175	95	89	59	295	151	139
Ca ²⁺	1343	1756	562	2817	1496	884	766	1398	1534	2133
Ag	0.02	0.01	0.02	0.03	0.01	0.00	< DL	< DL	0.00	< DL
Al	367	1305	484	455	229	-	-	-	-	-
As	0.14	0.05	0.22	0.15	0.05	0.02	0.04	0.02	0.03	0.00
Ba	5.1	7.5	7.8	6.0	3.8	< DL	54.3	36.6	3.3	0.7
Cd	0.02	0.04	0.08	0.03	0.02	< DL	< DL	< DL	0.003	0.000
Ce	0.40	1.4	0.54	0.40	0.21	0.04	0.41	0.11	0.04	0.02
Co	0.15	0.31	0.25	0.14	0.10	0.01	0.05	0.02	0.02	0.00
Cr	0.61	0.98	1.0	0.74	0.41	3.1	10.6	17.2	4.6	3.8
Cu	61	16.2	48.7	76	34.7	54.2	18.1	50	56	83
Fe	255	724	355	278	150	62	442	159	150	277
La	0.31	0.84	0.61	0.32	0.23	0.02	0.17	0.03	0.05	< DL
Mn	3.8	9.4	5.4	4.0	2.2	1.4	6.9	4.1	2.8	5.3
Nb	0.13	0.51	0.16	0.07	0.03	0.02	0.19	0.06	0.02	0.01
Ni	1.8	2.0	3.1	1.0	0.5	0.26	0.05	0.49	0.40	0.02
P	22.9	22.2	36.5	27.2	18.3	10.8	22.3	18.5	16.2	36.2
Pb	3.8	0.7	21.9	10.7	4.9	3.6	9.8	28.7	8.5	13.3
Rb	0.39	1.3	0.47	0.43	0.20	0.04	0.32	0.10	0.03	0.02
Sb	0.30	0.09	0.82	0.41	0.21	0.02	< DL	0.00	0.09	0.02
Sc	0.07	0.22	0.08	0.08	0.03	0.01	0.07	0.02	0.01	0.00
Se	1.0	2.0	1.5	1.1	0.63	0.09	0.12	0.35	0.11	0.04
Sn	0.29	0.12	0.81	0.36	0.20	0.02	< DL	< DL	0.09	< DL
Sr	9.1	18.3	12.9	12.2	7.7	5.9	24.0	24.0	10.3	12.8
Te	0.27	0.31	0.59	0.56	0.23	0.01	0.02	0.00	0.05	< DL
Ti	26.6	79.2	35.8	28.0	15.2	2.6	18.8	6.9	2.6	0.9
V	2.8	3.6	4.7	2.5	1.7	7.3	8.2	20.0	9.1	14.4
Zn	6.5	5.6	20.8	10.1	8.9	20.5	47.7	103	26.3	31.5
TC	1798	1696	3293	2146	1504	2804	3316	6861	6383	5246

< DL: under the detection limit

- : not determined

APPENDIX 16: Concentrations of inorganic ions, major elements, trace elements and total carbon in individual samples of fine and ultrafine particles collected in Kaftoun (Rural site)

ng/m ³	Rural fine particulate (FP-R)				Rural quasi-ultrafine particulate (UFP-R)			
	FP-R1	FP-R2	FP-R3	FP-R4-5	UFP-R1	UFP-R2	UFP-R3	UFP-R4-5
F ⁻	4.9	14.2	2.6	11.1	46.0	40.8	43.9	16.5
Cl ⁻	105	1059	210	685	200	145	150	123
NO ₃ ⁻	658	2200	563	2993	850	660	726	539
PO ₄ ³⁻	2.4	10.3	3.8	2.8	10.1	12.8	11.3	11.8
SO ₄ ²⁻	204	2026	540	1590	3545	5261	5955	3172
Na ⁺	108	802	230	817	638	310	450	415
NH ₄ ⁺	6.1	28.4	3.8	25.2	611	1453	1786	700
K ⁺	13.6	105	14.9	78	78	60	66	33.0
Mg ²⁺	20.2	163	35.0	117	83	43.2	71	54
Ca ²⁺	461	2950	511	1694	522	628	428	532
Ag	0.01	0.02	0.00	0.01	0.00	< DL	< DL	0.001
Al	206	4569	173	362	-	-	-	-
As	0.04	0.51	0.04	0.04	0.02	0.03	< DL	0.01
Ba	2.7	26.2	2.8	4.5	25.9	30.7	4.2	5.4
Cd	0.01	0.07	0.01	0.01	0.00	< DL	< DL	< DL
Ce	0.23	5.1	0.19	0.33	0.12	0.45	0.07	0.01
Co	0.07	0.99	0.07	0.12	0.03	0.07	0.00	0.00
Cr	0.39	4.3	0.36	0.35	5.2	7.0	2.0	1.9
Cu	6.1	14.1	6.8	38.9	12.5	20.3	4.0	23.7
Fe	143	2569	128	227	86	823	6.7	60
La	0.16	2.8	0.15	0.25	0.07	0.22	0.02	0.002
Mn	2.3	34.3	2.2	3.7	2.1	11.8	< DL	1
Nb	0.09	2.2	0.05	0.07	0.06	0.22	0.04	0.002
Ni	0.60	3.1	0.62	0.70	0.32	0.16	0.05	0.02
P	10.9	60	14.2	20.9	14.4	12.0	7.2	6.5
Pb	0.8	1.2	1.1	5.4	6.3	5.7	3.7	6.9
Rb	0.21	4.6	0.18	0.33	0.11	0.42	0.07	0.01
Sb	0.13	0.06	0.11	0.33	0.05	< DL	< DL	0.01
Sc	0.05	0.82	0.03	0.05	0.03	0.08	0.01	0.003
Se	0.62	3.7	0.59	0.93	0.31	0.15	0.02	0.02
Sn	0.10	0.23	0.06	0.21	0.03	< DL	< DL	0.007
Sr	4.9	74	5.3	8.9	8.8	27.4	1.2	4.3
Te	0.11	0.94	0.12	0.21	0.03	0.01	< DL	0.02
Ti	15.5	275	13.2	22.9	7.8	23.7	3.4	0.6
V	1.0	8.3	1.1	1.8	5.8	6.3	3.0	4.7
Zn	2.5	11.1	3.0	7.5	27.6	30.4	11.0	13.4
TC	748	3239	996	1417	2631	2700	2938	4014

< DL: under detection limit

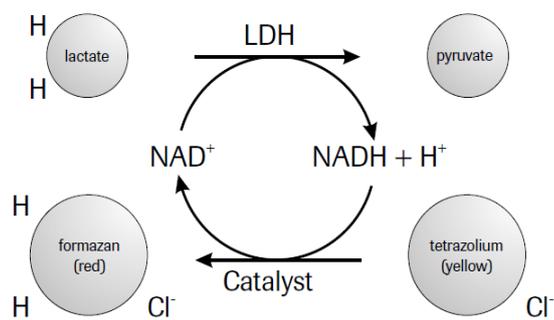
- : not determined

APPENDIX 17: PAHs concentrations in the quasi-ultrafine particles collected under industrial (UFP-I) and rural (UFP-R) influence expressed in pg/m^3

pg/m^3		UFP-I					UFP-R			
		UFP-I1	UFP-I2	UFP-I3	UFP-I4	UFP-I5	UFP-R1	UFP-R2	UFP-R3	UFP-R4-5
3 rings	Naphtalene	-	-	-	-	-	-	-	-	-
	Acenaphtylene	-	-	-	-	-	-	-	-	-
	Acenaphtene	-	-	-	-	-	-	-	-	-
	Fluorene	127	483	518	197	179	-	-	-	-
	Phenanthrene	101	374	406	153	141	188	-	-	57
4 rings	Anthracene	122	478	510	194	178	-	-	-	-
	Fluoranthene	126	454	510	187	171	228	-	198	66
	Pyrene	108	390	435	160	146	201	191	177	56
	Benz[a]Anthracene	180	684	756	278	257	331	339	293	96
5 rings	Chrysene	132	435	492	179	165	213	216	187	63
	Benzo[b]fluoranthene	258	697	913	303	273	366	336	291	110
	Benzo[k]fluoranthene	151	501	597	207	191	-	-	-	70
	Benzo[a]pyrene	177	692	755	274	253	359	336	304	99
6 rings	Dibenz[a,h]anthracene	271	990	1089	404	376	-	-	-	-
	Indeno[1,2,3-c,d]pyrene	363	1052	1306	440	404	527	495	433	172
	Benzo[g,h,i]perylene	345	970	1269	419	371	499	438	385	164

APPENDIX 19: Microplate planning for the cytotoxicity tests

	1	2	3	4	5	6	7	8	9	10	11	12
A	200µL of LHC-9 medium	LHC-9 medium with 240 µg/ cm ² of PM _{2.5-0.3}	Negative control: 200µL of cells		Cells with 3.75 µg/ cm ² of PM _{2.5-0.3}	Cells with 7.5 µg/ cm ² of PM _{2.5-0.3}	Cells with 15 µg/ cm ² of PM _{2.5-0.3}	Cells with 30 µg/ cm ² of PM _{2.5-0.3}	Cells with 60 µg/ cm ² of PM _{2.5-0.3}	Cells with 120 µg/ cm ² of PM _{2.5-0.3}	Cells with 180 µg/ cm ² of PM _{2.5-0.3}	Cells with 240 µg/ cm ² of PM _{2.5-0.3}
B												
C												
D												
E		Cells with the positive control Triton 4%										
F												
G												
H												

APPENDIX 20: Principle of the test of the activity of lactate dehydrogenase (LDH)

APPENDIX 21: Principle of RT-qPCR

Real-time quantitative polymerase chain reaction method (qPCR), preceded by a step of Reverse Transcription (RT) of the mRNA (RT-qPCR), is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. This technique became possible after introduction of an oligonucleotide probe which was designed to hybridize within the target sequence. Cleavage of the probe during PCR because of the 5' nuclease activity of Taq polymerase can be used to detect amplification of the target-specific product and allows the determination of the expression level of target genes in cells. This method comprises several steps: RNA extraction, reverse transcription of mRNA into cDNA (RT) and amplification and quantification of cDNA (qPCR).

Each amplification cycle involves three steps: denaturation of double-stranded DNA by heating, primer annealing and finally the synthesis of the sequence complementary to the template strand. This cycle is repeated n times.

TaqMan assay, the used quantitative detection system, is adopted for both the quantification of mRNAs and for detecting variation. The method exploits the 5' endonuclease activity of *Taq* DNA polymerase to cleave an oligonucleotide probe during PCR amplification, thereby generating a detectable signal. The probes are fluorescently labeled at their 5' end (reporter) and are non-extendable at their 3' end by chemical modification (a fluorochrome suppressor, the quencher). Specificity is conferred at three levels: via two PCR primers and the probe. Probes also include a minor groove binder (MGB[®]) for added specificity, which increases the melting temperature (T_m) of the probe, stabilizes its interaction with the DNA template and avoids mismatches. The primers are chosen exon-exon junction, thus avoiding the amplification of any residual genomic DNA and ensure the specificity of the reaction. Because the close proximity between the quench molecule and the fluorescent probe normally prevents fluorescence from being detected through FRET, the decoupling results in the increase of intensity of fluorescence proportional to the number of the probe cleavage cycles. It is therefore based on the detection and quantification of a fluorochrome during the amplification process. The increase in fluorescent emission signal is directly proportional to the amount of amplicons produced during the reaction. It is thus possible to use smaller probes consisting of 12-20 bases compared to the 25 to 30 bases conventional TaqMan[®] probes. The method is then more sensitive because the quenching efficiency is improved by the short length of the probe.

APPENDIX 22: Principle of flow cytometry

Flow cytometry is a technology that is used to analyze the physical and chemical characteristics of particles in a fluid as it passes through at least one laser. Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths. The fluorescence can be measured to determine various properties of single particles, which are usually cells. Up to thousands of particles per second can be analyzed as they pass through the liquid stream. Examples of the properties measured include the particle's relative granularity, size and fluorescence intensity as well as its internal complexity. An optical-to-electronic coupling system is used to record the way in which the particle emits fluorescence and scatters incident light from the laser.

Three main systems make up the flow cytometer instrument and these are the fluidics, the optics and the electronics. The purpose of the fluidics system is to transport the particles in a stream of fluid to the laser beam where they are interrogated. The optics system is made up of lasers which illuminate the particles present in the stream as they pass through and scatter light from the laser. Generally, the light, scattered from up to six or more fluorescences, is determined for two different angles. Optical filters and beam splitters then direct the light signals to the relevant detectors, which emit electronic signals proportional to the signals that hit them. Data can then be collected on each particle or event and the characteristics of those events or particles are determined based on their fluorescent and light scattering properties. Fluorescent light is filtered so that each sensor can detect a single fluorescence at a specific wavelength. These sensors are called photomultiplier tubes (PMT). A PMT in front of the laser beam captures light scattered in the forward (FS). Fluorescence and transmitted light scattered laterally (SS) are captured by the PMT located on the side. FS is correlated to cell size while SS depends on their density (number of cytoplasmic granules). In this manner, cell populations can often be distinguished on the basis of differences in size and density. The electronics system is used to change the light signals detected into electronic pulses that a computer can process.

The data are usually presented in the form of single parameter histograms or as plots of correlated parameters, which are referred to as cytograms. Cytograms may display data in the form of a dot plot, a contour plot or a density plot.

APPENDIX 23: Western blot

A. Principle

Western blot is used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight and by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. Non-protein binding areas on the membrane are blocked to prevent non-specific binding of antibodies. The membrane is then incubated with labeled antibodies specific to the protein of interest. The membrane is incubated with a primary antibody that specifically binds to the protein of interest. Unbound antibodies are removed by washing and a secondary antibody conjugated to an enzyme, a fluorophore or an isotope is used for detection. The detected signal from the protein: antibody complex is proportional to the amount of protein on the membrane. The most commonly used method for detection is chemiluminescence, based on secondary antibodies conjugated with horseradish peroxidase enzyme. In addition to peroxide-based reagent, the enzyme catalyses the oxidation of luminal leading to the emission of light. The light signal can be captured either using a charge-coupled device (CCD) camera-based imager or by exposure to X-ray film. A more recent detection method is fluorescence. The secondary antibodies are labeled with a fluorophore such as a CyDye™. A fluorescent light signal can be detected directly using a laser scanner or a CCD camera-based imager equipped with appropriate light sources and filters. Regardless of the detection method, the signal intensity correlates with the amount of protein and can be visually estimated as well as quantitated using analysis software. The thickness of the band corresponds to the amount of protein present; thus running a standard can help quantify the amount of protein present.

B. Detailed protocol

Protein expression of PARP1, OGG1 and DNMT1 was studied in BEAS-2B cells exposed (or not) to PM_{2.5-0.3} for 72 h, under industrial and rural influences at 3.75 or 15 µg/cm² or B[a]P as described in *Paragraph 4.1.3.3.b*.

- Sample preparation

Proteins were extracted, quantified by BCA method and stored at -80°C as described in *Paragraph 4.1.5*. Proteins samples were thawed on ice and then 15 µg of proteins, prepared in distilled water to a volume of 10 µL, were added to 10 µL of Tris glycine SDS (Novex® Tris-Glycine SDS Sample Buffer (2X)). The mixture was heated for 5 min at 95°C to denature the proteins.

- Gel electrophoresis

Electrophoresis is a separation technique based on the mobility of charged molecules in an electric field. Electrophoresis is normally carried out by loading a sample containing the molecules of interest into a well in a porous matrix to which a voltage is then applied. Molecules of different size,

shape and charge in the sample move through the matrix at different velocities. At the end of the separation, the molecules are detected as bands at different positions in the matrix. Protein expression of PARP1 (115 kDa), DNMT1 (185 kDa), OGG1 (39 kDa) were done on prepared Novex™ 4-12 % Tris- glycine gels (Invitrogen). Actin (42 kDa) was used as a reference protein when testing DNMT1 and OGG1. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight, the higher the percentage that should be used.

The comb was removed gently from the gel and the wells were washed with ultrapure water and then filled with running buffer (1X) (100 mL running buffer 25X + 900 mL distilled water). After placing the gel in the electrophorator (XCell SureLock Mini (Invitrogen)), the running buffer (1X) was poured until it covered the gel completely. A volume of 5 µL of the protein ladder (molecular weight marker: PageRuler Prestained™ Protein Ladder, 10 to 250 kDa) and 20 µL of the prepared samples were loaded into the wells. Markers are composed of different proteins of known size and the distances migrated over the time course of the run provide a logarithmic scale by which to estimate the size of unknown proteins. After connecting the electrophorator to a power supply, the gel was run with high voltage (500 V) and constant amperage (50 mA) for approximately 90 minutes, or until the dye front runs off the bottom of the gel.

- Electrotransfer

On completion of the separation of proteins by polyacrylamide gel electrophoresis (PAGE), the proteins were transferred from the gel to a solid support membrane, usually made of a chemically inert substance, such as nitrocellulose or polyvinylidene difluoride (PVDF). Blotting makes it possible to detect the proteins on the membrane using specific antibodies. The transferred proteins were next immobilized at their respective relative migration positions at the time point when the electric current of the gel run was stopped. Electrotransfer is almost exclusively the contemporary transfer method of choice due to its speed, uniformity of transfer, and transfer efficiency. Electrotransfer relies on the same electromobility principles that drive the migration of proteins during separation in PAGE. The gel, membrane, and electrodes are assembled in a sandwich so that proteins move from gel to membrane, where they are captured, in a pattern that perfectly mirrors their migration positions in the gel. It is important that transfer is as uniform as possible across the entire area of the gel: membrane sandwich in order to ensure that large and small proteins are transferred with similar efficiency. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. The use of gradient gels can optimize uniformity of transfer but the disadvantage is that it is more difficult to transfer the proteins, as they are more strongly “locked” into the polyacrylamide matrix. This is a particularly important consideration for the quantitative analysis of proteins. Two types of electrotransfer, known as wet transfer and semidry transfer, are in common usage in laboratories today. In wet transfer, the gel and membrane are both fully immersed in transfer buffer and a current is applied in the direction of the gel to the membrane. Generally, wet

transfer requires cooling of the unit and internal recirculation of the transfer buffer by the presence of a stirring magnet. Wet transfer is recommended for large proteins, but it is a relatively slow technique, requiring large volumes of buffer. Wet transfer should be applied in preference to semidry transfer when it is important to obtain blots of the highest quality in terms of distinct, sharp bands and efficient transfer. Therefore, we used the wet transfer in our experiment.

The glass plates containing the gel were separated and the gel was removed and equilibrated in transfer buffer (1X) (40 mL transfer buffer (25X), 200 mL methanol and 760 mL distilled H₂O). The PVDF membrane, the filters and the sponges were equilibrated in transfer buffer.

The transfer stack was assembled to allow the migration of proteins toward the membrane and built on the black half side of the cassette, as follow: one sponge, one filter, the gel, the PVDF membrane (Press gently to remove air bubbles), one filter, one sponge and the other side of the cassette. The assembled cassette should hold the gel in firm contact with the membrane without squeezing the gel. The blotting cassette was placed in a tray filled with chilled transfer buffer. Transfer buffer should act as an electrically conducting medium in which proteins are soluble and that does not interfere with binding of the proteins to the membrane. During wet transfer, most buffers become heated, increasing in temperature to a point where proteins may be irreversibly damaged. It is therefore important to start the transfer process using cooled buffer and to maintain a low temperature. For this reason, the transfer apparatus was placed on ice. Electrodes were placed on top of the sandwich, ensuring that the PVDF membrane was between the gel and a positive electrode. After connecting the apparatus to a power supply, the transfer was done for 90 minutes at 800 mA and a constant voltage (30 V).

- Confirmation of protein transfer to the membrane:

After electrotransfer, it may be necessary to confirm that all the proteins in the gel have been completely eluted. This can be achieved by staining the gel using a total protein stain after electrotransfer. In addition, in order to ensure that all the proteins are on the membrane (and have not passed through or migrated in the opposite direction), the membrane can also be treated with a total protein stain.

For gel staining, Coomassie Brilliant Blue was used. It is generally applied to polyacrylamide gels but they are also applicable to membranes. Coomassie Blue tablets from can detect most proteins at levels of 50 to 100 ng/band. The membranes are stained using 0.1% Coomassie dye dissolved in a mixture of 45% methanol, 45% water, and 10% acetic acid. The background is then destained using a mixture of 10 mL of the stock solution of Blue dye and 10 mL of acetic acid 20%. The stain will not bind to the acrylamide, and will wash out (leaving a clear gel). However, it remains strongly bound to the proteins in the gel, and these take on a deep blue color.

The membrane was stained with Pierce™ kit Reversible Protein Stain Kit or with Ponceau S. After the transfer, the membrane was washed with ultra pure water. The Pierce™ kit Reversible Protein Stain was added on the membrane and incubated for 30 seconds and then rinsed with ultrapure

water for two or three times with agitation to destain. This step is to remove the blue membrane proteins for a better view. A stain eraser (provided with the kit) was added; the membrane was shaken for two minutes to remove the dye and then rinsed with ultrapure water.

Ponceau S is a quick and easy way to visualize proteins transferred to membranes following PAGE. Ponceau S is easily removed with water and is regarded as a “gentle” treatment that does not interfere with subsequent immunological detection steps.

- **Blocking and antibody incubation:**

Western blotting involves the immobilization of biomolecules on a membrane via hydrophobic interactions. As non-specific binding of antibodies to the membrane is detrimental to the specificity and sensitivity of the assay, it is essential to “block” spaces not already occupied by proteins. The membrane was blocked with a solution of PBS-Tween (0.05%) (100 mL PBS 10X, 900 mL H₂O and 0.5 mL Tween 20) with 5% milk powder for 90 minutes. After blocking, the membrane was rinsed with PBS-Tween 0.05% (3 times for 5min, with agitation).

Following the blocking step, the protein of interest can be detected using antibodies. The primary antibody, which is specific for the target protein, can be labeled or unlabeled. To maximize sensitivity and signal-to-noise ratio, most Western blotting procedures use an unlabeled primary antibody that is specifically recognized by a labeled secondary antibody. Depending on the type of detection system used, the secondary antibody will generate a signal that can be quantitated by chemiluminescent, chemifluorescent, chromogenic, fluorescent, or radiolabeling methods.

The primary antibody was prepared at a concentration of 1/1000 in blocking solution, added to the membrane in a way to cover it and incubated overnight at 4°C with agitation. In case of determining the protein expression of PARP1, only anti-PARP1 (rabbit monoclonal IgG, Abcam ab191217) was added to the membrane. While in the case of both DNMT1 and OGG1, anti-β-Actin (a housekeeping protein, R and D systems) was tested in the same membrane as anti-DNMT1 (polyclonal IgG, Abcam ab19905) or anti-OGG1 (Abcam), and therefore the membrane was cut according to the ladder and the target membrane’s size in a way to add on each fragment the specific antibody. The next day, the membrane was rinsed with PBS-Tween 0.05% (3 times for 5min, with agitation).

A wide variety of secondary antibodies are commercially available and your choice will depend firstly on the species in which the primary antibody was produced. If, for example, the primary antibody was of the IgG isotype and produced in goat, the secondary antibody must be an anti-goat IgG antibody produced in another species as it will bind to the Fc region of the primary antibody.

The secondary antibody was prepared at 1/1000 in blocking solution, added to the membrane in a way to cover it and incubated for one hour with shaking. Anti-mouse secondary antibody was used for β-Actin while anti-rabbit second antibody was used for PARP1, DNMT1 and OGG1. The membrane was rinsed with PBS-Tween 0.05% (3 times for 5 min, with agitation).

- **Detection and imaging:**

A variety of detection systems, based on chemiluminescence, chemifluorescence, fluorescence, chromogenic or radioisotopic detection are available. Enzyme-based chemiluminescent and chemifluorescent techniques, as well as direct fluorescence have been extensively developed and are now usually the methods of choice for detection due to their improved sensitivity and wider dynamic range. Enzymatic detection methods, such as chemiluminescence and chemifluorescence require the addition of a reagent that emits light when it reacts with an enzyme conjugated to a secondary antibody. Fluorescence-based detection, on the other hand, requires no additional reagents after binding of the labeled secondary antibody.

In this study, chemiluminescence detection was used. The HRP-conjugated secondary antibody binds to the primary antibody, specifically bound to the target protein on the membrane. After the addition of a luminol peroxide detection reagent, the HRP enzyme catalyzes the oxidation of luminol in a multi-step reaction. The reaction is accompanied by the emission of low intensity light at 428 nm. In the presence of certain chemicals, the emitted light is enhanced up to 1000-fold, making it easier to detect, and thus increasing the sensitivity of the reaction in a process known as enhanced chemiluminescence (ECL). The intensity of signal is a result of the number of reacting enzyme molecules and is thus proportional to the amount of antibody, which is related in turn to the amount of protein on the blot. Pierce[®] ECL 2 Western Blotting Substrate kit (Thermo Scientific) was used to prepare the ECL mixture by adding 2 mL of the solution A and 50 μ L of the solution B provided in the kit. Excess reagent was drained from the membrane by tapping the membrane's edge with forceps against an absorbent tissue. The membrane was incubated with ECL and the light signal was detected by digital imaging with a charge-coupled device (CCD) camera-based imager: Gbox (SynGene) and Snap Gene software was the used.

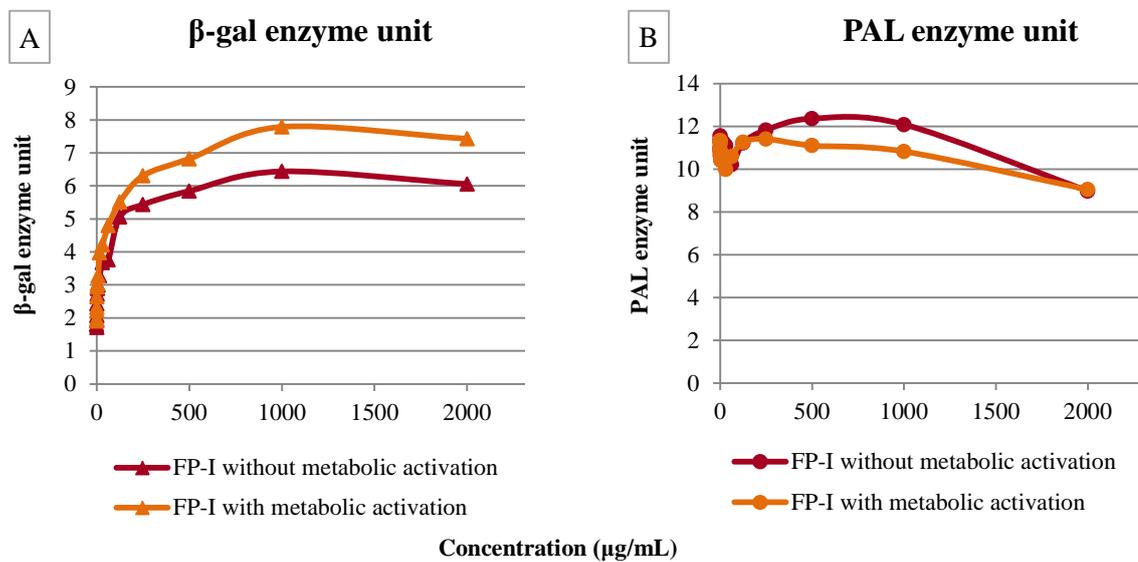
Another type of detection, CN/DAB Substrate Kit (Thermo Scientific) was also used. It is for chromogenic detection of horseradish peroxidase in immunohistochemistry and immunoblotting applications. The formed product is a black precipitate that photographs well and is useful in double staining applications. The colored product is soluble in xylenes but not soluble in aqueous buffer such as Tris or phosphate. This combination of DAB and CN has a synergistic effect that results in greater sensitivity than either reagent alone. 125 μ L of the (10X) CN/DAB Concentrate were combined with 1.125 mL of the Stable Peroxide Substrate Buffer. The substrate solution was applied to the membrane and incubated for 2 to 5 minutes until significant color develops. To stop the reaction, the membrane was washed with ultrapure water.

APPENDIX 24: Dose-Response of the SOS Chromotest of FP-I with and without Metabolic Activation

Positive controls: 10 µg/mL 4NQO without S9 and 100 µg/mL 2AA with S9

Concentration (µg/mL)	Genotoxic activity		Induction factor	
	-S9	+S9	-S9	+S9
0	0.1558	0.1681	1.0000	1.0000
0.122	0.1514	0.1768	0.9716	1.0513
0.244	0.1702	0.1951	1.0920	1.1603
0.488	0.1883	0.2149	1.2082	1.2782
0.976	0.2232	0.2422	1.4321	1.4406
1.9	0.2561	0.2762	1.6436	1.6429
3.9	0.2670	0.2914	1.7135	1.7328
7.89	0.2745	0.3053	1.7616	1.8160
15.625	0.2916	0.3811	1.8709	2.2668
31.25	0.3305	0.4225	2.1207	2.5126
62.5	0.3685	0.4533	2.3648	2.6958
125	0.4510	0.4894	2.8939	2.9107
250	0.4594	0.5528	2.9476	3.2881
500	0.4728	0.6146	3.0339	3.6554
1000	0.5329	0.7197	3.4193	4.2805
2000	0.6745	0.8213	4.3281	4.8844
4NQO	0.8585	-	5.5086	-
2AA	-	0.9112	-	5.4193

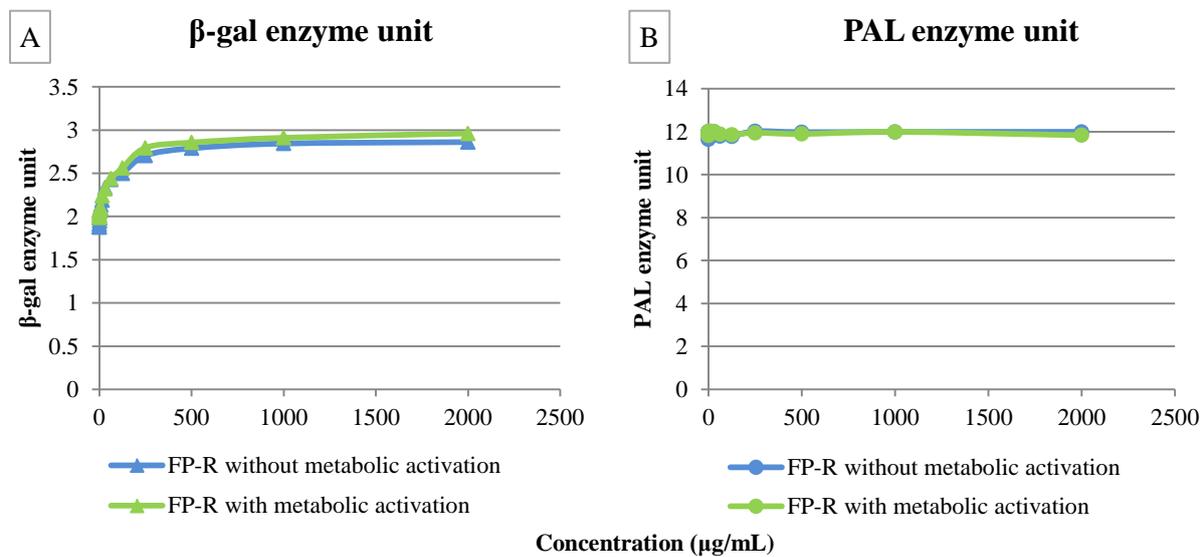
APPENDIX 25: β -gal enzyme unit (A) and PAL enzyme unit (B) at different concentrations of FP-I with and without metabolic activation



APPENDIX 26: Dose-Response of the SOS Chromotest of FP-R with and without Metabolic Activation

Positive controls: 10 µg/mL 4NQO without S9 and 100 µg/mL 2AA with S9

Concentration (µg/mL)	Genotoxic activity		Induction factor	
	-S9	+S9	-S9	+S9
0	0.1612	0.1682	1.0000	1.0000
0.122	0.1572	0.1713	0.9753	1.0188
0.244	0.1597	0.1692	0.9908	1.0059
0.488	0.1625	0.1688	1.0082	1.0036
0.976	0.1649	0.1721	1.0230	1.0232
1.9	0.1674	0.1739	1.0384	1.0341
3.9	0.1728	0.1747	1.0721	1.0388
7.89	0.1793	0.1751	1.1122	1.0413
15.625	0.1831	0.1863	1.1359	1.1080
31.25	0.1947	0.1933	1.2080	1.1498
62.5	0.2059	0.2051	1.2777	1.2200
125	0.2122	0.2160	1.3163	1.2847
250	0.2252	0.2338	1.3969	1.3907
500	0.2328	0.2401	1.4446	1.4277
1000	0.2373	0.2427	1.4720	1.4433
2000	0.2385	0.2502	1.4799	1.4881
4NQO	0.9064	-	5.6233	-
2AA	-	0.8873	-	5.2765

APPENDIX 27: β -gal enzyme unit (A) and PAL enzyme unit (B) at different concentrations of FP-R with and without metabolic activation

Résumé

L'exposition à la pollution atmosphérique, notamment aux particules fines ($PM_{2.5}$), représente un risque majeur pour la santé dans le monde entier, et d'autant plus dans les pays en développement. Le Nord du Liban est ainsi affecté par plusieurs sources de pollution d'origine anthropique, urbaine et industrielle. Pourtant, dans cette région, aucune étude ne s'est intéressée à l'impact des $PM_{2.5}$ sur la santé publique. Il faut également souligner que les mécanismes de toxicité des $PM_{2.5}$ ne sont pas totalement identifiés. Le but de ce travail est d'étudier la nature et l'impact sanitaire de la pollution atmosphérique particulaire dans le Nord du Liban. Nous avons procédé à une enquête épidémiologique, et prélevé des particules fines que nous avons caractérisées sur les plans physico-chimique et toxicologiques. Deux régions ont été considérées dont une est située à proximité d'activités industrielles.

L'étude épidémiologique et de perception menée dans les deux zones du Nord du Liban, (310 questionnaires / zone traitée), a montré une relation entre gêne, maladies respiratoires et proximité des industries. Cette enquête a ainsi confirmé l'intérêt de mener une étude toxicologique dans cette région.

Afin de renforcer les connaissances sur la toxicité pulmonaire des aérosols atmosphériques particulaires avec une attention toute particulière portée à l'étude de certains des mécanismes d'action suspectés d'être impliqués dans la cancérogénicité, les caractéristiques physico-chimiques et toxicologiques des particules fines ($PM_{2.5-0.3}$) prélevées sur les deux sites ont été étudiées. Les particules collectées ont montré une composition similaire sur les deux sites concernant les espèces majeures. La contribution des activités industrielles a été mise en évidence par des teneurs légèrement plus élevées de certains éléments traces, d'HAP et surtout par une teneur jusqu'à 125 fois plus élevée en dioxines. Nos résultats ont mis en évidence l'influence de nombreuses sources de combustion (diesel, essence, charbon et biomasse) ; la combustion de déchets et d'autres procédés industriels sont également suspectées.

Un potentiel génotoxique et mutagène plus prononcé a été mis en évidence pour les particules collectées sur le site sous influence industrielle par rapport aux particules provenant du site sous influence rurale, à l'aide du test d'Ames en milieu liquide et le SOS chromotest. Les effets observés sont très probablement influencés par la fraction organique des particules. Afin d'approfondir la recherche des mécanismes génotoxiques des PM, des cellules bronchiques humaines (BEAS-2B) ont été exposées à différentes concentrations de particules. Les mécanismes de toxicité, tels que l'activation métabolique des HAP (*CYP1A1*) et les cassures double-brins (quantification de γ H2AX par cytométrie en flux et in-cell western), ont été induits par les deux échantillons de $PM_{2.5-0.3}$, avec un effet plus prononcé pour les particules industrielles. Par ailleurs, les PM ont montré une tendance à perturber le fonctionnement du système de réparation de l'ADN (par l'expression des gènes *OGG1*, *NTH1*, *APE1*, *NUDT1*, *DNMT1*, *MGMT*, *XPA* et *XRRC1*, et l'expression des protéines PARP1, DNMT1 et OGG1). Les mécanismes de réparation des dommages de l'ADN ont ainsi été réprimés jusqu'à 48h d'exposition aux PM, notamment aux $PM_{2.5-0.3}$ collectées sous influence industrielle, et réactivés après 72h d'exposition. Ces dommages concernent les adduits encombrants à l'ADN, et ceux causés par le stress oxydant, des cassures des brins d'ADN et la méthylation. Nos résultats suggèrent des mécanismes d'action mutagènes, génotoxiques et épigénétiques impliqués dans la cancérogénicité des particules fines, en partie liés à la composition de la fraction organique.

Mots-clés: Pollution atmosphérique; Influences industrielle et rurale; Perception et épidémiologie; Particules fines ($PM_{2.5-0.3}$); Particules quasi-ultrafines ($PM_{0.3}$); Physico-chimie; Activation métabolique; Génotoxicité; Réparation de l'ADN.

Abstract

Exposure to air pollution, especially fine particulate matter (PM_{2.5}), remains a major health risk, mainly in the developing countries. Northern Lebanon is affected by several sources of anthropogenic, urban and industrial pollution. However, no studies have examined the impact of PM_{2.5} on public health in this region. In addition, it should be noted that the toxicity mechanisms of PM_{2.5} are not fully identified. The aim of this work is to study the composition and the health impact of the atmospheric particulates in Northern Lebanon. An epidemiological survey was performed and fine particles were extracted and characterized physico-chemically and toxicologically. This study was conducted in two sites, one of which is influenced by industrial activities.

Epidemiological survey, conducted in two areas in Northern Lebanon, rural and industrial (310 treatable questionnaires/area), showed a relationship between annoyance, respiratory diseases and living in proximity to industrial activities. Moreover, results confirmed the interest in conducting a toxicological study in this region. Hence, to contribute to fulfill the gap of knowledge about the pulmonary toxicity of particulate matter and the mechanisms of action involved in the carcinogenicity, the study of physicochemical characteristics and toxicological endpoints of PM_{2.5-0.3} from both sites were performed.

Physicochemical analyses of the collected particles evidenced similar characteristics in major species. In particular, we have shown slightly higher levels of PAHs and trace metals and up to 125 times' higher dioxins concentrations at the vicinity of industries. Our results evidenced the influence of numerous combustion sources (diesel, gasoline, coal and biomass burning); waste combustion and other industrial processes are also suspected.

A more pronounced genotoxic and mutagenic potential was evidenced after exposure to particles collected at the vicinity of industries when compared to the rural ones, using the Ames fluctuation test and SOS chromotest. The effects of the collected particles are probably related to their organic composition. In order to assess the underlying toxic mechanisms, human bronchial epithelial cells (BEAS-2B) were then exposed to different concentrations of the sampled PM_{2.5-0.3}. Genotoxicity mechanisms such as metabolic activation of organic compounds (*CYP1A1*) and consecutive DNA damages such as DNA strands breaks (γ H2AX quantification by flow cytometry analysis and in-cell western assay) were induced by the two samples of PM_{2.5-0.3}, with a more pronounced effect of industrial particles. Moreover, PM showed tendency to alter the DNA repair process (*OGG1*, *NTH1*, *APE1*, *NUDT1*, *DNMT1*, *MGMT*, *XPA*, *XRRC1* gene expression and PARP1, DNMT1, OGG1 proteins expression). DNA repair mechanisms were repressed up to 48h of exposure to PM especially to the industrial influenced PM_{2.5-0.3} and reactivated after 72h of exposure. The DNA damages involve bulky DNA adducts, oxidative stress damages, DNA strand breaks and methylation.

These results suggest mutagenic, genotoxic and epigenetic mechanisms of action involved in the carcinogenicity of fine particles, partly related to their organic composition.

Keywords: Air pollution; Industrial and rural influences; Perception and epidemiology; Fine particulate matter (PM_{2.5-0.3}); Quasi-ultrafine particulate matter (PM_{0.3}); Physico-chemistry; Metabolic activation; Genotoxicity; DNA repair.