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AIR POLLUTION AND HUMAN HEALTH RISK: EVALUATION OF CARCINOGENIC POTENTIAL OF URBAN AIRBORNE PARTICULATE MATTER

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Introduction and aim of the study

Air pollution constitutes a major public health concern because of its ubiquity and of its potential short-term and long-term health impact. Polluted air contains a complex mixture of particles and gas phase pollutants so that individuals are exposed to many air pollutants at once. Most pollutants are highly correlated to each other, so that an additive or synergic effect cannot be excluded.

Fine particulate matter ($PM_{2.5}$, PM_1), which is generated by combustion, has been suggested to induce mutagenic and toxic effects related to particle size and PM concentration and composition. The effects of inhaled PM on human health have been widely studied in humans as well as in animal models and include asthma, cardiovascular issues and premature death due to respiratory diseases and probably lung cancer (Pope *and Dockery*, 2006; WHO 2000; Rueckerl *et al.*, 2011; Teixeira *et al.*, 2012)

Recently, the International Agency for Research on Cancer (IARC) classified outdoor air pollution as "carcinogenic to humans" (Group 1) (Loomis *et al*., 2013; Hamra *et al*., 2014). The IARC classification was based on the evidence of lung tumours as a consequence of lifetime exposure to 10-30 $\mu g/m^3$ $PM_{2.5}$.

It has been also suggested that PM short and long term effects are related to particles concentration, chemistry and size (Valavanidis *et al*., 2008). The field of mixture toxicology and the experimental analysis of chemical mixtures have undergone a significant expansion, which is driven by the need to clarify the effects of exposure on human health. Historically, this approach is being superseded in favour of investigating the effects of complex

mixtures and understanding of the interactions between different chemicals.

Predicting the toxicological risk associated with the exposure to environmental samples, such PM extracts, still shows some critical issues. The environmental samples are characterized by the simultaneous presence of a large number of pollutants, showing different mechanisms of action and toxicity profiles.

The current EU regulation establishes the list of chemicals that should be identified and characterized in the airborne PM and set the acceptable concentration levels for reference compounds, whose toxicological profile has been evaluated in standard tests (EU, 2008). The acceptable concentration levels are derived from experimental studies. Below these levels, the exposure is considered as safe. However, this approach may be inadequate to estimate the real risk from several environmental carcinogens co-present in a complex mixture at low doses.

Among all of the strategies seeking for the relationships between exposure to chemicals and the effects on human health, the predictive toxicology approach has the potential to better identify biological effects from exposure to environmental mixtures and predict the final health outcome, by using *in vitro* methods supported by high throughput approaches and linking them to known key steps in disease progression.

Alternative methods to animal testing are considered as suitable tools to support hazard identification and are also of growing interest for predicting the toxicological risks. The simultaneous presence of a huge number of different chemicals at low concentrations could often result in misleading characterization of

the hazard associated with complex mixtures, leading to underestimation of the risk, since the possible additive or more than additive interactions among chemicals could not be properly identified (Jarvis, *et al*., 2014).

Airborne particulate matter (PM) could be regarded as the prototypical example, as it is nearly impossible to identify and measure all components in the PM extracts. Moreover, individual pollutants in airborne PM samples are often under the acceptable concentration level established by the current legislations and near or even under the method detection limits. In this context, the establishment of *in vitro* methods able to characterize the toxic effects and the carcinogenic potential of mixtures could be relevant for hazard and risk assessment.

Cancer may arise from the exposures to these environmental mixtures as the consequence of the interaction among single chemicals, each one affecting one (or more) cancer hallmarks. The adverse outcome may be reached at doses much lower than those at which the effect has been observed in traditional toxicological studies. Also, not all components in a complex mixture may be equally identified and characterized (Vaccari *et al*., 2015).

In recent years, a shift from *in vivo* costly and time consuming animal studies to short term *in vitro* assays has been proposed to assess the hazard of single chemicals or complex mixtures (Collins *et al*., 2008; Kohonen *et al*., 2014). The UE current regulations on the Registration, Evaluation, Authorization and Registration of Chemicals (REACH) are prompting the use of alternative test methods including *in vitro* methodologies (EU, 2003, 2006).

Among *in vitro* tests reproducing several stages of the multistep process of carcinogenesis, the cell transformation assays (CTAs) appear to be the most suitable tools to predict the carcinogenic properties of chemicals (Lilienblum *et al*., 2008; Vasseur and Lasne, 2012) and to evaluate the carcinogenic risk associated with environmental samples (Colacci *et al*., 2007; Mascolo *et al.*, 2010; Colacci *et al.*, 2014)

The aim of this thesis is to highlight the toxic and carcinogenic potential of airborne particulate matter from different seasons at a site that is located in the northern area of the city of Bologna by using alternative *in vitro* tests, such as the cell transformation assay with BALB/c 3T3 -clone A31-1-1- and Bhas 42 cells. The purpose is also to evaluate the lifetime cancer risks associated with air inhalation in different sites, (rural and urban) by using the relative potency of compounds belonging to the same chemical class (PAHs and nitro-PAHs) and the specific unit of carcinogenic risk.

CHAPTER 1

Alternative tests *in vitro*

1.1 Introduction

Carcinogenesis is a multistage process that can take many years before clinical symptoms are manifested. The prediction and the assessment of the carcinogenic potential of a new compound is thus an essential component of toxicity testing. Historically, the evaluation of cancer hazard and potency is assessed using the chronic carcinogenicity bioassay in rodents (OECD, 2009) and based upon the expectation that the potential to induce tumours in rodent can be extrapolated to humans. However, rodent carcinogenicity assays are costly, time consuming and use a high number of animals and the extrapolation of the results to man is a challenging and often imprecise exercise (Mascolo *et al*., 2010).

For the last 20 years the scientific community has paid great attention to alternative strategies in compliance with common moral and ethical values. The European Union began a policy of development and use of alternative methods, defined as "systems that can be used to replace, reduce or refine the use of animal testing in biomedical research, testing and education".

This definition goes back to a text of 1959 (Russell and Burch, 1959), and is commonly known as the definition of the "*3 R: replace, reduce, refine*". An alternative to animal testing is defined as any technique that:

- replace animals with non-sentient alternatives (*replacement*);
- reduce to a minimum the number of animals used (*reduction*);
- refine experiments which used animals so that they caused the minimum pain and distress (*refinement*).

Recently, the new European chemical regulation, aiming at the Registration, Evaluation and Authorization of Chemicals (REACH), which strongly supports the development and use of alternative tests to reduce and eventually replace animal bioassays, gave added momentum (Reg.EC 1907/2006) and recommends that the registration of chemicals is achieved through the least possible use of animal testing, to be considered as a last "resort" for obtaining information (Lilienblum *et al*., 2008).

In vitro studies have considerable advantages for the study of carcinogens, such as speed, cost-effectiveness and reproducibility, in addition to the possibility of evaluating the dose-response relationship.

1.2 Cell transformation assay test

Among *in vitro* testing methods, cell transformation assay (CTA) appears to be one of the most appropriate approaches to predict the carcinogenic properties of single chemicals, complex mixtures and environmental pollutants (Lilienblum *et al.,* 2008; Mascolo *et al*, 2010; Corvi *et al*, 2012; Vanparys *et al*, 2012; Vasseur and Lasne, 2012).

They are proposed as a second-level screening for carcinogens and as a screening test of choice for non-genotoxic carcinogens, which are not detected in mutagenicity assays.

In vitro Cell Transformation Assays (CTAs) have been shown to involve a multistage process that closely models some stages of the *in vivo* carcinogenesis. The cellular and molecular changes that are involved in the *in vitro* process of cell transformation are similar to those of *in vivo* carcinogenesis and arise from cellular responses to direct and indirect damage to DNA, genes and cellular systems (Vanparys *et al*., 2012; Vasseur and Lasne, 2012).

Several authors have defined the *in vitro* cell transformation assay as "unique system that offers potential benefit in such screening" (Kakanuga, 1985; Montesano *et al*., 1986; Sakai, 2007).

These assays measure the induction of malignant features in mammalian cells after the treatment with the tested chemicals and entail morphological, biochemical and molecular changes in behaviour and growth control of cultured cells, such as alteration of cell morphology, disorganized pattern of colony growth, and

acquisition of anchorage-independent proliferation (Combes et al., 1999).

Later on, transformed cells become able to grow in semi-solid agar (anchorage-independent growth), produce autocrine growth factors and can cause tumours when injected in susceptible animals (Combes *et al*, 1999; Sakai, 2007).

The transformed cells acquire the ability to divide indefinitely (immortalization) which is associated with other alterations like aneuploid karyotype and altered genetic stability.

Chemical carcinogens can be classified into two categories according to their ability to interact directly or indirectly with DNA:

genotoxic carcinogens (or their metabolites) are able to initiate cells to carcinogenesis via direct interaction with DNA. These interactions result in DNA damages and/or structural/numerical chromosomal aberrations which can be detected by genotoxicity tests. Generally, an evaluation of genotoxic potential focuses on the assessment of gene mutations and structural/numerical chromosomal aberrations;

non-genotoxic carcinogens are carcinogenic agents that are, at least initially, devoid of direct interaction with DNA. The indirect modifications to DNA structures, amount or function may induce altered gene expression and/or signal transduction.

The evaluation of the genotoxic potential of a compound can be assessed by a variety of tests that focus on gene mutations and chromosomal damage (structural and numerical aberrations). These genotoxicity tests are not suitable for the detection of the carcinogenic potential of non-genotoxic carcinogens that exhibit

indirect modifications to DNA structures/functions and alterations in signal transduction pathways and cellular communication.

It has been shown that the established *in vitro* cell transformation assays are responsive to chemicals acting via genotoxic or non-genotoxic mechanisms (Sakai, 2007).

Various types of cell transformation assays have been developed for the detection of the carcinogenic potential of chemicals. The *Syrian hamster embryo cells* (SHE) CTA is a primary cell system, while C3H 10T1/2, BALB/c 3T3 and Bhas 42, derived by BALB A31-1-1, are established cell lines.

Syrian hamster embryo cells are "normal cells" since they are diploid, genetically stable, non tumorigenic cells and are theorized to measure the initial stages of transformation, whereas the BALB/c 3T3 and C3H 10T1/2 are aneuploid, immortalized cells and are hypothesized to measure later stages in the carcinogenic process. Bhas 42 cells are supposed to be initiated cells.

International validation studies of SHE and BALB/c 3T3 CTAs were performed by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM). Afterwards an international validation study of Bhas 42 CTA was performed by the Japanese Centre for the Validation of Alternative Methods (JaCVAM) in conjunction with the New Energy and Industrial Technology Development Organization (NEDO). This validation study ensured the use of standardized Bhas 42 CTA protocol, confirmed its transferability between and within laboratories, and established its intra and inter laboratory reproducibility.

1.2.1 BALB/c 3T3 A31-1-1 Cell Transformation Assay

BALB/c 3T3 Cell Transformation Assay is one of the three available models to investigate the cell transformation *in vitro* as a consequence of the exposure to possible carcinogens. This model has been reported to show good predictability of mammalian carcinogenicity (IARC/NCI/EPA Working Group 1985, Mascolo *et al*., 2010, Creton *et al*., 2012). Moreover, a modified protocol of the CTA on BALB/c 3T3 has been validated in the EURL-ECVAM (Sasaki *et al*., 2012 a,b).

BALB/c 3T3 cells are immortalized embryonic mouse fibroblast. These aneuploid, contact-inhibited cells are able to grow as a monolayer culture until confluent. The chemical transformation of 3T3 cells results in the induction of morphologically aberrant foci, shaped with cells that do not stop proliferating at confluence but grow over contact-inhibited normal cells. Only foci that show basophilic dense multilayering of cells, random orientation at the focus edge, invasion into the surrounding contact-inhibited monolayer and domination of spindle shaped cells are recognized as positive transformed foci (Kakanuga, 1985; Sakai, 2007)

1.2.2 Bhas 42 Cell Transformation Assay

The Bhas 42 cell line was established by the transfection with a plasmid pBR322 containing vHa-*ras* oncogene into the BALB/c 3T3 A31-1-1 cell line (Sasaki, *et al.*, 1988; Sasaki *et al*, 2010). Similar to the parental BALB/c 3T3 cell line, untransformed Bhas 42 cells grow to confluence forming a contact-inhibited monolayer and lack tumorigenicity upon transplantation *in vivo*. After exposure to carcinogenic stimuli, Bhas 42 cells can become

morphologically altered and form independent aberrant colonies, referred to as transformed foci, capable of invading the surrounding non-transformed contact-inhibited monolayer.

The Bhas 42 cell transformation assay has recently been evaluated by comparing it with the BALB/c 3T3 transformation assay (Muramatsu, *et al*., 2009), by analyzing the performance of detection of chemicals carcinogenicity (Ohmori, *et al*., 2004; Sakai, *et al*.,2010) in interlaboratory collaborative studies (Ohmori, 2005; Tanaka, *et al*., 2009), and in an international validation study (Sakai, *et al*., 2011).

These studies show that the Bhas 42 cell transformation assay is reproducible and reliable and has the following advantages, when compared to the BALB/c 3T3 cell transformation assay:

- it is a *simple assay*: treatment with a tumour initiator can be omitted to detect tumour promotors
- it is a *short – term assay*: the culture period is shortened from 4 to 6 weeks to 2.5 to 3 weeks;
- it is a *sensitive assay*: specificity is high
- it is *economical assay*: six wells of 6-wells plates are required for each dose, instead of 8 to 12 60-mm dishes (Ohmori, *et al*., 2009).

Several comprehensive studies were performed to assess the relevance and predictive reliability of the Bhas 42 CTA. These included:

- extensive analysis of 98 chemicals (Sakai *et al*., 2010),
- a multi-laboratory collaborative study (Ohmori *et al*., 2005),
- a prevalidation study (Tanaka *et al*., 2009),

- two international validation studies (Sakai, *et al*., 2011)

The results of all of these studies confirmed the applicability, transferability, reproducibility and reliability of the Bhas 42 CTA protocol and the assay was found to be sufficiently sensitive to predict both initiating activity and promoting activity of carcinogens.

1.3 Application of the CTAs in various industry sectors

1.3.1 Pharmaceutical industry

For carcinogenicity testing, the rodent bioassay is still a required method within the pharmaceutical field. Long-term rodent carcinogenicity studies are associated with high cost and time making them unsuitable for carcinogenic screening (Vanparys *et al*., 2012). For these reasons, the cell transformation assays (CTAs) have been used by some in pharmaceutical industry. The CTAs are mentioned in the FDA guidance as a recommended approached for the integration of genetic toxicology study results (FDA, 2006; Vanparys *et al*., 2012). Cell transformation assays could be considered in the pharmaceutical industry as another tool for screening for potential carcinogens during early phases of drug development. They can also be used to show similarities or differences through compounds of the same family.

1.3.2 Chemical industry

The development of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) policy requires the registration of thousands of individual substances (EU, 2006).

CTAs are used in the chemical industry to investigate the carcinogenic potential of genotoxic compounds or to determine whether a non genotoxic chemical is a potential non genotoxic carcinogen (Vanparys *et al.*, 2012).

1.3.3 Cosmetic industry

Due to the 7th Amendment to the EU Cosmetics Directive 76/768/EEC testing ban that was promulgated March 2009 (EU, 2003) it is not possible to perform acute *in vivo* genotoxicity testing for cosmetic ingredients. However, until 2013, *in vivo* genotoxicity testing may be still conducted if integrated in repeated dose toxicity studies (Pfuhler *et al*., 2009). In the absence of the possibility of performing *in vivo* testing after 2013, CTAs provide a useful approach to obtain additional hazard information with the data obtained in the conventional *in vitro* genotoxicity test batteries (Adler *et al*., 2011).

1.3.4 Food industry

The European Food Safety Authority (EFSA) published in 2011 a scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011). The EFSA opinion also suggests that CTAs can be useful for investigation of substances with structural alerts for carcinogenicity and to demonstrate similarities or differences across chemical categories of food ingredients (Vanparys *et al*., 2012).

CHAPTER 2

2.1 Risk assessment

Risk assessment is the process that scientists and government officials use to estimate the increased risk of health problems in people who are exposed to different amounts of toxic substances.

Risk assessment is a four-step process, including *hazard identification*, *dose-response modelling*, *exposure assessment and risk characterization*. (**Figure 2.1**)

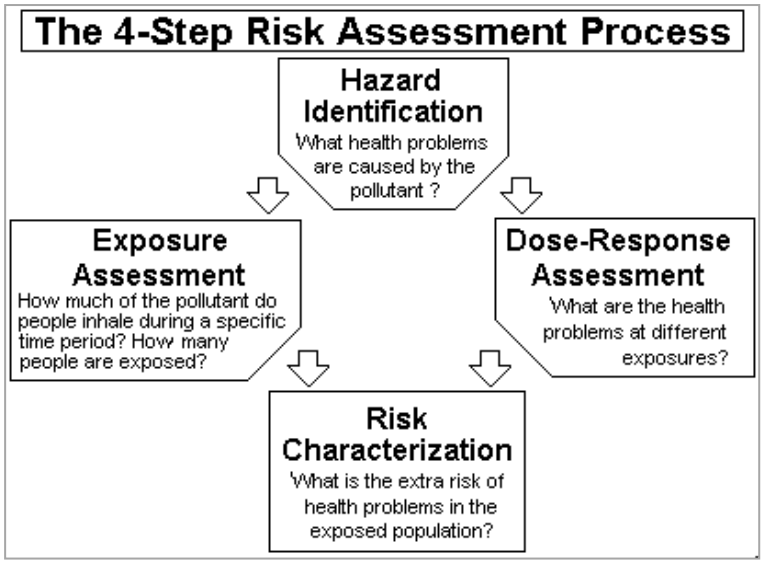


Figure 2.1: The four-step process in the risk assessment

source http://www3.epa.gov/airtoxics/3_90_024.html

The dose-response modelling and the exposure assessment are combined to quantify the risk associated with current and anticipated exposures. The risk characterization step presents both

the qualitative likelihood that the hazard will occur and the quantitative estimates of risk.

The hazard identification is the description of the toxic potential of the analyzed agent. It is based on the evaluation of all available data (e.g., epidemiology, animal bioassay studies, and *in vivo* and *in vitro* studies) to characterize the strength of evidence indicating potential health effects that might occur in exposed human populations.

The first step in cancer risk assessment is to determine the carcinogenicity to humans of a specific exposure. The degree of uncertainty in the classification process depends on the availability of adequate and acceptable data. The best type of evidence comes from human studies.

The international institutions which are mainly involved in identifying and classifying the risk of carcinogenicity of agents and exposures are:

- the *International Agency for Research on Cancer* (IARC) which publishes the *Monographs on the Evaluation of Carcinogenic Risks to Humans*,
- the *US National Toxicology Program* (NTP) which publishes every two months, the *Report on Carcinogens*,
- the *U.S. Environmental Protection Agency* (U.S. EPA) that develops assessment of carcinogenic risk.

The IARC categorizes agents, mixtures and exposures into five categories (IARC, 2010b):

Group 1: "*the agent (mixture) is carcinogenic to humans. The exposure circumstance entails exposures that are carcinogenic to humans*". This category is used when there is sufficient evidence of carcinogenicity in humans. Exceptionally, an agent (mixture) may be placed in this category when evidence of carcinogenicity in humans is less than sufficient but there is sufficient evidence of carcinogenicity in experimental animals and strong evidence in exposed humans that the agent (mixture) acts through a relevant mechanism of carcinogenicity.

Group 2 A "*the agent (mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans*". This category is used when there is limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals. In some cases, an agent (mixture) may be classified in this category when there is inadequate evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans.

Group 2 B: "*the agent is possible carcinogenic to humans*". This category is used for agents, mixtures and exposure circumstances for which there is limited evidence of carcinogenicity in humans and less than sufficient evidence of carcinogenicity in experimental animals. It may also be used when there is inadequate evidence of

carcinogenicity in humans but there is sufficient evidence of carcinogenicity in experimental animals.

Group 3: *"the agent (mixture) is unclassifiable as to carcinogenicity in humans"*. This category is used most commonly for agents, mixtures and exposure circumstances for which the evidence of carcinogenicity is inadequate in humans and inadequate or limited in experimental animals. Exceptionally, agents (mixtures) for which the evidence of carcinogenicity is inadequate in humans but sufficient in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Group 4: the agent (mixture) is probably not carcinogenic to humans. This category is used for agents or mixtures for which there is evidence suggesting lack of carcinogenicity in humans and in experimental animals.

The Italian CCTN classification (CCTN, 1991) was similar to that from IARC and EPA. The CCTN classification distinguished, until recently, 5 groups:

- *Group A*: carcinogenic to humans
- *Group B*: probably carcinogenic to humans (B1 limited evidence of carcinogenicity in epidemiological studies; B2 sufficient evidence of carcinogenicity in experimental animals)
- *Group C*: possible carcinogenic to humans
- *Group D*: unclassifiable as to carcinogenicity in humans.
- *Group E*: probably not carcinogenic to humans, based on animals studies

The general categories recognized by the 1986 EPA guidelines were (U.S. EPA, 1986):

- *Group A* - Carcinogenic to Humans:
- *Group B* - Probably Carcinogenic to Humans: Agents with sufficient evidence (i.e., indicative of a causal relationship) from animal bioassay data, but either limited human evidence (i.e., indicative of a possible causal relationship, but not exclusive of alternative explanations; Group B1), or with little or no human data (Group B2).
- *Group C* - Possibly Carcinogenic to Humans: Agents with limited animal evidence and little or no human data.
- *Group D* - Not Classifiable as to Human Carcinogenicity: Agents without adequate data either to support or refute human carcinogenicity.
- *Group E* - Evidence of Non-carcinogenicity for Humans: Agents that show no evidence for carcinogenicity in at least two adequate animal tests in different species or in both adequate epidemiologic and animal studies.

The 2005 EPA Guidelines recommend expressing Weight of Evidence (WOE) by narrative statements rather than only hierarchical categories, and expressing them separately for the oral and inhalation routes. The statements are (U.S. EPA, 2005):

- Carcinogenic to Humans
- Likely to be Carcinogenic to Humans
- Suggestive Evidence of Carcinogenic Potential
- Inadequate Information to Assess Carcinogenic Potential
- Not Likely to be Carcinogenic to Humans

The evaluation of the dose-response analysis, through the calculation of the carcinogenic potential, is required to look at the quantitative aspect of the cancer risk. Dose-response analysis determines the relationship between the dose and the type of adverse response and/or the probability or the incidence of the effect (dose-response assessment). The complexity of this step in many contexts derives mainly from the need to extrapolate results from experimental animals (mouse, rat) to humans, and/or from high to lower doses, including the extrapolation from high acute occupational levels to low chronic environmental levels.

In the absence of human data to describe low-dose effects, two different approaches are most frequently used for dose-response analysis:

- approach for "threshold" endpoint;
- approach for "non threshold" endpoint.

For non-cancer effects, it has been assumed that at low doses the body's natural protective mechanisms repair any damage caused by the pollutant, so a dose may exist below the minimum health effect level for which no adverse effects occur. The first dose of an agent which induces a response above zero (or above the control response) is usually referred to as a "threshold-dose". The safe doses of chemicals associated with human diseases that are thought to act by a "threshold dose mechanism" are most frequently defined by applying safety factors to the no-observed-effect levels (NOEL) in animal bioassay studies. These so-called safe exposure levels are reported as acceptable daily intakes (ADIs) or reference doses (RfD).

For non-threshold effects (cancer), meaning that there may be some risks associated with any exposure, dose-response relationships based on observed incidence in humans and animals exposed at high doses are established and then extrapolation models are used to estimate the cancer risk associated with low-dose exposure.

Essentially all chemicals can cause non-cancer adverse health effects if given at high enough doses. However, when the dose is sufficiently low, typically no adverse effect is observed. Thus, in characterizing the non-cancer effects of a chemical, the key parameter is the threshold dose at which an adverse effect first becomes evident. Doses below the threshold are considered to be safe, while doses above the threshold are likely to cause an effect (Grilli, 1992).

The threshold dose is typically estimated from toxicological data (derived from studies of humans and/or animals) by determining the highest dose that does not produce an observable adverse affect and the lowest dose which produces an effect. These are referred to as the "*no-observed-adverse-effect-level*" (NOAEL) and the "*lowest-observed-adverse-effect-level*" (LOAEL), respectively.

However, in order to be conservative (protective), non cancer risk evaluations are not based directly on the threshold exposure level, but on a value referred to as the Reference Dose (RfD). The RfD is an estimate of a daily exposure to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime.

The RfD is derived from the NOAEL, (or LOAEL if a reliable NOAEL is not available) by dividing the NOAEL by an "uncertainty factor". If the data were from studies in humans and the observations were considered to be very reliable, the uncertainty factor may be as small as 1.0. However, the uncertainty factor is normally at least 10 and can be much higher if data are limited. The purpose of dividing the NOAEL or the LOAEL by an uncertainty factor is to ensure that the RfD is not higher than the true threshold level for adverse effects. Thus, there is always a "margin of safety" built into an RfD, and doses equal to or less than the RfD are nearly certain to be without any risk of adverse effect. Doses higher than the RfD may carry some risk, but because of the margin of safety, a dose above the RfD does not mean that an effect will necessarily occur.

For cancer effects, the toxicity assessment process has two components. The first one is a qualitative evaluation of the weight of evidence that the chemical does or does not cause cancer in humans. For chemicals that are believed to be capable of causing cancer in humans, the second part of the toxicity assessment is to describe the carcinogenic potency of the chemicals. This is done by quantifying how the number of tumours observed in exposed animals or humans increases as the dose increases.

Usually, it is assumed that the dose-response curve for cancer has no threshold (there is no dose other than zero that does not increase the risk of cancer), arising from the origin and increasing linearly until high doses are reached. Thus, the most convenient descriptor of cancer potency is the slope of the dose-response curve at low dose (where the slope is still linear). Estimating the cancer slope

factor is often complicated by the fact that observable increases in cancer incidence usually occur only at relatively high doses, frequently in the part of the dose-response curve that is no longer linear. It is necessary to use mathematical models to extrapolate from the observed high dose data to the desired slope at low doses.

For carcinogen pollutants, such as dioxins (PCDDs/PCDFs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and some heavy metals (HMs), the EPA model (2005) assumes there is no safe threshold dose below which there is no health risk. The health risk, measured as the probability of developing cancer during the entire lifetime (assumed to be 70 years), is estimated by multiplying the exposure dose by the Cancer Slope Factor (CSF), which represents the estimate of the carcinogenic potency of the chemical.

Three approaches have been recommended by WHO (WHO/IPCS, 1998) for the quantitative assessment of cancer risk by inhaled PAHs:

1. Benzo(a)pyrene (B(a)P) Toxicity Equivalence Factors (TEFs) approach, based on individual potencies relative to B(a)P in order to obtain a benzo(a)pyrene equivalent ,
2. Benzo(a)pyrene surrogate epidemiological approach, which assumes that B(a)P is an indicator of all PAHs in the mixture,
3. comparative Potency Approach, which does not identify or quantify the individual compounds.

The current study was based on the first approach. To evaluate the risk for human health of mixtures of PAHs, international and national Regulatory Agencies suggest to use equivalence factors (Potency Equivalence Factors, PEFs) (**Table 2.1**), which are

derived from carcinogenicity studies in small rodents and, where available, correlate the carcinogenic potential of each PAH or Nitro-PAH (NPAH) to that of B(a)P. The carcinogenic potential of B(a)P is assumed as 1 (WHO/IPCS, 1998).

B(a)P was chosen as the reference compound as it is considered the most appropriate indicator for the carcinogenic fraction of the large number of PAHs in air (WHO, 2000).

The potency of many individual PAHs and NPAHs relative to that of B(a)P was estimated in order to obtain B(a)P equivalents (B(a)Peq) (WHO/IPCS, 1998). The B(a)Peqs represent the relative carcinogenic potential of the corresponding mixture of PAHs and NPAHs to B(a)P.

The B(a)P equivalents are calculated multiplying the concentration of each component of the mixture by its PEF. Then the carcinogenic potency of the complete mixture of PAHs and NPAHs is estimated as the sum of each individual B(a)Peq with the following equation:

$$B(a)Peq = \sum (PAHi \times PEFi) + \sum (NPAHi \times PEFi)$$

In Table 2.1 the list of PEFs, which are currently available, is reported:

PAH	**PEF**
Benzo(a)pyrene	**1**
Anthracene	0.28*
Benz(a)anthracene	0.1
Benzo(c) Phenanthrene	0.023*
Benzo(b)fluoranthene	0.1
Benzo(j)fluoranthene	0.1
Benzo(k)fluoranthene	0.1
Benzo[rst]pentaphene	1.1*
Benzo(g.h.i)perilene	0.01#
Ciclopenta(c.d)pyrene	0.012*
Chrysene	0.01
Dibenzo(a.h)acridine	0.1
Dibenzo(a.j)acridine	0.1
Dibenzo(a.h)anthracene	1#
7H-Dibenzo(c.g)carbazole	1
Dibenzo(a.e)pyrene	1
Dibenzo(a.h)pyrene	10
Dibenzo(a.i)pyrene	10
Dibenzo(a.l)pyrene	10
Phenanthrene	0.00064*
Indeno[1.2.3-c.d]pyrene	0.1
5-Methylcrhysene	1
NPAH	**PEF**
Benzo(a)pyrene	**1**
1,6-Dinitropyrene	10
1,8-Dinitropyrene	1
6-Nitrochrysene	10
2-Nitrofluorene	0.01
1-Nitropyrene	0.1
4-Nitropyrene	0.1

Table 2.1: Potency Equivalency Factors (PEFs) for PAHs and NPAH in B(a)P equivalent. Data from California EPA 1999 except (*) from MOE (Ministry of the Environment- Toronto-Canada) 1997 and (#) from CCME (Canadian Council of Ministers of the Environment) 2008.

The Cancer risk was assessed for each carcinogenic compound detected in the samples, whose *Unit Risk* (UR) value has been established by International Agencies (WHO, 2000 and CA-EPA, 2005).

The lifetime cancer risk (LCR) attributable to inhalation exposure was calculated by multiplying each UR estimated value by the average daily concentration of each compound:

$$LCRi = Ci \times URi$$

The UR of each chemical corresponds to the excess lifetime cancer risk calculated as a result of the continuous exposure to B(a)Peq at a concentration of 1 ng/m^3 in air over a lifetime of 70 years (U.S. EPA, 2011).

The UR used for calculations are reported in Table 2.2.

B(a)P	**Cancer UR** **1,1 x 10^{-6}/ng/m^3 (CA EPA, 2005)**
B(a)P equivalents	**Cancer UR** **8,7 x 10^{-5}/ng/m^3 [rounded to 1 x 10^{-4}/ng/m^3] (WHO, 2000)** **1 x 10^{-4}/ng/m^3 (CCTN, 1991)**

Table 2.2: Unit Risk for cancer risk assessment: B(a)P and B(a)P equivalents

CHAPTER 3

Particulate Matter

3.1 General characteristics of Particulate Matter

Air pollution is already known to increase risks for a wide range of diseases, such as respiratory and heart diseases (Pope C.A., *et al.*, 2009). Studies indicate that in recent years exposure levels have increased significantly in some part of the world, particularly in rapidly industrializing countries with large populations. The most recent data indicate that in 2010, 223.000 deaths from lung cancer worldwide resulted from air pollution (Straif, K., *et al*. 2013). In terms of the global burden of all diseases attributable to air pollution, lung cancers account for less than 7% of the 3.22 million estimated deaths (Hamra, G.B., *et al*, 2014).

Airborne air pollution is made up of particulate matter (PM) and hundreds of chemicals from natural sources and human activities (Brauer, M., *et al*, 2012; Fajerztajn et al., 2013)

In October 2013, the International Agency for Research on Cancer (IARC) classified outdoor air pollution and particulate matter from outdoor air pollution as carcinogenic to humans (*Group 1*) (Loomis, D., *et al*, 2013) based on sufficient evidence of carcinogenicity in humans and experimental animals and strong mechanistic evidence.

Particle pollution (also called particulate matter or PM) is the term for a mixture of solid particles and liquid droplets found in the air. PM is an air-suspended mixture of solid and liquid particles that

vary in time and space, in dependence on the source of emission, by the chemistry of the atmosphere and weather conditions (Pope and Dockery, 2006; Schauer *et al.*, 1996). Among these particles, there are different components, such as sand, ash, dust, soot, acidic substances of various nature, plant substances, metal compounds, natural and artificial textile fibres, salts and elements such carbon or lead.

Among the chemical-physical characteristics of particles, the size is the one used for classification due to its intrinsic importance. In fact the atmospheric deposition rates of particles, their residence times in the atmosphere and deposition patterns within the lung are strongly influenced by particles size.

The behaviour of particles in the atmosphere and within the human respiratory system is determined largely by the physical properties, which have a strong dependence on size, varying from a few nanometres to tens of micrometres.

The aerodynamic properties of particles determine how they are transported in air and how they can be removed from it. These properties also govern how far they get into the air passages of the respiratory system. Additionally, they provide information on the chemical composition and the source of particles.

Airborne particles have irregular shapes and their aerodynamic behaviour is expressed in terms of the diameter of an idealised spherical particle known as *aerodynamic diameter*. The sampling and description of particles is based on this aerodynamic diameter, which is usually simply referred to as “particle size”. The aerodynamic diameter of a particle is the diameter of a spherical

particle having unit density and the same settling velocity from an airstream as the particle under study (Schlesinger 1985).

Based on size, particulate matter is often divided into two main groups:

- *coarse fraction* contains the larger particles with a size ranging from 2.5 to 10 μm ($PM_{10} – PM_{2.5}$).

- *fine fraction* contains the smaller ones with a size up to 2.5 μm ($PM_{2.5}$).

The particles in the fine fraction which are included in the 1 μm – 0.1 μm range are called *ultrafine* particles (UFP or UP).

One of the most important distinctions of particulate pollution is based on how the particles are introduced into the atmosphere:

- primary particles, that are released directly from their source into the atmosphere. The main sources of primary PM are the land, the sea through soils carried by the wind and the generation of marine aerosol particles by the bursting of air bubbles in breaking waves. Road transport and industrial process are also major sources of primary particles. Primary PM and the precursor gases can have both man-made (*anthropogenic*) and natural (*non-anthropogenic*) sources. Anthropogenic sources include combustion engines (both diesel and petrol), solid-fuel (coal, lignite, heavy oil and biomass) combustion for energy production in households and industry, other industrial activities (building, mining, manufacture of cement, ceramic and bricks, and smelting), (Schwarze *et al*., 2006) and erosion of the pavement by road traffic and abrasion of brakes and tyres. Agriculture is the main source of

ammonium. Natural sources include sea salt, naturally suspended dust, pollen and volcanic ash (EEA, 2012).

- secondary particles are subsequently formed within the atmosphere as a result of chemical reactions, producing substances of low volatility, which consequently condense into solid or liquid phase, becoming PM. Examples include sulphates and nitrates, formed from the oxidation of sulphur dioxide (SO_2) and nitrogen dioxide (NO_2) in the atmosphere to acid, which are then neutralised by atmospheric ammonia derived from agricultural sources. Carbonaceous particulates also contain a secondary fraction formed from the oxidation of volatile organic compounds (VOCs). Compared to primary particles, the chemicals processes involved in the formation of secondary ones are relatively slow and their persistence in the atmosphere is prolonged. The secondary particles are composed almost exclusively of particles of diameter less than 2.5 µm (Kelly *et al*., 2012).

The process by which secondary particles are formed is termed nucleation (Hamed *et al.*, 2007); this term refers to the processes by which molecules of low volatility condense to form solid or liquid matter. There are two distinct types of nucleation process (Hinds, 1999). The first is heterogeneous nucleation. Most secondary particle formation in the atmosphere occurs by this process, newly formed substances condense onto existing particles causing the growth of those particles. The condensation processes are most effective on small and medium sized particles, causing these to grow larger. The second process is called homogeneous nucleation. The best known process of homogeneous nucleation occurs when sulphuric acid (H_2SO_4) is formed from the atmospheric oxidation of SO_2. Another phenomenon that leads to the growth of the

particles is the coagulation, which interact with those produced by nucleation. Coagulation can also affect products from homogeneous reactions occurring in the atmosphere.

Figure 3.1 shows, in schematic form, the typical size distribution of airborne particles. Sizes range over several orders of magnitude. The smallest, freshly nucleated, particles are only 1–2 nm in diameter and contain only tens of molecules. At the other extreme, particles may be up to ~100 μm in diameter, which is comparable to a human hair. Particles as large as this rapidly settle out of the air and are of minor health significance because, although they can be inhaled, they do not generally penetrate beyond the nose and mouth. Consequently, air pollution research generally focuses on particles ≤10 μm in diameter, although it is important to recognise that larger particles are also present and contribute mass to 'total suspended' particles (TSP).

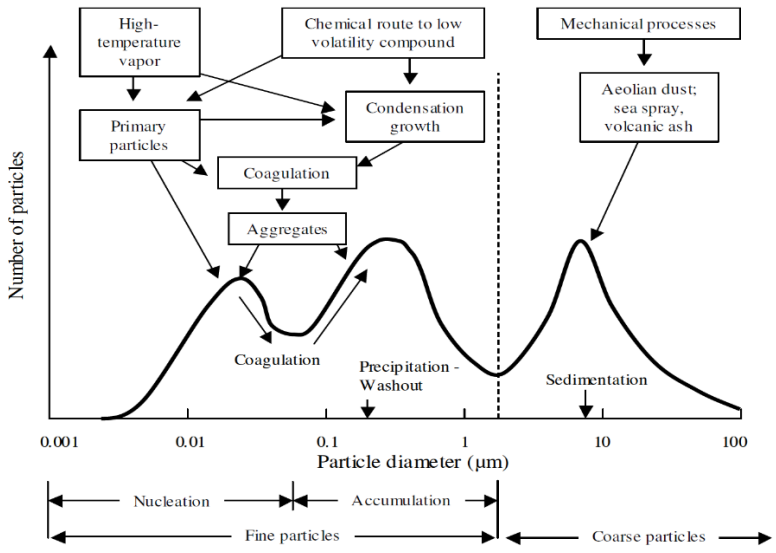


Figure 3.1 Schematic diagram of the size distribution of airborne particles
Adapted from: United Kingdom Department of Environment, Food, and Rural Affairs, Expert Panel on Air Quality Standards, 2004

3.2 Composition of Particulate Matter

Airborne particles have a very diverse chemical composition that varies both in time and space. Air samples of particulate matter from urban areas from around the world typically show the same major components, although in considerably different proportions according to the sampling location (Harrison and Yin 2000). Airborne particles contain both major and minor components. The major components, normally, include the following:

- *Sulphate* – arises mainly as a secondary component from atmospheric oxidation of SO_2, although there may be a small primary component that arises from sea salt or mineral matter such as gypsum;
- *Nitrate* – normally present as NH_4NO_3, which results from the neutralisation of HNO_3 vapour by NH_3, or as sodium nitrate ($NaNO_3$), due to displacement of hydrogen chloride from NaCl by HNO_3 vapour;
- *Ammonium* – generally present in the form of ammonium sulphate ($(NH_4)_2SO_4$) or NH_4NO_3;
- *Sodium and chloride* – in sea salt;
- *Elemental carbon* – black, graphitic carbon formed during the high temperature combustion of fossil and contemporary biomass fuels;

- *Organic carbon* – carbon in the form of organic compounds, either primary, resulting from automotive or industrial sources, or secondary, resulting from the oxidation of volatile organic compounds (VOCs);
- *Mineral components* – crustal materials (rock and soil) are rich in elements such as aluminium, silicon, iron and calcium. These are generally present in coarse dusts that arise from, for example, wind-driven entrainment processes, quarrying, construction and demolition processes;
- *Water*
- *Biological materials* - bacteria, spores, pollens, debris and plant fragments; generally coarse in size, considered as part of the organic
- *Carbon component* in most studies rather than as a separate biological component.

Minor components include the following:

- *Trace metals* – many metals such as lead, cadmium, mercury, nickel, chromium, zinc and manganese are used in metallurgical processes. Some occur as impurities or additives in fuels and others are used in industrial products. These and other uses cause emissions to the atmosphere, but concentrations are generally very small;
- *Trace organic compounds* – although the total mass of organic compounds can comprise a significant part of the overall mass of particles, it is made up of a very large number of individual organic compounds, each of which is present at a very low concentration. Such organic compounds vary greatly in

composition and include aliphatic and aromatic hydrocarbons, heterocyclics and oxygenates such as aldehydes, ketones and carboxylic acids. Generally speaking, organic compounds that arise directly from fuel combustion processes have relatively high hydrogen to carbon ratio, whereas secondary organic compounds are more oxidised and polyfunctional carbonyl and carboxylic acids species are often present (**Figure 3.2**).

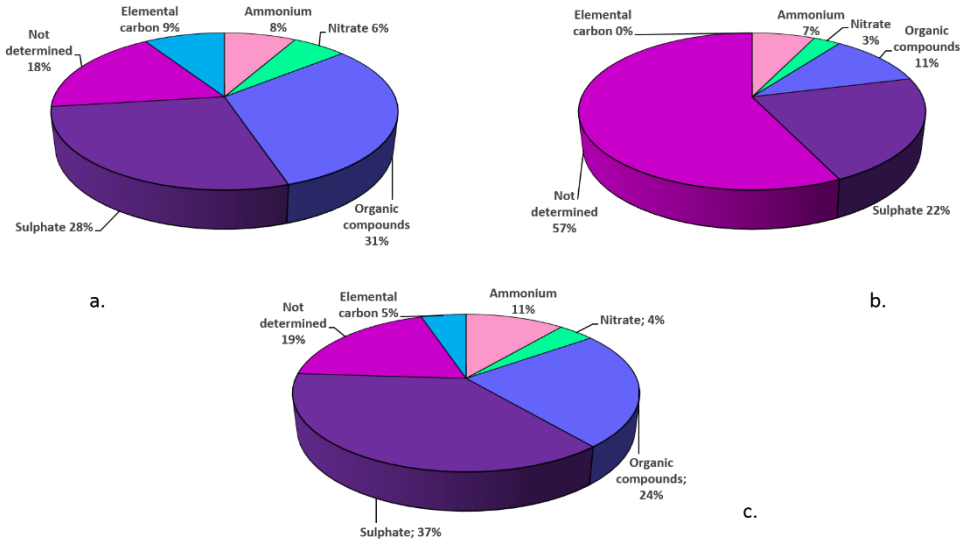


Figure 3.2: Composition (%) of particulate typical of urban source (a), natural (b) and rural (c). *(*source Ministry of the Environment, Land and Sea)

3.3 Effects of Particulate Matter

The respiratory system is the primary target of airborne particles which are inhaled and tend to accumulate in the airways. The comprehension of PM dosimetry is important to understand the toxic potential of PM. The respiratory tract can be divided into three main regions: the *extrathoracic region*, the *tracheobronchial region* and the *alveolar or pulmonary region*, where gas exchange occurs (U.S. EPA, 2004; **Figure 3.3**). The most important PM property driving deposition rate in the airways is particles size: in fact inhaled particles penetrate the respiratory system depending on mechanisms, such as inertial impact, sedimentation, diffusion, electrostatic attraction, which are related to particles size (U.S. EPA, 2004). Each region of the respiratory system presents typical clearance mechanisms that protect the organism from inhaled external agents. Filtration of the nasal passages is the main protective mechanism in the head region, while mucus secretion and ciliated cells act to remove particles and other external bodies in the tracheobronchial region. At the alveolar surface the clearance machinery is constituted by macrophages and type II pneumocytes that perform particles removal through their phagocytic activity. Particular attention should be finally paid to ultrafine particles which, thanks to their tiniest dimensions, may be able to escape macrophages removal and penetrate the alveolar-capillary barrier (Elder *et al*, 2006).

The size of the particle is a main determinant of where in the respiratory tract the particle will come to rest when inhaled. Simple nomenclature is used to indicate the different degrees of relative penetration of a PM particles into the cardiovascular system

(**Figure 3.3**). Larger particles are generally filtered in the nose and throat via cilia and mucus (*inhalable fraction*). The particulate matter smaller than about 10 micrometers, referred to as PM_{10}, can settle in the bronchi and lungs *(thoracic fraction).* Particles smaller than 2.5 micrometers, $PM_{2.5}$, tend to penetrate into the gas exchange regions of the lung (alveolus) and very small particles (<100 nanometers) may pass through the lungs to affect other organs *(respirable fraction*) (Nemmar *et al*, 2002).

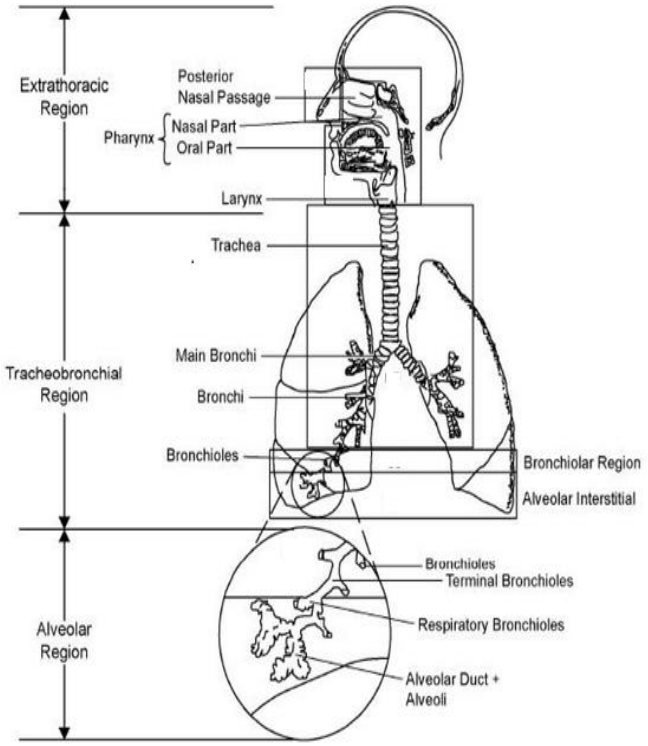


Fig 3.3 Disposition of particulate matter (PM) in the respiratory system (U.S. EPA 2004)

3.3.1 PM exposure and health effects

Numerous epidemiological studies have established an association between short or long-term exposure to various airborne particulates and human mortality and morbidity (Chen and Lippmann, 2009; Rueckerl e*t al*, 2011; Schwarze et al, 2006). Studies have found a significant correlation between excess mortality and short-term exposure to high concentration of ambient particulate matters (Bell and Davis, 2001; Schwartz and Markus 1990). A population-based study on data collected in 6 US cities suggested an association between long-term exposure and human mortality (Dockery et al. 1993; Laden et al., 2000). Studies conducted in European and United States cities reported short-term exposure to PM_{10} to increase mortality of a value ranging from 0.46 to 0.62 % (mean 0.5 %) for each 10 $\mu g/m^3$ increment in the daily concentration (Cohen *et al*., 2004; Katsouyanni *et al*., 2001).

Pope and colleagues (Pope et al., 2009) reported that $PM_{2.5}$ concentrations fell by a third from the early 1980s to 1990s across major US metropolitan areas, with each 10 $\mu g/m^3$ reduction associated with an increase in life expectancy of 0.61 years (Pope et al., 2009).

A report from the prospective Cancer Prevention II study of the American Cancer Society, including about 500.000 participants, showed that each 10 μg/m^3 increase in fine particulate air pollution was associated with a 6% increase in all-cause mortality, and with a 9% increase in risk of cardiopulmonary mortality and 14% increase in risk of lung cancer (Pope *et al*., 2002).

Recent long-term studies show associations between PM and mortality at level well below the current annual WHO air quality guideline level for $PM_{2.5}$ (10 μg/m^3). Scientific evidence does not suggest a threshold of exposure to PM below which no adverse health effects would be anticipated (WHO, 2006a, 2006b, 2013). Another important epidemiological project, ESCAPE (European Study of Cohorts for Air Pollution Effects), was recently published. In the first study the association between long-term exposure and lung cancer was confirmed unequivocally (Raaschou-Nielsen *et al*., 2013) whereas the second study was focused on the relationship between long-term mortality and air pollution (Beelen *et al.*, 2013). In the first study the authors did not explore the causes of death. Recently it was reported an 18% increase in lung cancer incidence for each 5 μg/m^3 increase in $PM_{2.5}$ concentration in this cohort (Raaschou-Nielsen *et al*., 2013). The second study presents an analysis of pooled data from 22 longitudinal cohort studies across Europe, including more than 360.000 people followed up for an average of 13.9 years (Beelen *et al*., 2013). The authors show a 7% increase in natural cause mortality with each 5 μg/m^3 increase in $PM_{2.5}$ concentration (Beelen *et al*, 2013). These results are important because they present contemporary evidence from

diverse European populations exposed to $PM_{2.5}$ concentration below the limits recommended in existing guidelines.

Toxicological studies widely described the genotoxic effect of PM in humans (Møller *et al*., 2008; Billet et al., 2008; Oh *et al.,* 2011). These results, together with the epidemiological evidences of lung cancer mortality for long-term exposure to $PM_{2.5}$, suggest a role for DNA damage in PM related health effects (U.S. EPA, 2009). In agreement with epidemiology, fine PMs usually have a higher genotoxic potential compared to coarse fractions, in *in vitro* and *in vivo* models (Chackra *et al*., 2007; de Kok et al., 2005; Park et al., 2005); moreover, PMs from industrialized, traffic and wood/biomass combustion areas have been found to exert a stronger effect on DNA (Shi *et al*., 2006; U.S. EPA, 2004).

The high genotoxicity of these PMs is due to the particles composition and source, since particles derived from combustion processes have been found to be rich in metals and organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), which are notorious genotoxic elements (Mehta *et al.,* 2008). Actually, different mechanisms have been reported for PM-induced DNA damage, which include oxidative stress, organics metabolites formation and direct interaction of particles organic compounds with the DNA (Møller *et al.,* 2008). Generation of ROS and consequent oxidative stress is widely recognized as an important toxicological mechanism in PM induced DNA double strand breaks/oxidation and possibly lung cancer (Shi *et al.,* 2006; Møller *et al*., 2008).

High level of ROS production could lead to cell apoptosis and tissue damage, which may contribute to lung injury as seen in human or animal models following acute exposure to PM. ROS are able to initiate a series of redox signalling cascades to induce inflammatory responses and cytokine production found in both acute and chronic PM exposures (Akthar *et al.*, 2010; Diabate *et al.*, 2011). It has been demonstrated that transition metals (Cr, Cd, Ni and arsenic) and aldehydes present in PM can inhibit DNA damage repair mechanisms and this effect may enhance the genotoxic effect of PM and contribute to lung carcinogenesis (Mehta *et al.*, 2008).

3.4 Polycyclic aromatic hydrocarbons (PAHs)

A major group of chemicals that are found in complex mixtures are the polycyclic aromatic hydrocarbons (PAHs), a family of more than 1500 compounds (NTP, 2012) comprised of two or more fused aromatic rings, found both in their native and substituted form. PAH molecules are made up of three or more benzene rings. PAHs are ubiquitous environmental contaminants that are formed as a result of incomplete pyrolytic processes and to which humans are exposed through inhalation, ingestion and dermal absorption.

In nature, PAHs are found in coal tar, crude oil, being also produced during combustion processes, such as forest fires and volcano eruptions (Rajput and Lakhani, 2009). In urban atmospheres, where the occurrence of PAHs in ambient air is of a particular concern, the majority of PAHs come from vehicular traffic emissions (Castellano *et al*, 2003; Gaga *et al*, 2012; Omar *et al*, 2002); however, PAHs are also emitted by trains and aircrafts. Stationary sources such as domestic heating, various industrial processes, waste incineration and energetic production systems are other significant contributors of PAHs to outdoor atmosphere (WHO, 1998). Due to their persistence in environmental matrices, as well as their adverse effects on human health, PAHs have been classified as priority pollutants by the US EPA – United States Environmental Protection Agency (U.S. EPA, 2004). PAHs are generally insoluble in water but can be solubilised in organic solvents or organic acids. This means that in aqueous environments PAH are generally found adsorbed on particulates and on humic matter; PAHs have an affinity for environmental matrices, such as

sediments and soils due to their greater hydrophobicity and through bioaccumulation and thus magnified in the food chain (WHO – IARC, 1998; Senthilkumar *et al.*, 2008).

Once released into the atmosphere, PAHs are partitioned between the PM and gas phases depending on their molecular weight (Chen *et al*., 2005; Furuuchi *et al*., 2007). As a result, larger molecular weight PAHs (< 4 rings) are almost exclusively bound to particulate matter, while low molecular weight PAHs (3 rings) can also be found dissolved in water (Wang *et al*., 2007; Khaiwal *et al.*, 2006).

Links between human exposure to complex PAH mixtures and development of diseases including cancer, and respiratory and cardiovascular diseases have been described previously (IARC, 2010; Pope *et al*, 2002). Despite their structural similarities, PAHs vary greatly in their carcinogenic potency, with both individual and complex mixtures of PAHs classified as possible or probable human carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 2010).

Subsequent to exposure, PAHs cross through cell membranes and, being lipophilic, undergo intracellular accumulation by concentrating in liquid droplets (Murphy et al., 2008). Studies from Ramesh et al have shown that PAHs can initiate and accelerate atherosclerosis and aneurysm in experimental animals through a cascade of biochemical events that involve oxidative stress and inflammation (Ramesh *et al*, 2015; Prins *et al*., 2012; Wang *et al*., 2009).

3.4.1 Carcinogenic effects of PAHs

The binding of PAHs to DNA, and the associated effects that occur as a result, is considered the major mechanism of PAH-induced mutagenesis and carcinogenesis. Like chemical carcinogens, PAHs require activation through a series of enzymatically-catalyzed reactions to form their active metabolites (Conney *et al*., 1982; Huberman *et al*.,1976; Sims *et al*., 1974). The cytochrome P-450 family of enzymes, in particular CYP1A1, CYP1A2 and CYP1B1, are primarily involved in bioactivation of PAHs and formation of their reactive intermediates (Pelkonen and Nebert, 1982; Shimada and Fujii-Kuriyama, 2004). Many PAHs are ligands for the aryl hydrocarbons receptor (AhR), which has different roles in metabolism including regulation of the different bioactivating enzymes (Baird *et al*., 2005; Pàlkovà *et al*, 2015). The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factor essential for adaptive responses to xenobiotics (Hao *et al*., 2013). Polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons (HaHs) are by far the most common classes of AhR ligands (Denison *et al*., 2003). For example, B(a)P is the most widely studied PAH. It is usually chosen as a marker for evaluating the toxicity of PAH mixtures, since the available toxicological data provide a sufficient basis for the conventional risk assessment, in which it can be assumed that the toxicity of all PAHs is equivalent to that of B(a)P. As a result of recent toxicological research, B(a)P has become classified as human carcinogen, group 1 (IARC 2012).

B(a)P is activated through a three step enzymatic mechanism involving (Jarvis *et al*., 2014) initial metabolism by CYP enzymes to B(a)P-7,8-epoxides, followed by conversion to B(a)P-7,8-diols by epoxide hydrolase, and final transformation to the ultimate reactive B(a)P-7,8-diol-9,10-epoxide metabolites, again by CYP enzymes (Conney, 1982) (**Figure 3.4**). This mechanism of activation has also been shown for other PAHs.

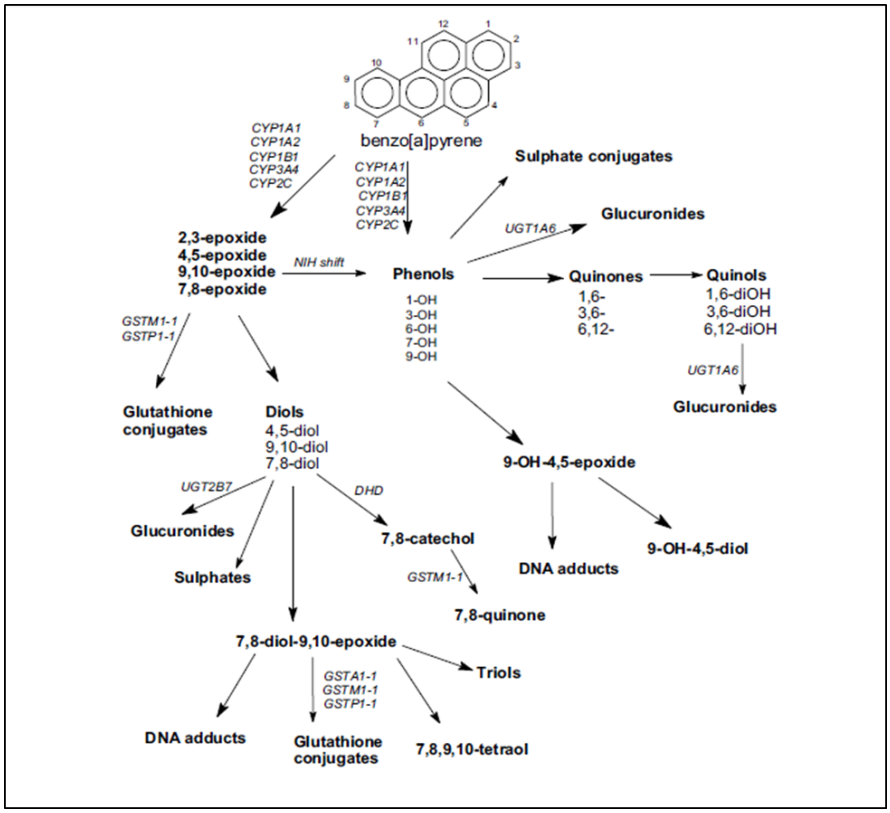


Fig.3.4: Metabolism of benzo(a)pyrene

The tumorigenic and mutagenic activities of many PAHs have been linked to the ability of their diol epoxide metabolites to bind covalently to exocyclic amino groups on purine bases to form either stable bulky adducts or depurinating adducts which are released from DNA, leaving abasic sites (Rogan *et al.*, 1993; Szeliga and Dipple, 1998). Following bioactivation and reaction with DNA, the PAH metabolites can induce mutation that either activate oncogenes or inactivate tumour suppressor gene as part of their carcinogenic mechanism (Jarvis *et al.*, 2014).

CHAPTER 4

Materials and Methods

4.1 Experimental design

Six different monitoring campaigns were performed by Arpa ER in the period 2011-2013 (**Table 4.1**).

For each campaign, the toxicological characterization in BALB/c 3T3 cells and the cancer risk assessment were performed. During my training period at Harlan Cytotest Research, (Rossdorf, Germany), I have had the opportunity to perform the Bhas 42 CTA. Two different campaigns (Winter 2012 and Summer 2013) were evaluated with both cell transformation assays in order to compare the results obtained by the two testing methods.

CAMPAIGNS	SEASONS	CELL TRANSFORMATION ASSAY BALB/c 3T3 A31.1.1		CELL TRANSFORMATION ASSAY Bhas 42		CANCER RISK
		CYTOTOXICITY	TRANSFORMATION	INITIATION	PROMOTION	
I	AUTUMN 2011	✓	✓			✓
II	SUMMER 2012	✓				✓
III	AUTUMN 2012	✓	✓	✓	✓	✓
IV	WINTER 2013	✓	✓			✓
V	SUMMER 2013	✓	✓	✓	✓	✓
VI	AUTUMN 2013	✓	✓			✓

Table 4.1: Scheme of the tests used in the different monitoring campaigns

The experimental design for the toxicological characterization is reported in Figure 4.1.

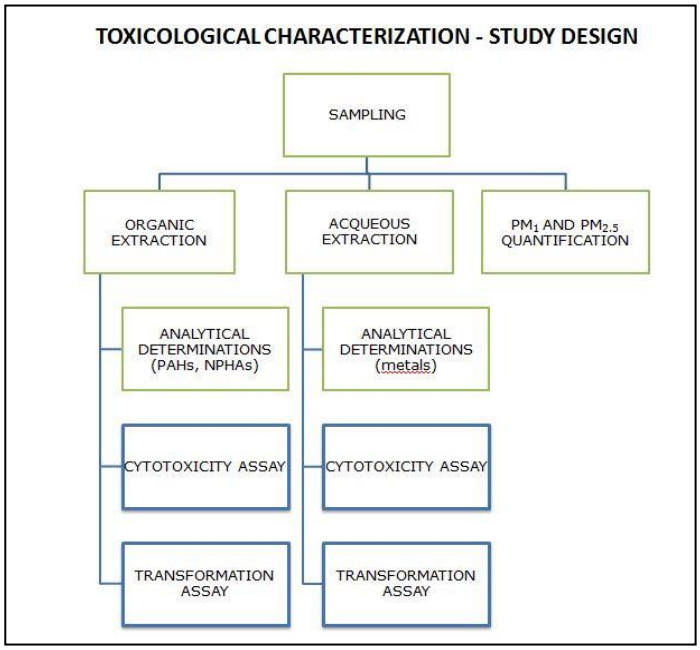


Figure 4.1 Study design for the toxicological characterization. Green lines: activity performed by Arpa ER

In Figure 4.2, the experimental workflow for cancer risk assessment is reported.

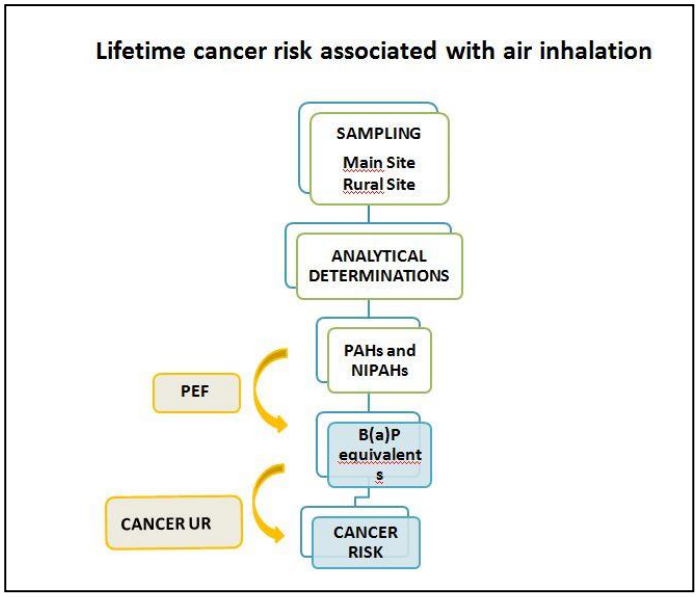


Figure 4.2 Experimental workflow for cancer risk assessment. Green lines: activity performed by Arpa ER.

4.2 Air samples collection

For the toxicological characterization, the $PM_{2.5}$ fraction was collected by the high volume air Flow $PM_{2.5}$-HVS sampler, whereas Air Flow polyurethane foam (PUF), which could collect simultaneously gas (using PUF-polyurethane foam) and PM_1, was used for PM_1 sampling. Each filter was weighed daily before and after $PM_{2.5}$ and PM_1 collection in order to obtain the gravimetric data.

For cancer risk assessment, the $PM_{2.5}$ samples were collected at two sites located in the surroundings of Bologna (Emilia Romagna, Italy). The sites were representative of different levels of environmental pollution.

$PM_{2.5}$ was collected daily on quartz-fiber filters (47 mm diameter) by low-volume air flow samplers (Skypost TCR TECORA), during the sampling campaigns I-IV, whereas high volume air flow sampler were used during campaigns V and VI.

4.3 Preparation of treatment solutions

All the filters collected in one season were pooled to obtain a unique sample that was representative of the season.

To obtain organic extracts, each pooled sample was extracted with acetone, using a Soxhlet apparatus, then reduced to dryness and dissolved in dimethylsulfoxide (DMSO) at 800 m^3 equivalents/ml. The $PM_{2.5}$ and PM_1 inorganic fractions were extracted by sonication in ultrapure water and diluted in order to obtain stock solutions at 200 m^3 equivalents/ml.

The treatment solutions were prepared by diluting the stock solutions in the culture media immediately before use, at final doses ranging 1.5-12 m^3 equivalents / 60 mm plate (0.5-4 m^3 equivalents/ml) for BALB/c 3T3 or 2-8 m^3 equivalents/6 well plate (1-4 m^3 equivalents/ml) for Bhas 42. The final concentrations of the vehicles DMSO or ultrapure water were 0.5% or 0.2%, respectively.

4.4 BALB/c A31-1-1 Cells

The BALB/c 3T3 A31-1-1 cell line was purchased from the *Health Science Research Resource Bank* (Osaka, Japan). The cells were grown in Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS), cryoconserved in MEM 10% FBS solution containing 5% DMSO and used for the CTAs at passages 3-5 from arrival. For the transformation assays, only sub-confluent cells (about 70% confluence) were used. The target cells were not maintained in culture beyond the third passage after thawing (**Figure 4.3**).

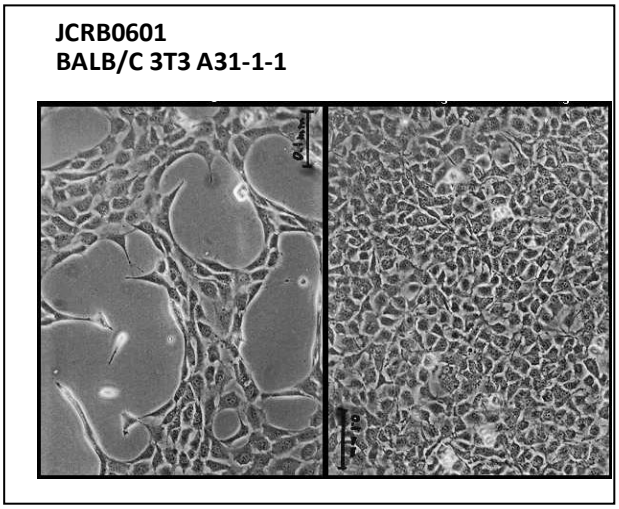


Figure 4.3: Balb/c 3T3 A31-1-1 cells at different degrees of confluence. 10X magnification (from cellbank.nibio.go.jp/celldata/jcrb0601.htm)

4.5 BALB/c A31-1-1 Cell Transformation Assay

4.5.1 Cytotoxicity test

The cytotoxicity assay was performed by seeding exponentially growing BALB/c 3T3 A31-1-1 cells at 250 cells/60-mm dish in five replicates for each treatment. Cells were then exposed to different concentrations of the environmental samples (1.5 – 12 m^3 equivalents) for 48 h. At the end of this period, the treatment was removed and fresh culture medium was added to the plates. Cells were maintained in culture for 8-10 days, then fixed with methanol, stained with 10% aqueous Giemsa and scored for colony formation. Only colonies containing more than 50 cells were counted (IARC/NCI/EPA Working Group, 1985; Franken et al., 2006). Untreated BALB/c 3T3 A31-1-1 cells and solvent-treated cells were used as negative controls.

The results were expressed as the mean number of colonies/plate ± standard error (SE), the absolute clonal efficiency (ACE), i.e. the fraction of cells which survived chemical treatment with respect to the number of seeded cells, and the relative clonal efficiency (RCE), which estimates the percent reduction of cell clonal efficiency in treated groups as compared to that of the relative control (vehicle-treated cells).

4.5.2 Morphological Cell transformation assay

Cells were seeded at a density of 3 x 10^4 cells/60 mm dish, incubated for 48 h and then exposed to the $PM_{2.5}$ and PM_1 extracts at concentrations ranging from 1.5 to 12 m^3 equivalents. Ten replicates were carried out. Untreated BALB/c A31-1-1 cells and solvent-treated cells were used as negative controls. Positive controls were represented by cells treated with the well-known carcinogen, 3-MCA, final concentration 4 µg/ml. After 48 h, the treatment medium was removed and replenished with fresh normal culture medium. Cells were maintained in culture for 4 weeks, with medium changes twice a week, then fixed with methanol, stained with 10% aqueous Giemsa and scored for foci formation (**Figure 4.4**).

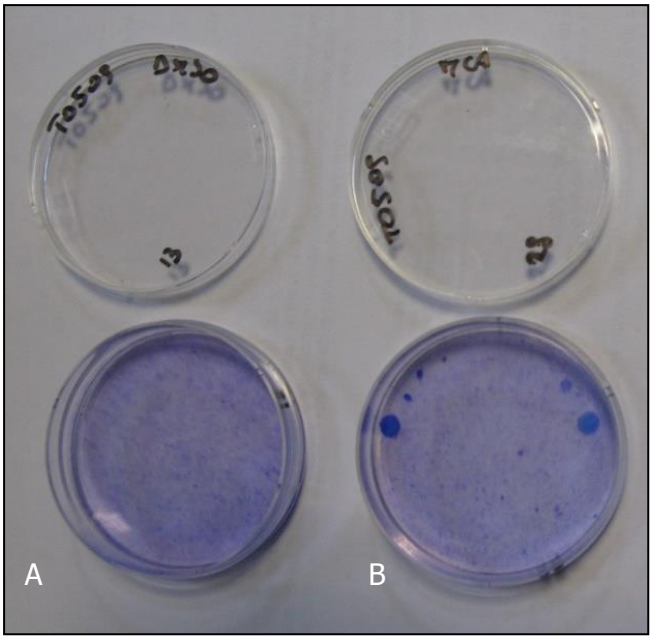


Figure 4.4: Plates from a cell transformation experiment
A – negative control - cells treated with solvent (DMSO).
B – positive control - cells treated with carcinogen (3-MCA).

4.5.3 Evaluation Criteria for Morphological Cell Transformation

The scoring of foci was carried out according to the recommended guidelines (Sasaki *et al*., 2012; Colacci *et al*., 2011). Only foci considered as positive (type III) (**Figure 4.5**) showing deeply basophilic, dense multilayering of cells, random cell orientation at all parts of the focus edge, invasion into the surrounding contact-inhibited monolayer and domination of spindle-shaped cells were counted. Unlike the parental cells, cells isolated from foci type III are tumorigenic when injected into the host mice (Kakanuga *et al*., 1985; Colacci *et al.*, 1993).

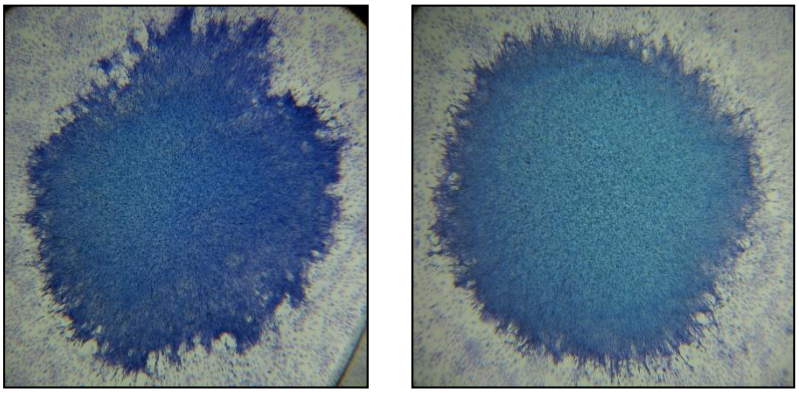


Figure 4.5: transformed foci induced by 3-MCA (40X).

The result of the CTAs are considered acceptable when: (i) the cell transformation was significantly increased in the positive controls; (ii) no significant increase in the cell transformation was observed in the negative control (DMSO); (iii) the total number of transformed foci detected in the positive control plates was at least 2-time higher than the number of foci detected in the negative control.

Results were reported as the number of positive control (plate with foci/scored plates); the mean number foci/plate ± standard error (SE); and the transformation frequency (TF), calculated on the cells that survived after chemical exposure. TF is expressed as a function of the total number of foci for treatment divided by the number of surviving cells estimated from the clonal efficiency observed in the cytotoxicity assay performed in parallel with the transformation test (Schechtman, 1985).

4.5.4 Classification criteria

According to the classification criteria as previously established (Ohmori *et al*., 2005; Hayashi *et al*., 2008; Mascolo *et al*., 2010; Sakai *et al*., 2010), the CTA test is considered positive when the following criteria are fulfilled:

- the increase of the mean number of transformed foci/plate was statistically significant, at 99% confidence level, when two or more doses induced positive effects;
- the increase of the TF in the treatment groups was at least two-fold higher than that detected in the solvent control;

– the toxicity in the treatment groups was less than 90%, i.e. the surviving cells in the treatment group were more than 10% of the treated population.

If only the first criterion but not the second one was fulfilled, the test would be considered equivocal. If either the first criterion or the second criterion were fulfilled but not the third one, the test would be considered as misleading positive. Negative samples were those where no statistically significant increase of transformed foci was observed at any assayed dose.

4.5.5 Statistical analysis

The difference between the mean colony numbers in the treated groups compared to the control group was evaluated by the Student t-test. Significant differences in the ACE of cells exposed to the chemical treatments were tested by the z-test for comparison of two proportions. The RCE was analyzed by the Chi-square test of significance in 2 x 2 contingency tables. The significant percentage of plates with foci with respect to scored plates was calculated according to the Fisher – Yates test of significance in 2 x 2 contingency tables. The statistical analysis of the *foci* distribution was performed by the Mann-Whitney test (*Mann – Whitney unpaired t-test*). The transformation frequency (TF) significance was analyzed by the comparison of the Poisson rates, after verifying that the TF values would fit the Poisson distribution. The linear regression analysis and the Cochran – Armitage test for positive trends were applied to the TF dose-response curve in the cell transformation assay.

4.6 Bhas 42 Cells

The Bhas 42 cell line was purchased from the *Health Science Research Resource Bank* (Osaka, Japan). The cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and 1% 100x Penicillin/Streptomycin solution, cryoconserved in MEM 10% FBS solution containing 5% dimethylsulfoxide (DMSO) and used for the CTAs. Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 100 units/ml of penicillin, 100µg/ml of streptomycin and 5% FBS was used for the transformation assay.

4.7 Bhas 42 Cell transformation assay

The current protocol consist of two assay components, the initiation assay and promotion assay for examining tumour-initiating activity and tumour-promoting activity of chemicals, respectively (Asada *et al*., 2005; Sakai *et al*., 2010).

In the initiation assay the cells are treated at the beginning of the growth phase to allow for fixation of the induced DNA damage, whereas in the promotion assay the cells are repeatedly treated at stationary phase to provide growth advantages for anomalous cells.

4.7.1 Cell growth assay

Initiation assay

Cells were seeded at a density of 2 x 10^3 cells/ml and distributed into each well of 6-well micro plates in a 2 mL volume (4000 cells/well, day 0), incubated for 24 h and then exposed to the $PM_{2.5}$ and PM_1 organic extracts at concentrations ranging from 2 to 8 m^3 equivalent/well (1-4 m^3/ml). Three wells were prepared for each treatment group. On day 4, the medium containing the test chemical was replaced with the fresh medium DF5F. On day 7 the cells were fixed with 10% formalin and stained with a 0.1% Crystal Violet (CV) solution for 15 min. CV was extracted from the stained cells with 2 ml of dye extraction solution (containing 0.02 mol/HCl and 50% ethanol), and the OD is measured at a wavelength between 540-570 nm. The relative cell growth of cultures treated with a chemical is calculated as follows:

relative cell growth (%) = [(Treatment – Blank)/(Control – Blank)] X 10

Promotion assay

In the cell growth component of the promotion assay, cells were seeded at a density of 7 x 10^3 cells/ml (14 x 10^3 cells/well, day 0) and on day 4 the culture medium was replaced with a medium containing the different doses (2 - 8 m^3/well) of PM extracts. On day 7 the cells were fixed, stained with CV and the CV extracted from cells was measured as previously described.

4.7.2 Cell transformation test

Initiation assay

Cells were seeded at a density of 2 x 10^3 cells/ml and distributed into each well of 6-well micro plates in a 2 ml volume (4 x 10^3 cells/well, day 0). On day 1, 24 h after seeding, the cells were treated for three days in the same way as the cell growth assay. The medium was changed on day 4, 7, 10, and 14. On day 21, the cells were fixed with methanol and stained with 5% Giemsa solution (**Figure 4.6**)

Promotion assay

The promotion assay was carried out as described for the initiation assay, except for the number of cell plated and timing of chemical treatment. Cells were plated at 14 x 10^3 cells/well and cultured for 4 days without a medium exchange. On day 4, day 7, day 10 the culture medium was replaced with different concentrations of the test chemical and the treatment in the promotion phase was continued until day 14 (**Figure 4.7**).

The known tumour initiator MCA (1 µg/ml final concentration) and the known tumour-promoter TPA (50 ng/ml final concentration) were used as positive controls in the initiation and the promotion assay, respectively. MCA and TPA were dissolved in dimethyl sulfoxide (DMSO), which serves as the vehicle for these two positive agents.

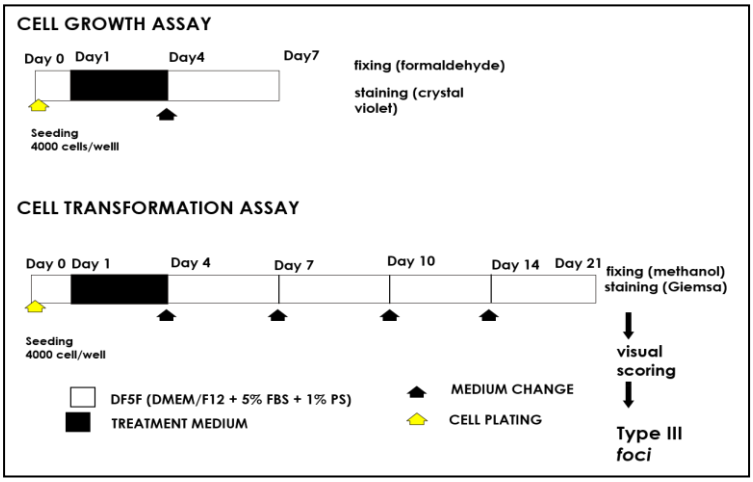


Figure 4.6*:* Schematic protocol of cell growth assay and transformation assay in the initiation assay.

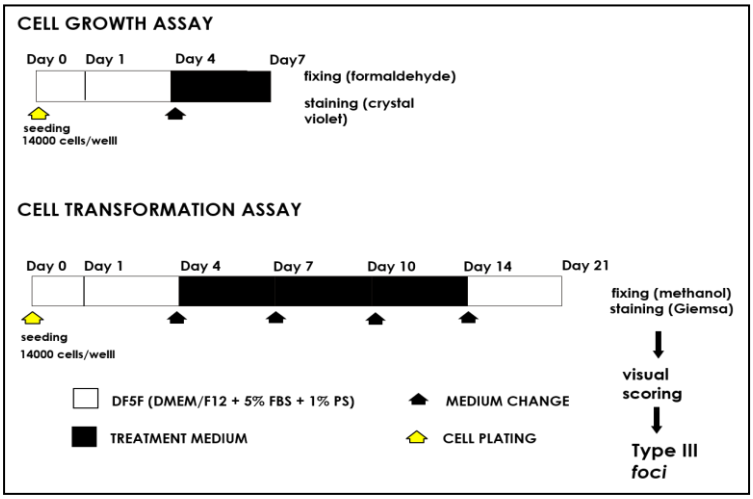


Figure 4.7*:* Schematic protocol of cell growth assay and transformation assay in the promotion assay.

4.7.3 Counting of transformed foci and statistical analysis

The transformed foci were judged on the basis of the following morphological characteristics:

- more than 100 cells;
- spindle-shaped cells whose morphology was distinctly different from the contact – inhibited monolayer cells;
- deep basophilic staining;
- random orientation of cells;
- dense multilayering of cells;
- invasive growth into the monolayer of surrounding contact – inhibited cells

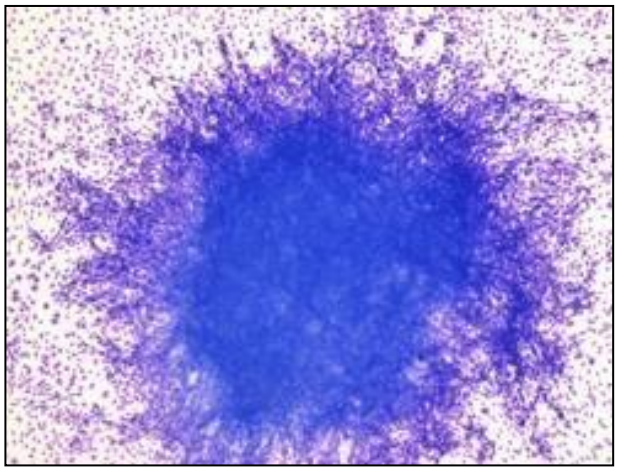


Figure 4.8: Transformed foci induced by 3-MCA (40X).

Test substance – induced transformation frequency (number of transformed foci/well) in the 6-well method is statically analyzed by multiple comparison using the *one-sided Dunnett test* ($p<0.05$, upper side).

4.8 Risk Assessment

Cancer risk analysis associated with lifetime inhalation of airborne environmental pollutants was performed on chemical parameters measured by Arpa ER during six different sampling campaigns, which are carried out in different seasons. The cancer risk assessment was focused on several chemicals extracted from $PM_{2.5}$, such as polycyclic aromatic hydrocarbons (PAH, ng/m^3) and nitroPAHs (NPAH, ng/m^3) extracted from $PM_{2.5}$.

The following 33 PAHs and NPAHs were included in the total PHAs estimation (**Table 4.2**). Among the 33 PAHs which were included in the total PHAs estimation, 15 PAHs were assessed using the potency equivalency factor (PEF) approach. The same PAHs and NPAHs were measured in all sampling campaigns.

Chemical name	IARC classification	PEF
PAHs		
Benzo[*a*]pyrene	1	**1**
Acenaphthene	3	- [a]
Acenaftilene		- [a]
Anthracene	3	- [a]
Benz[*a*]anthracene	2B	0.1
Benzo(b+j)fluoranthene	2B	0.1
Benzo[*e*]pyrene	3	- [a]
Benzo[*ghi*]perylene	3	0.01[#]
Benzo[*k*]fluoranthene	2B	0.1
Cyclopenta[*cd*]pyrene	2A	0.012[*]
Chrysene	2B	0.01
Dibenz[*a,c*]anthracene	3	1[#]
Dibenz[*a,h*]anthracene	2A	- [a]
Dibenzo[*a,e*]fluoranthene	3	- [a]
Dibenzo[*a,e*]pyrene	3	1
Dibenzo[*a,h*]pyrene	2B	10
Dibenzo[*a,i*]pyrene	2B	10
Dibenzo[*a,l*]pyrene	2A	10
Fluoranthene	3	- [a]
Fluorene	3	- [a]
Indeno[1,2,3-*cd*]pyrene	2B	0.1
Naphthalene	2B	- [a]
Phenanthrene	3	- [a]
Pyrene	3	- [a]
Perylene	3	- [a]
N-PAHs		
9-Nitroanthracene	3	- [a]
3-Nitrobenzanthrone	2B	- [a]
7-Nitrobenz[*a*]anthracene	3	- [a]
6-Nitrobenzo[*a*]pyrene	3	- [a]
6-Nitrochrysene	2A	10
9-Nitrofenantrene		- [a]
2,3-Nitrofluoranthene		- [a]
1-Nitropyrene	2A	0.1

Table 4.2: List of PAHs and NPAHs with relative PEFs (Potency Equivalency Factors) a: absence, data from California EPA 1999 except (*) from MOE (Ministry of the Environment- Toronto-Canada) 1997 and (#) from CCME (Canadian Council of Ministers of the Environment) 2008.

Such determinations were used in order to evaluate the lifetime cancer risks associated with air inhalation in two different sites (rural site and urban site), by the relative potency of compounds belonging to the same chemical class and the specific unit of carcinogenic risk.

The daily mean concentrations of chemicals in $PM_{2.5}$ were measured in different sampling periods (2011-2014). When the amount of the chemical present in the analyzed sample was lower than the detection limit (LOD), the half of the LOD (LOD/2) was taken into account, in accordance with US EPA criteria (US EPA, 2000). The LODs for PAHs and NPAHs were rounded to 0.2 ng and 5 ng, respectively.

For the determination of the inhalation exposure, the daily measurements of each chemical belonging to the same chemical class were summed together to obtain daily class total values.

Statistical analyses were performed by Student t-Test to compare the daily mean concentrations ± standard deviation (SD) from the different sites (urban vs rural sites).

Furthermore, in order to properly assess health risks, certain groups of chemicals were assessed as mixtures. Specifically, carcinogenic PAHs were assessed using the toxic equivalency factors (TEF) approach. TEFs for carcinogenic PAHs represent their toxicity relative to B(a)P (WHO/IPCS, 1998).

So, the air concentrations of pollutants from each site were evaluated as B(a)Peq. The potency of many individual PAHs and NPAHs relative to that of B(a)P was estimated in order to obtain the B(a)P equivalents.

All these data were compared to limits and UR (*unit risk*) values for inhalation exposures derived from the available literature (WHO, 2000 and CA-EPA, 2005) (**Table 2.2**).

CHAPTER 5

Results

5.1 Cell Transformation Assay in BALB/c 3T3 A31-1-1 cells

The organic and aqueous extracts from the collected samples were evaluated for cytotoxicity and transforming activity in order to identify dose-related effects possibly associated with different aerodynamic diameters or airborne chemicals component. The cell transformation assay (BALB/c 3T3 CTA) was performed treating cells with equivalent doses (m^3) of organic and aqueous extracts of $PM_{2.5}$ and PM_1 in order to highlight possible differences in the transforming or cytotoxic potential of the mixtures of chemicals associated with particles with different aerodynamic diameters. The results from CTA conducted with $PM_{2.5}$ and PM_1 samples have been accepted, as all of the established acceptability criteria were fulfilled.

Before the experimental study, particular attention was paid to the choice of the cell model and the reference protocol

The validation study of the cell transformation assay *in vitro* with BALB/c 3T3 cells, coordinated by EURL-ECVAM (Corvi *et al*., 2012), has in fact lead to the development of a standardized, transferable and repeatable protocol, which will form the basis of the testing Guideline OECD.

The protocol used by the Laboratory of Experimental Toxicology in previous projects of air quality monitoring protocol (in-house protocol) (Mascolo et al., 2010) differed from the ECVAM validated protocol (**Figure 5.1**) for the following features:

- seeding - the cell number is increased (3×10^4 instead of 1×10^4 in 60 mm diameter plates) (Matthews *et al*., 1993);
- time treatment – the cells were treated on Day 2 instead of Day 1 (*i.e*. 48 h after seeding instead of 24h) (Matthews *et al*., 1993);
- culture medium – the cells were maintained in standard media from Day 7 to the end of the experiment instead of the ECVAM protocol modified media (enriched basal medium supplemented with 2% fetal bovine serum) (Tsuchya *et al*., 1995; 2010).
- number and type of plates.

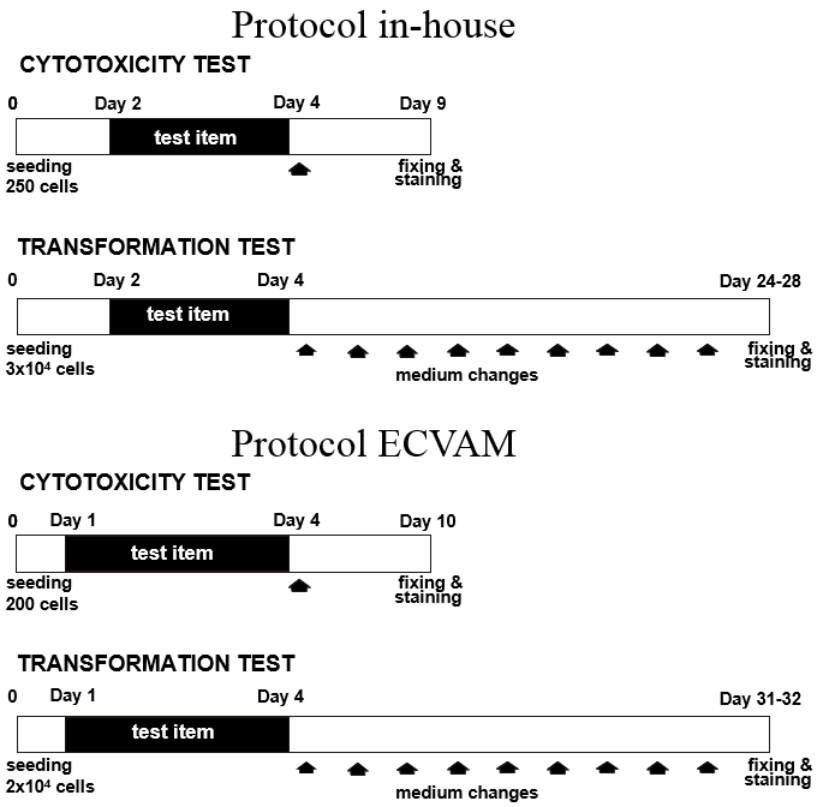


Figure 5.1: Comparison of the "in house" protocol and the ECVAM protocol

The performance of the two protocols of the cell transformation assay was compared by using the organic extract $PM_{2.5}$ collected during the I autumn campaign (**Figures 5.2** and **5.3**).

The results obtained by treating cells with the collected $PM_{2.5}$ samples have been reported in Figure 5.2 and 5.3. On the basis of the results obtained both in term of the cytotoxic effect that in term of mean number of detected foci (**Figures 5.2** and **5.3**), no significant differences between the two protocols under comparison have been demonstrated. For this reason, the *in-house protocol* for cell transformation assay was chosen to perform the

evaluation of each collected sample, in order to compare with the results obtained in previous projects. The study continued analyzing the cytotoxic and transformation effects of the organic and inorganic extract of $PM_{2.5}$ and PM_1 collected during the various monitoring campaigns.

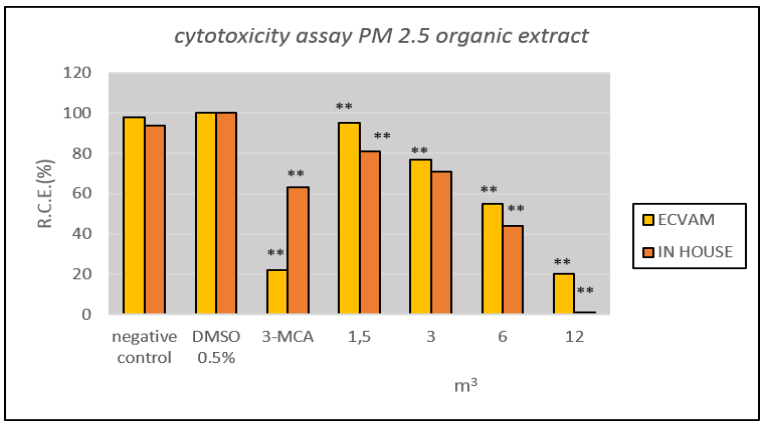


Figure 5.2*:* I autumn campaign - Cytotoxic effects induced by organic extract of $PM_{2.5}$; RCE = relative clonal efficiency

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

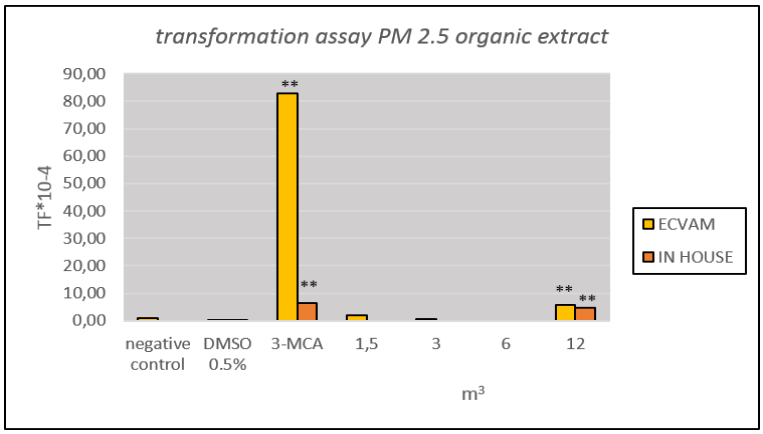


Figure 5.3*:* I autumn campaign - Transformation frequency (TF) induced by organic extract of $PM_{2.5}$

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Poisson test*

5.2 Evaluation of toxicity in BALB/c 3T3 A31-1-1

The study continued analyzing the cytotoxicity and transformation effects induced by the organic and inorganic extract of $PM_{2.5}$ and PM_1 collected during the various monitoring campaigns.

The following figures (**Figures 5.4 – 5-9**) report the relative clonal efficiency (RCE), which estimates the percent reduction of cell clonal efficiency in treated groups as compared to that of the relative control (DMSO).

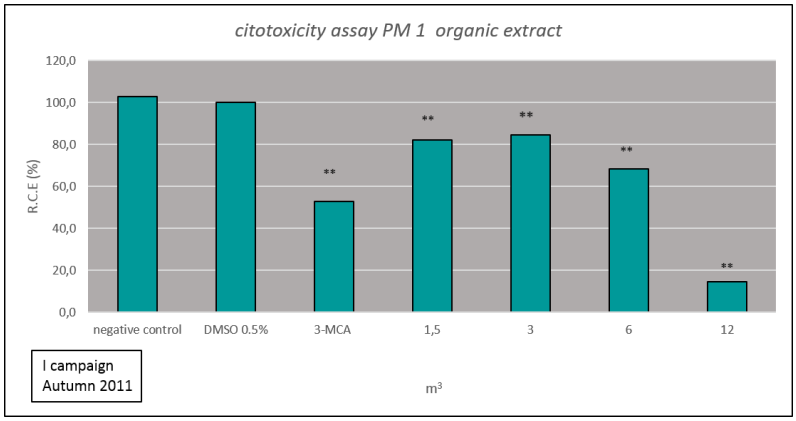


Figure 5.4*:* RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_1$ I autumn campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

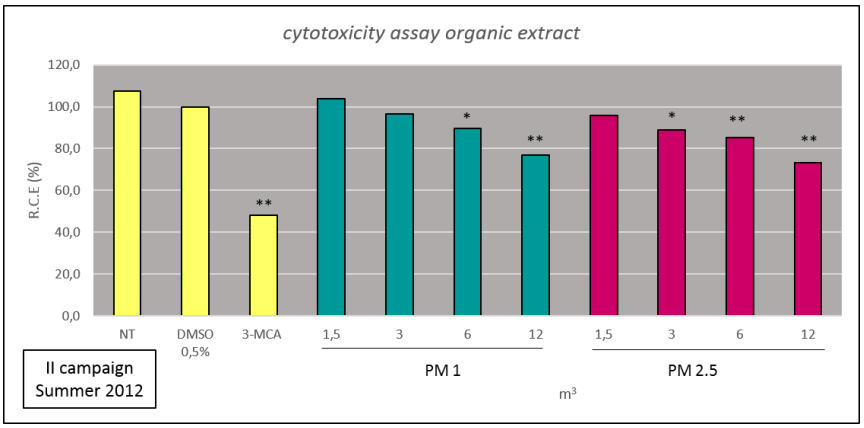


Figure 5.5: RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_{1}$ and PM $_{2.5}$ II summer campaign 2012

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

** significantly different (p<0.05) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

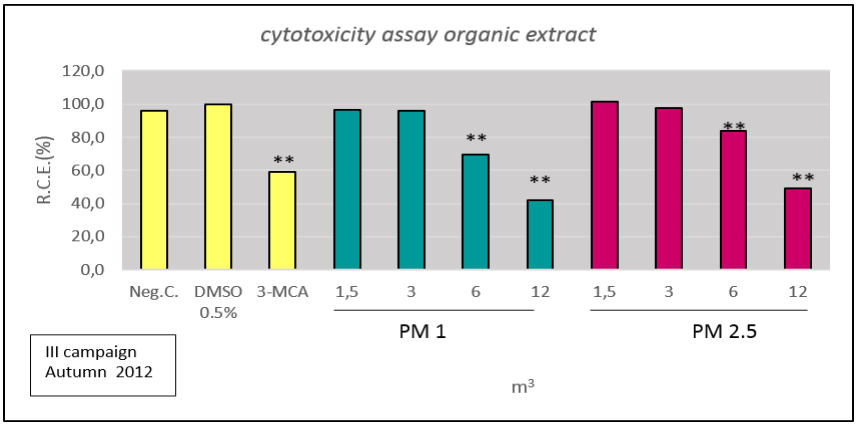


Figure 5.6*:* RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_{1}$ and PM $_{2.5}$ III Autumn campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

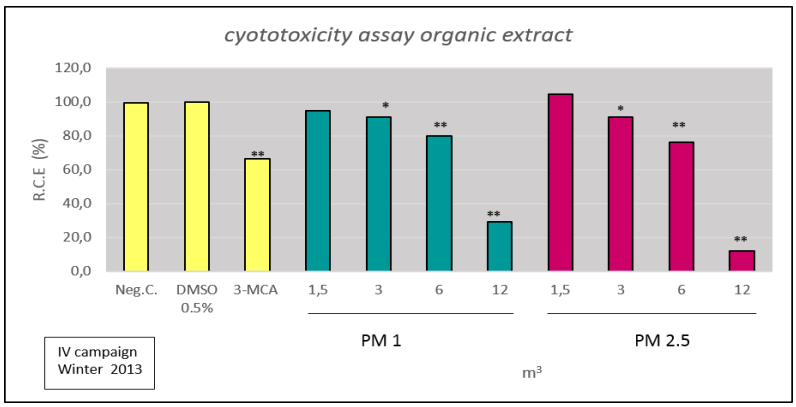


Figure 5.7*:* RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_{1}$ and PM $_{2.5}$ IV Winter campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

** significantly different (p<0.05) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

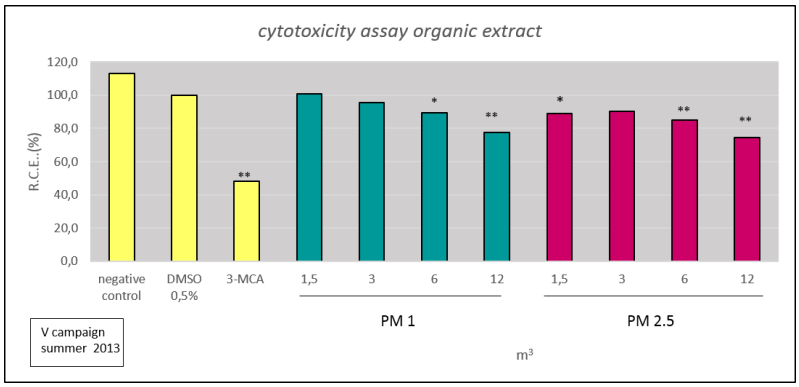


Figure 5.8*:*RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_{1}$ and PM $_{2.5}$ V Summer campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

** significantly different (p<0.05) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables.*

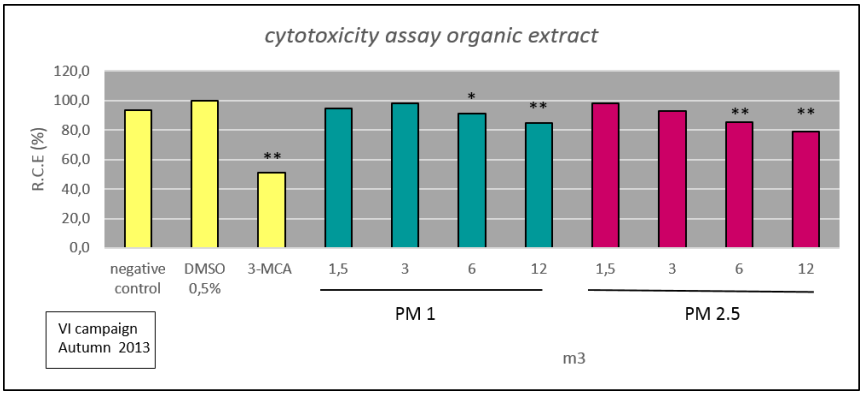


Figure 5.9*:* RCE relative clonal efficiency cytotoxicity effects induced by organic extract of PM $_{1}$ and PM $_{2.5}$ VI Autumn campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

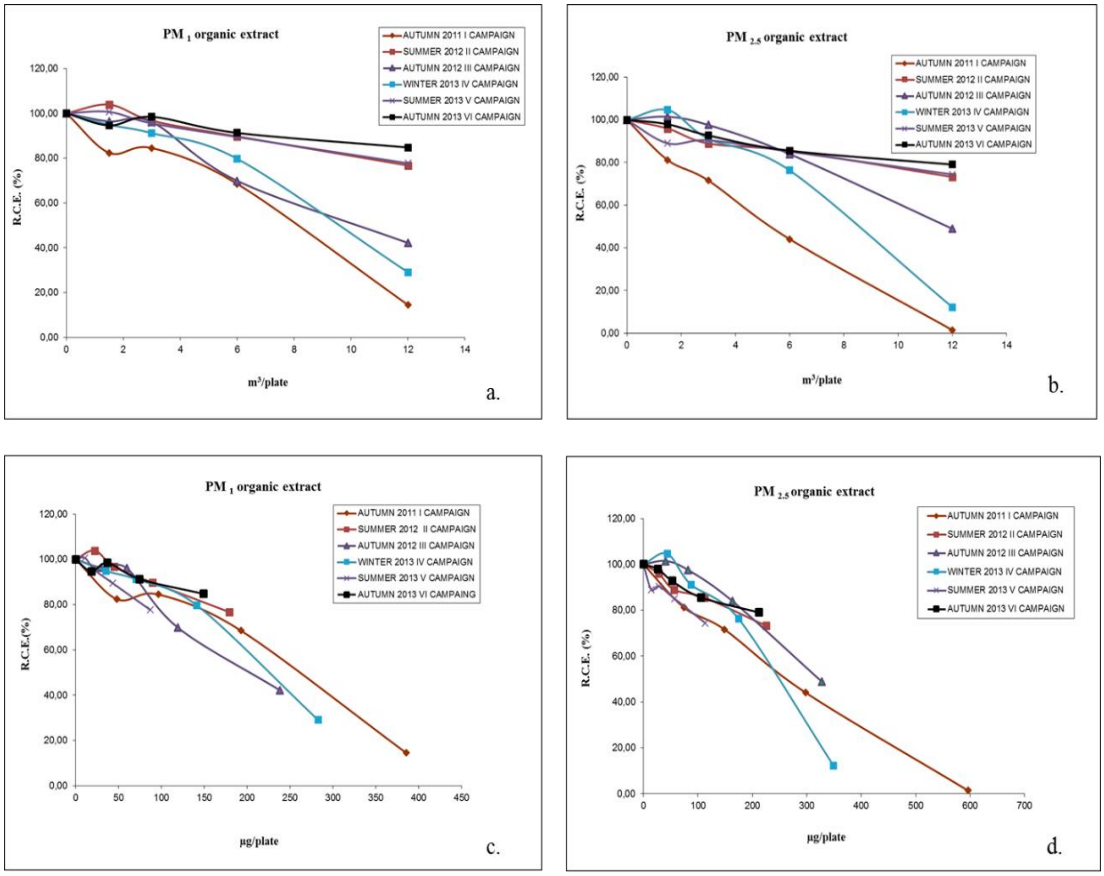
** significantly different (p<0.05) from control DMSO (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

All the assayed $PM_{2.5}$ and PM_{1} organic extract samples were able to induce significant dose-dependent cytotoxicity. All the winter and summer samples of organic extract of PM_{1} and $PM_{2.5}$ were toxic at least at the two higher doses (6 m^3 and 12 m^3) (**Figures 5.2** and **5.4 - 5.9**).

The samples collected during the I campaign (autumn 2011) were the most toxic, as all the treatment doses were found to induce highly significant cytotoxic effects (**Figure 5.2**).

All the assayed organic extracts induced a dose-related reduction in the cloning efficiency, but the degree of the toxic response which was provided depended on the analyzed sample and was mainly associated with the season of sampling (**Graphic 5.1 a-b**). In general, the worst scenario seems to be associated with autumn 2011 both for PM_{1} and $PM_{2.5}$ organic extracts.

The differences in behaviour between the samples derived from different campaigns in term of cellular toxicity were confirmed when the RCEs were related with the amount of particulates (PM, µg/plate) corresponding to the m^3 equivalents present in the treatment solutions (**Graphic 5.1 c-d**).



Graphic 5.1: Cytotoxic effects of organic extracts of PM_1 and $PM_{2.5}$ in BALB/c 3T3 A31-1-1.

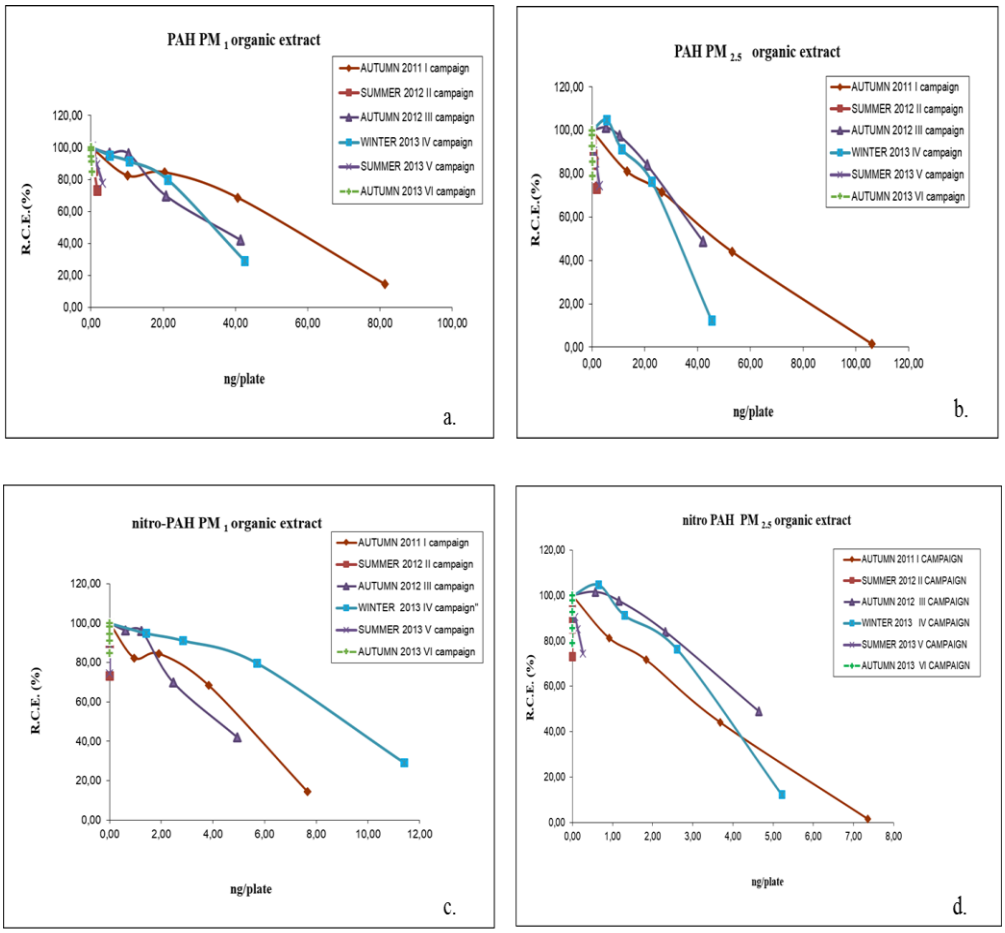
a-b: m3 equivalents/plate; c-d: PM, µg/plate

Overall, the data demonstrated that the observed toxicity was associated with the concentrations of PM present in the sample, which varied with the sampling season. Referring to the organic extracts, the winter samples were always associated with the worst scenarios, in terms of both toxicity and concentration of particles.

Results from our experiments did not show any significant difference between $PM_{2.5}$ or PM_1 organic extracts in eliciting cytotoxic or transforming effects, even if biological effects are usually associated with the fine and ultrafine fractions.

In order to possibly relate the observed toxic effects to the presence and/or the amount of micro-pollutants in the collected PM samples, the extracts were chemically characterized, focusing on micro-pollutant such as PAHs and NPHAs, which have been associatedwith the adverse effects of the inhalable fraction of PM (toxicity,mutagenicity and carcinogenicity) and are of concern for public health (Sun *et al*., 2014). In numerous studies the concentrations of PAHs or NPAHs measured in air samples have also been linked with the toxicological effects of particulate matter and organic extracts of particulates (Colacci *et al.*, 2007; Kang *et al*., 2010). It was observed that some PAHs and NPAHs induced transformation in BALB/c 3T3 cells or in Bhas 42 cells (Asada *et al.*, 2005; Sasaki *et al.*, 2010; Ohmori *et al.*, 2013).

When the cell toxicity observed in the different campaigns was related to the overall amount of PAHs and NPAHs (ng/plate) present in each treatment extract, the seasonal trends previously observed were confirmed (**Graphic 5.2**).



Graphic 5.2: Cytotoxic effects of organic extracts of PM_1 and $PM_{2.5}$ in BALB/c 3T3 A31-1-1

a-b: PAH (ng/plate) ; c-d: NPAHs (ng/plate)

As for the aqueous extracts, cytotoxic effects and even dose-dependent responses were not observed after the treatment with both the PM_1 and $PM_{2.5}$.. The values of ECR which were significantly different from the controls (**Figure 5.10 - 5.15**) were not related to the treatment doses and were higher than the 80%.

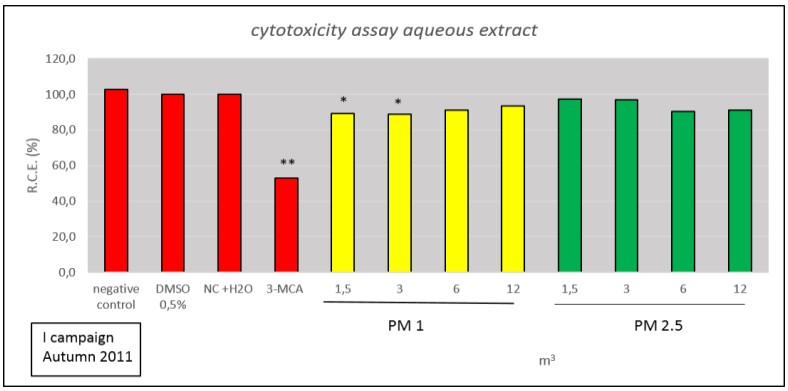


Figure 5.10*:* Cytotoxicity induced by aqueous extracts of PM $_1$ and PM $_{2.5}$ - I autumn campaign. RCE= relative clonal efficiency

** significantly different (p<0.05) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

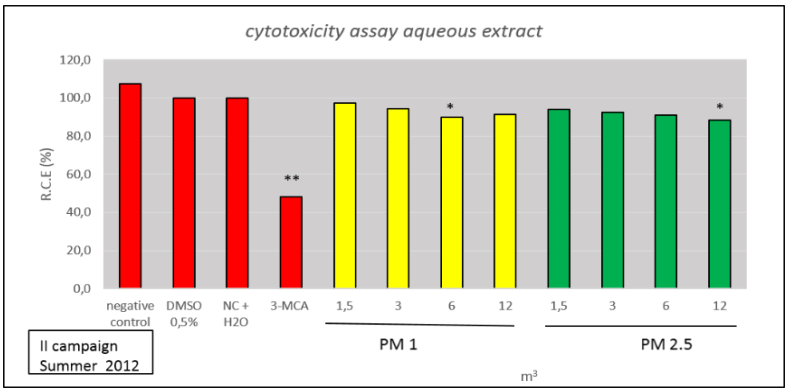


Figure 5.11*:* Cytotoxicity induced by aqueous extracts of PM $_1$ and PM $_{2.5}$ - II Summer campaign. RCE= relative clonal efficiency.

** significantly different (p<0.05) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables.*

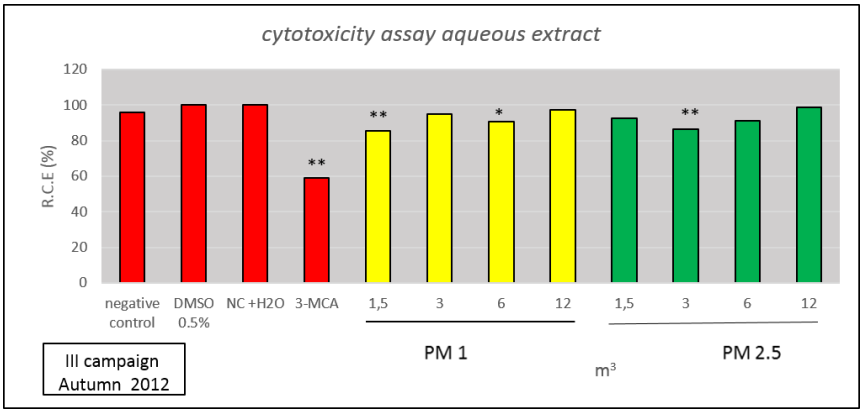


Figure 5.12*:* Cytotoxicity induced by aqueous extracts of PM $_{1}$ and PM $_{2.5}$ - III Autumn campaign. RCE= relative clonal efficiency.

** significantly different (p<0.05) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

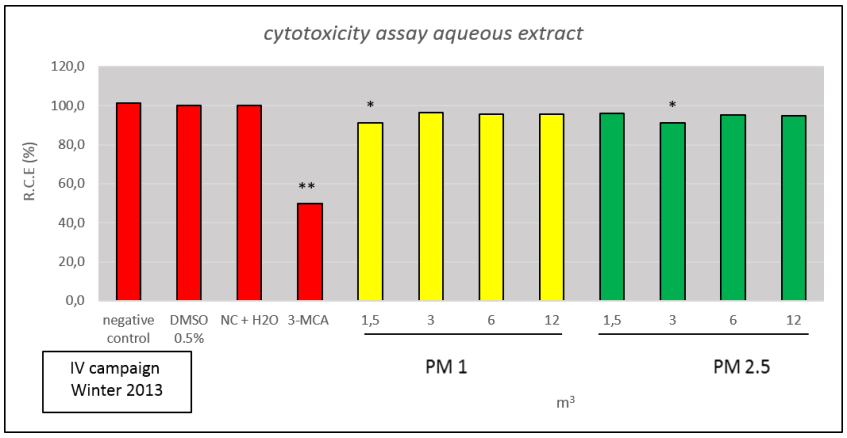


Figure 5.13*:* Cytotoxicity induced by aqueous extracts of PM $_{1}$ and PM $_{2.5}$ - IV Winter campaign. RCE= relative clonal efficiency.

** significantly different (p<0.05) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

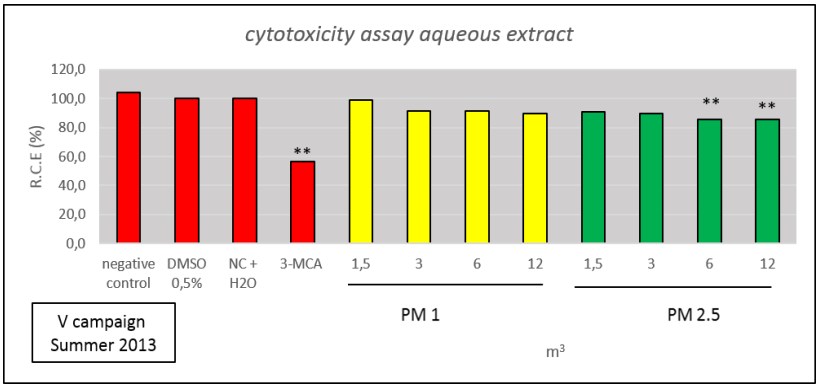


Figure 5.14*:* Cytotoxicity induced by aqueous extracts of PM $_{1}$ and PM $_{2.5}$ – V Summer campaign. RCE= relative clonal efficiency.

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

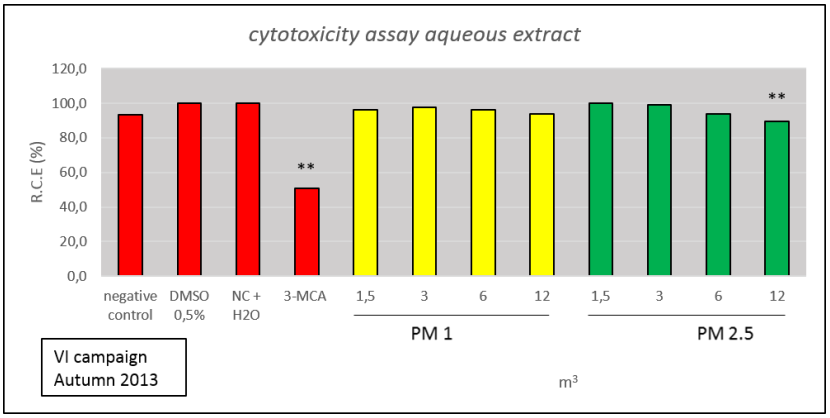


Figure 5.15: Cytotoxicity induced by aqueous extracts of PM $_{1}$ and PM $_{2.5}$ - VI Autumn campaign. RCE= relative clonal efficiency.

*** significantly different (p<0.01) from control (solvent-treated-plates) at the Chi-square test of significance in 2x2 contingency tables*

5.3 Evaluation of the transforming potential in BALB/c 3T3 A31-1-1 cells

The results from the CTAs performed with the $PM_{2.5}$ and PM_1 organic samples were accepted, since all the established acceptability criteria had been fulfilled. The positive control (3-MCA 4 µg/ml) induced a significant increase of the transformation frequency, while the negative control (DMSO 0.5%) did not induce any significant increase of the cell transformation. Also, the total number of transformed foci detected in the positive control plates was at least 2 times higher than the number of foci observed in DMSO-treated plates.

No significant increase in the average number of transformed foci/plate or in the transformation frequency was observed in the plates treated with summer and winter-autumn $PM_{2.5}$ organic samples. The treatment with the extract of $PM_{2.5}$ collected during the I campaign (autumn 2011) did not induce any significant increase in the mean number of foci/plate; however, the transformation frequency was increased in cell treated with the highest dose of the sample (**Figure 5.16**).

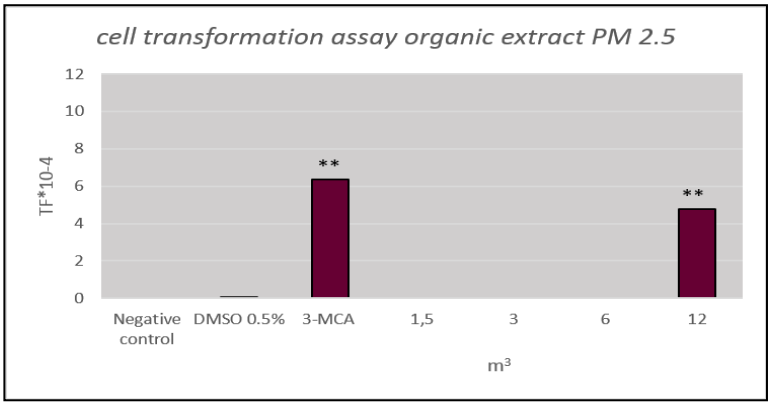
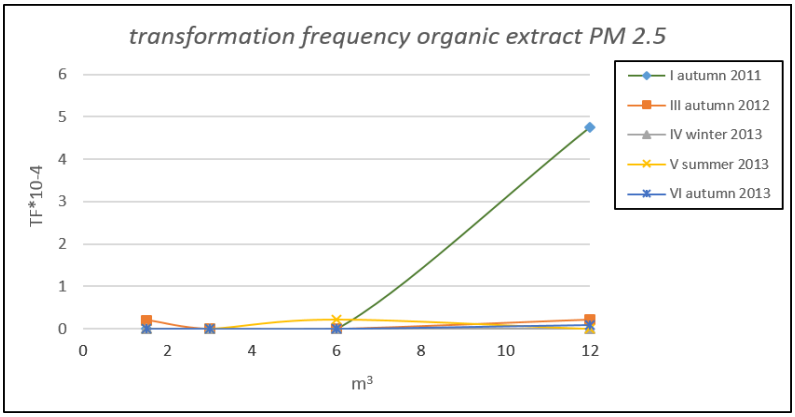


Figure 5.16: I autumn campaign - Transformation frequency (TF) induced by organic extract of PM 2.5 I autumn campaign

*** significantly different (p<0.01) from control DMSO (solvent-treated-plates) at the Poisson test*

This result may suggest that the increase in the transformation frequency was a consequence of the high toxicity of this sample. Therefore, all the $PM_{2.5}$ organic samples (**Graphic 5.3**) were considered negative in the BALB/c 3T3 A31-1-1 cell transformation assay.



Graphic 5.3*:* Transformation frequency (TF) induced by organic extract of PM 2.5 in in BALB/c 3T3 A31-1-1

All the aqueous extracts, as well as the PM_1 organic extracts, failed in inducing cell transformation in our study (data not shown).

5.4 Bhas 42 Cell Transformation Assay

Two different monitoring campaigns (summer 2013 and autumn 2012) were analyzed with the Bhas 42 Cell Transformation Assay. The samples were assayed to detect the initiating potential of organic particulate extracts (PM_1 and $PM_{2.5}$). Moreover, the promoting potential of environmental samples was evaluated by the determination of the occurrence of transformed foci as a consequence of the repeated treatment (total: 10 days) of Bhas 42 cells.

The highest final concentration of the treatment solutions was 4 m^3/ml, which is the same that was used in the BALB 3T3 CTA. So, the highest dose of PM that was provided in each well was 8 m^3. In the initiation assay, the Bhas 42 cells were exposed to equal volumetric concentrations (2-8 m^3) of organic extracts from $PM_{2.5}$ or PM collected during autumn 2012 (III campaign) and summer 2013 (V campaign). In the promotion assay, the same final concentrations of PM extracts were provided at each administration of the treatment.

The cytotoxicity was determined by crystal violet staining. The transformation frequency was calculated as the mean number foci/plate ± standard deviation.

5.4.1 Initiation assay: results from the autumn campaign 2012

The results from the initiation phase of the cell transformation assay for the III campaign (autumn 2012) were shown in Table 5.1 and Figure 5.17.

cell growth assay (a)		
samples	***Absorbance in % of solvent control***	
INITIATION	**PM 1**	**PM 2.5**
medium control	104	104
DMSO 0,5%	100	100
2 m^3	88	79
4 m^3	76	66
6 m^3	55	53
8 m^3	45	43

Table 5.1: Cell growth assay PM_1 and $PM_{2.5}$ III campaign autumn 2012

(a) % of cell growth compared to that of solvent control

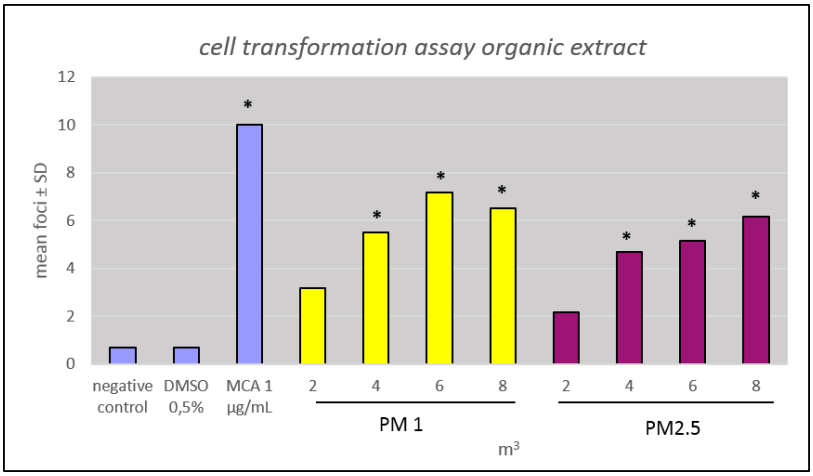


Figure 5.17: Effects of the treatment with $PM_{2.5}$ and PM_1 III campaign autumn 2012 on the transformation rate of Bhas 42 cells (initiation*)*

**Significantly different (p<0.05) from control DMSO at the Dunnet test*

The initiating treatment with the organic extracts of the III campaign (autumn 2012) induced a cytotoxic dose-related effect. The reduction of the cell viability was about 50% at the highest doses (6 m^3 and 8 m^3) (**Table 5.1**).

The transformation frequency was affected by the PM organic extracts, as demonstrated by the significant increase of type III foci at the doses 4, 6 and 8 m^3 ($p < 0.05$, Dunnet test) (**Figure 5.17**).

5.4.2. Promotion assay: results from the autumn campaign 2012

cell growth assay (a)	
samples	***Absorbance in % of solvent control***
PROMOTION	**PM 2.5**
medium control	104
DMSO 0,5%	100
2 m^3	95
4 m^3	97
6 m^3	85
8 m^3	91

Table 5.2: Cell growth assay $PM_{2.5}$ 5 III campaign autumn 2012

(a) % of cell growth compared to that of solvent control

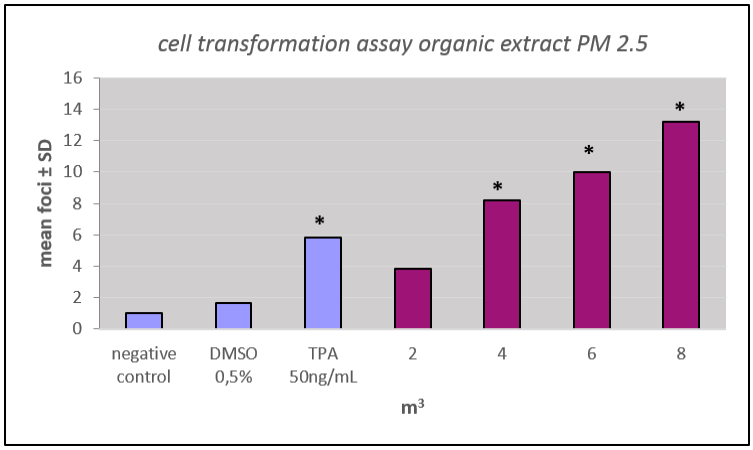


Figure 5.18: Effects of the treatment with $PM_{2.5}$ III campaign autumn 2012 on the transformation rate of Bhas 42 cells (promotion)

**Significantly different ($p<0.05$) from control DMSO at the Dunnet test*

The promotion phase assay was conducted only for $PM_{2.5}$ extracts. No cytotoxic effects were observed at the end of the treatment period up to the highest applied concentration as evaluated both with crystal violet staining and microscopical observation. The significant increase of type III foci was induced at doses 4, 6 and 8 m^3 ($p < 0.05$, Dunnet test) (**Table 5.2** and **Figure 5.18**).

5.4.3 Initiation assay: results from the summer campaign 2013

cell growth assay (a)		
samples	***Absorbance in % of solvent control***	
INITIATION	**PM 1**	**PM 2.5**
medium control	97	97
DMSO 0,5%	100	100
2 m^3	91	97
4 m^3	92	91
6 m^3	89	96
8 m^3	82	91

Table 5.3: Cell growth assay $PM_{2.5}$ and PM_1 1 V campaign summer 2013

(a)% of cell growth compared to that of solvent control

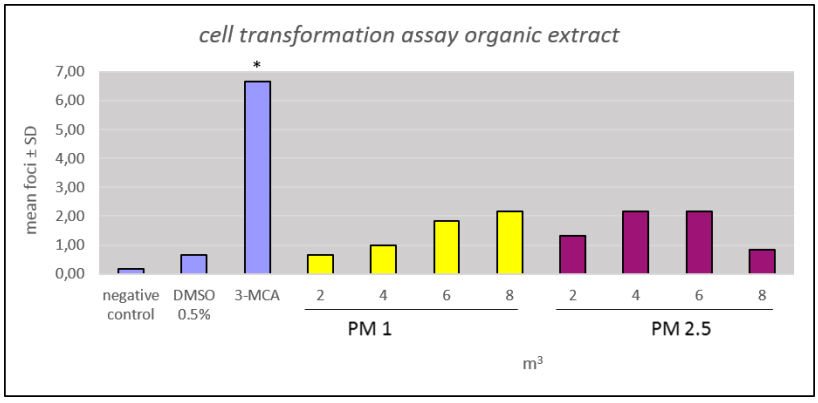


Figure 5.19: Effects of the treatment with $PM_{2.5}$ and PM_1 V campaign summer 2013 on the transformation rate of Bhas 42 cells (initiation)

**Significantly different ($p<0.05$) from control DMSO at the Dunnet test*

The summer extracts did not induce any toxic effect in Bhas 42 cells treated from Day 1 to Day 4 in the initiation assay. In these experimental conditions, neither a statistically significant nor a concentration-dependent increase of type III foci was observed in the transformation assay after treatment with the test items. The negative and the solvent control did not affect the number of type III foci, whereas the positive control MCA (4µg/ml) induced a statistically significant increase in the number of type III foci, thus ensuring the validity of the study.

5.4.4 Promotion assay: results from the summer campaign 2013

cell growth assay (a)		
samples	***Absorbance in % of solvent control***	
PROMOTION	**PM 1**	**PM 2.5**
medium control	107	107
DMSO 0,5%	100	100
2 m^3	99	96
4 m^3	100	97
6 m^3	94	99
8 m^3	103	97

Table 5.4: Cell growth assay $PM_{2.5}$ and PM_1 V campaign summer 2013

(a) % of cell growth compared to that of solvent control

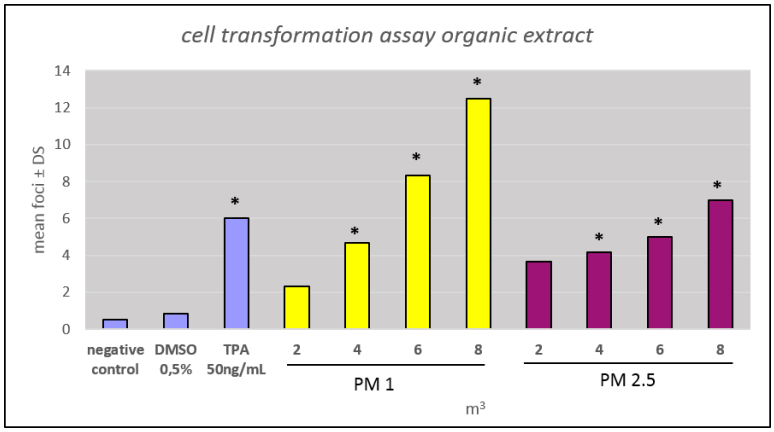


Figure 5.20: Effects of the treatment with $PM_{2.5}$ and PM_1 V campaign summer 2013 on the transformation rate of Bhas 42 cells (promotion)

**Significantly different ($p<0.05$) from control DMSO at the Dunnet test*

In the promotion phase, no cytotoxicity was observed while the number of type III foci was increased after the repeated treatment with both PM_1 and $PM_{2.5}$ extracts at doses 4 m^3, 6 m^3 and 8 m^3 ($p < 0.05$) (**Table 5.4, Figure 5.20**).

5.5 Risk assessment

5.5.1 Chemical characterization

The cancer risk assessment was focused on several chemicals extracted from $PM_{2.5,}$ such as polycyclic aromatic hydrocarbons (PAH, ng/m^3) and nitroPAHs (NPAH, ng/m^3) (**Table 4.2**), which were measured at the urban and rural sites during different seasons.

The urban site, which is located in the northern area of the city of Bologna, is assumed as representative of the urban background. The rural site, which is located in the surrounding of Bologna, in an area far from relevant pollution sources, is considered as representative of the rural background of the eastern Po Valley.

In Table 5.5 the results from the chemical characterization of samples collected during the different monitoring campaigns were reported. The daily mean concentration of pollutants represented the human inhalation exposure during the sampling periods at different sites, assuming that all the inhaled pollutants reached the target tissue. For each sample, the daily concentrations of the individual chemicals belonging to the PAH class were summed together. Then, these values were averaged in order to calculate the daily mean concentration of the PAH mixture. As the LOD/2 was assumed to conservatively overestimate the exposure, the daily mean concentrations were not representative of the average distribution in the different periods, but they were considered as representative of the toxicological worst scenario. The sum of the daily mean concentrations of PAHs for which a PEF was available were also shown in Table 5.5, together with the B(a)P concentration, which is commonly considered a marker for evaluating the toxicity of PAH mixtures.

The micropollutants concentrations in the winter samples were always higher than that determined in summer, as expected for airborne samples collected in the Po valley area. Moreover, the comparison between the urban and rural samples demonstrated that the urban samples were significantly enriched in PAHs and B(a)P (Student t-test, $p<0.01$), except for the IV campaign (winter 2013).

The B(a)P daily mean concentrations were lower than the established EU annual target value (1 ng/m^3) in all the sampling campaigns (**Table 5.8**).

As far as the general trend during the sampling period is concerned, it can be observed that the 2011 autumn represents the worst scenario, as all the values analyzed (total PAHs, PAHs bearing PEF and B(a)P) were higher than all the other campaigns ($p<0.01$, Student t-test) (**Graphic 5.5 and 5.6**)

The NPAHs measurements were always near or below the LOD, so the daily mean concentrations mainly resulted from the implementation of LOD/2, which was used as a precautionary measure. As a consequence, the NPAHs concentrations were very similar to each other (**Table 5.6**). The V summer campaign represented an exception, as the calculations returned a very low NPAHs concentration. The air collection was performed by low volume samplers from I to IV campaign, then high volume samplers were used. As the LODs were unchanged, the concentrations, expressed as ng/m^3, lowered as a consequence of the sampling procedures.

The daily mean concentrations of NPAHs determined at the urban site differed from that estimated for the rural site in the III, IV and V campaigns (**Table 5.6**).

Among the several NPAHs analyzed in this study, 1-nitro pyrene and 6-nitrochrysene were the only two compounds for which the relative Potency Equivalence Factors PEFs were available (**Table 4.2**). So, their concentrations were reported in Table 5.6.

In Table 5.7 the concentrations (ng/m^3) of B(a)P equivalents are shown. Even if the NPAH measurements were always near or below the LOD, the contribution of NPAHs to the B(a)P equivalent calculation was taken into account. The potency for 1-nitropyrene is extremely low (PEF = 0.1), while for 6-nitrochrysene the relative PEF is 10. So, the NPAHs equivalents were mainly attributable to the 6-nitrochrysene B(a)P equivalents transformation (**Table 5.7**).

B(a)P equivalents were very similar in the urban and rural sites during the same season. Some differences, however, were detected during the I and V campaigns (Student t-test, $p<0.01$and $p<0.05$ respectively), even if the leading trend was associated with the season of sample collection.

The contribution to B(a)P equivalents due to PAHs was higher than that of NPAHs, except for the 2012 summer campaign, where the concentration of PAHs B(a)P equivalents was very low compared to the concentration of NPAHs B(a)P equivalents (**Table 5.7**).

PM 2.5 CAMPAIGNS		TOTAL PAHs (ng/mc/24 h) (media ± SD)		PAHs with PEF (ng/mc/24 h)(media ± SD)		B(a)P alone (ng/mc/24h)(media ± SD)	
		URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE
AUTUMN 2011	I	13.15 ± 5.177 [a]	8.152 ± 4.344	10.247 ± 4.092 [a]	6.372 ± 3.471	1,084 ± 0,496 [b]	0,715 ± 0,448
SUMMER 2012	II	0.256 ± 0.089 [a]	0.178 ± 0.061	0.146 ± 0.070 [a]	0.067 ± 0.039	0,015 ± 0,009 [a]	0,007 ± 0,003
AUTUMN 2012	III	3.837 ± 2.163 [a]	2.041 ± 1.403	3.018 ± 1.759 [a]	1.617 ± 1.142	0,401 ± 0,260 [b]	0,215 ± 0,191
WINTER 2013	IV	3.585 ± 2.509 [c]	4.309 ± 2.914	2.996 ± 2.073 [c]	3.162 ± 2.369	0,391 ± 0,476 [c]	0,465 ± 0,407
SUMMER 2013	V	0.256 ± 0.107 [a]	0.077 ± 0.076	0.171 ± 0.076 [a]	0.053 ± 0.056	0,020 ± 0,012 [b]	0,007 ± 0,011
AUTUMN 2013	VI	0.63 ± 0.52 [c]	0.584 ± 0.334	0.473 ± 0.424 [c]	0.370 ± 0.207	0.068 ± 0,073 [c]	0.046 ± 0.031

Table 5.5: Data are expressed as mean concentration ± SD of total PAHs, PAHs with PEF and B(a)P collected during the monitoring campaigns.

[a] Significantly different ($p<0.01$) from rural site at the Student t-test.

[b] Significantly different ($p<0.05$) from rural site at the Student t-test.

[c] Not significantly different from rural site at the Student t-test

PM 2.5 CAMPAIGNS		TOTAL NPAHs (ng/mc/24 h) (mean ± SD)		1-nitropyrene B(a)P eq. (ng/mc/24 h)(mean ± SD)		6-nitrochrysene B(a)P eq. (ng/mc/24 h)(mean ± SD)	
		URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE
AUTUMN 2011	I	0.410 ± 0.071 [c]	0.593 ± 0.650	0.005 ± 0.001 [c]	0.004 ± 0.000	0.497 ± 0.090 [b]	0.449 ± 0.048
SUMMER 2012	II	0.378 ± 0.019 [c]	0.380 ± 0.029	0.005 ± 0.000 [c]	0.005 ± 0.000	0.460 ± 0.005 [c]	0.465 ± 0.025
AUTUMN 2012	III	0.631 ± 0.179 [a]	0.430 ± 0.105	0.005 ± 0.000 [c]	0.005 ± 0.000	0.461 ± 0.260 [c]	0.458 ± 0.007
WINTER 2013	IV	0.435 ± 0.079 [a]	0.970 ± 0.607	0.005 ± 0.000 [c]	0.005 ± 0.000	0.467 ± 0.024 [c]	0,464 ± 0.019
SUMMER 2013	V	0.044 ± 0.012 [a]	0.03 ± 0.003	0.001 ± 0.001 [c]	0.000 ± 0.000	0.037 ± 0.004 [c]	0.035 ± 0.00
AUTUMN 2013	VI	0.332 ± 0.266 [c]	0.201 ± 0.171	0.000 ± 0.000 [c]	0.000 ± 0.000	0.035 ± 0.00 [c]	0.035 ± 0.00

Table 5.6 Data are expressed as mean concentration ± SD of total NPHAs, 1-nitropyrene B(a)P equivalent and 6-nitrochrysene B(a)P equivalent collected during the monitoring campaigns.

[a] Significantly different ($p<0.01$) from rural site at the Student t-test.

[b] Significantly different ($p<0.05$) from rural site at the Student t-test.

[c] Not significantly different from rural site at the Student t-test

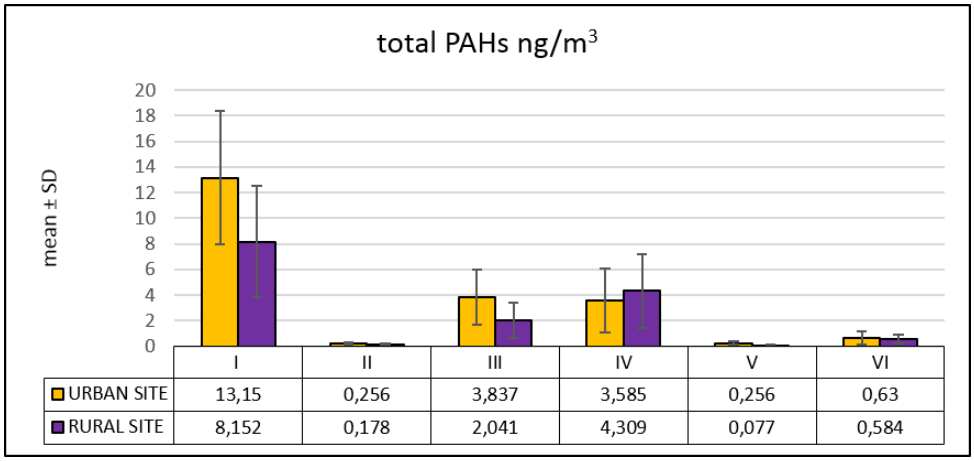
PM 2.5 CAMPAIGNS		PAH B(a)P equivalent (ng/mc/24 h) (media ± SD)		NPAH B(a)P equivalent (ng/mc/24 h) (media ± SD)		PAH + NPAH B(a)P equivalent (ng/mc/24h) (media ± SD)	
		URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE	URBAN SITE	RURAL SITE
AUTUMN 2011	I	4,059 ± 2,460 [a]	2,129 ± 1,090	0,502 ± 0,091 [b]	0,454 ± 0,049	4,561 ± 2,462 [a]	2,582 ± 1,095
SUMMER 2012	II	0,097 ± 0,034 [c]	0,087 ± 0,038	0,464 ± 0,005 [c]	0,469 ± 0,026	0,561 ± 0,038 [c]	0,557 ± 0,044
AUTUMN 2012	III	1,395 ± 0,731 [c]	1,003 ± 1,260	0,465 ± 0,011 [c]	0,463 ± 0,007	1,860 ± 0,731 [c]	1,466 ± 1,261
WINTER 2013	IV	1,163 ± 0,921 [c]	1,626 ± 1,231	0,472 ± 0,024 [c]	0,468 ± 0,019	1,635 ± 0,936 [c]	2,094 ± 1,227
SUMMER 2013	V	0,072 ± 0,030 [a]	0,026 ± 0,018	0,038 ± 0,004 [b]	0,035 ± 0,000	0,110 ± 0,028 [a]	0,061 ± 0,017
AUTUMN 2013	VI	0,297 ± 0,288 [c]	0,249 ± 0,197	0,035 ± 0,000 [c]	0,035 ± 0,000	0,333 ± 0,288 [c]	0,285 ± 0,197

Table 5.7: Data are expressed as mean concentration ± SD of PAH B(a)P equivalent, NPAH B(a)P equivalent and PAH + NPAH B(a)P equivalent collected during the monitoring campaigns.

[a] Significantly different ($p<0.01$) from rural site at the Student t-test.

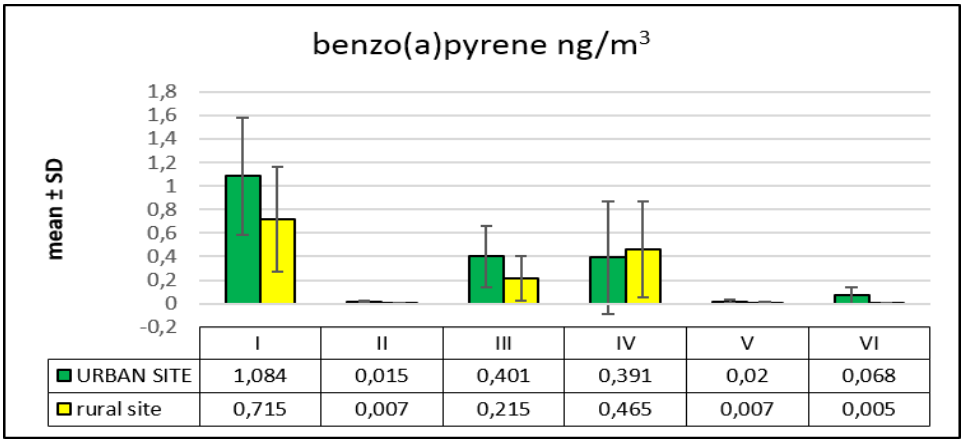
[b] Significantly different ($p<0.05$) from rural site at the Student t-test.

[c] Not significantly different from rural site at the Student t-tes



	I	II	III	IV	V	VI
URBAN SITE	13,15	0,256	3,837	3,585	0,256	0,63
RURAL SITE	8,152	0,178	2,041	4,309	0,077	0,584

Graphic 5.5: mean ± SD of total PAHs in six different campaigns



	I	II	III	IV	V	VI
URBAN SITE	1,084	0,015	0,401	0,391	0,02	0,068
rural site	0,715	0,007	0,215	0,465	0,007	0,005

Graphic 5.6: mean ± SD of B(a)P in six different campaigns

5.5.2 Cancer risk: PAHs and NPAHs

In order to calculate the cancer risk, the values derived from the transformation of PAHs and NPAHs in B(a)P equivalents were used. These values were usually evaluated to obtain the assessment of the carcinogenic risk associated with complex mixtures. The cancer risk was calculated multiplying the exposure (concentration of pollutants in the air expressed as ng/m^3) for the cancer risk unit ($1 \times 10^{-4}/ng/m^3$) determined for B(a)P equivalents (CCTN, 1991; WHO, 2000).

The cancer risk estimates from B(a)P life-time inhalation and from PAHs and NPAHs life-time inhalation, expressed as B(a)P eq, are shown in Table 5.8 and Table 5.9.

A comparison between the cancer risk associated with PM inhalation at the urban and rural sites during the same season was carried out first. Then, the comparison was extended to all the different monitoring campaigns.

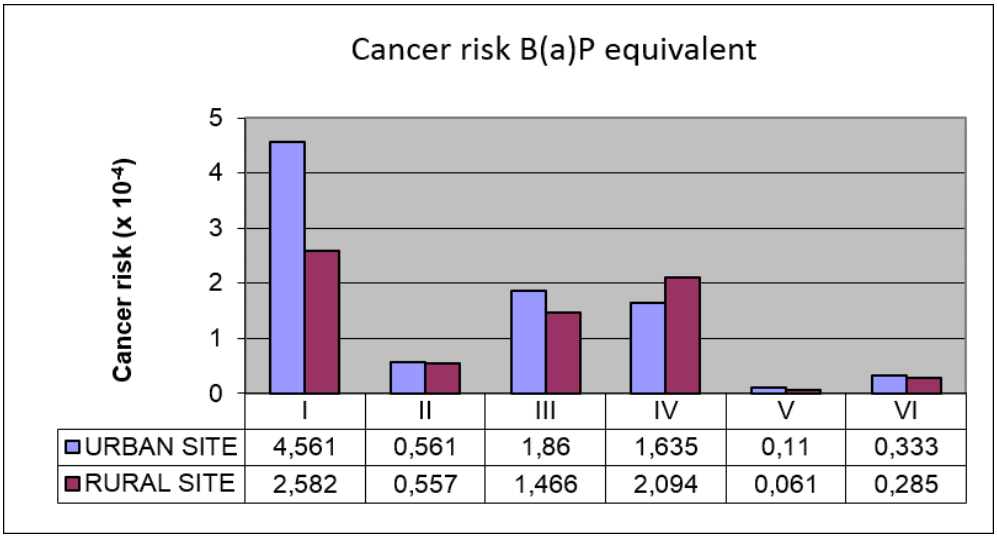
The human exposure to polluted air in the summer seasons was not associated with an excess of cancer risk. The cancer risk associated with chronic inhalation of complex mixtures estimated on B(a)P equivalents appears to be about 1×10^{-5} for the summer campaigns and 1×10^{-4} for the autumn-winter campaigns, except for the I autumn campaign 2011 (4×10^{-4}) and the VI autumn campaign 2013 (3×10^{-5}) (**Table 5.9 and Graphic 5.7**).

$PM_{2.5}$	B(a)P(ng/m^3)	Cancer risk
Autumn 2011 I		
URBAN	1,084	**$1,192 \times 10^{-6}$**
RURAL	0,715	**$0,787 \times 10^{-6}$**
Summer 2012 II		
URBAN	0,015	**$0,016 \times 10^{-6}$**
RURAL	0,007	**$0,007 \times 10^{-6}$**
Autumn 2012 III		
URBAN	0,401	**$0,441 \times 10^{-6}$**
RURAL	0,215	**$0,236 \times 10^{-6}$**
Winter 2013 IV		
URBAN	0,391	**$0,430 \times 10^{-6}$**
RURAL	0,465	**$0,511 \times 10^{-6}$**
Summer 2013 V		
URBAN	0,02	**$0,022 \times 10^{-6}$**
RURAL	0,007	**$0,007 \times 10^{-6}$**
Autumn 2013 VI		
URBAN	0.068	**$0,074 \times 10^{-6}$**
RURAL	0.046	**$0,050 \times 10^{-6}$**

Table 5.8: Cancer risk assessment B(a)P

$PM_{2.5}$	B(a)P eq. (ng/m^3)	Cancer risk
Autumn 2011 I		
URBAN	4,561	**4,561 x 10^{-4}**
RURAL	2,582	**2,582 x 10^{-4}**
Summer 2012 II		
URBAN	0,561	**0,561 x 10^{-4}**
RURAL	0,557	**0,557 x 10^{-4}**
Autumn 2012 III		
URBAN	1,86	**1,860 x 10^{-4}**
RURAL	1,466	**1,466 x 10^{-4}**
Winter 2013 IV		
URBAN	1,635	**1,635 x 10^{-4}**
RURAL	2,094	**2,094 x 10^{-4}**
Summer 2013 V		
URBAN	0,11	**0,110 x 10^{-4}**
RURAL	0,061	**0,061 x 10^{-4}**
Autumn 2013 VI		
URBAN	0,333	**0,333 x 10^{-4}**
RURAL	0,285	**0,285 x 10^{-4}**

Table 5.9: Cancer risk assessment B(a)P equivalents



	I	II	III	IV	V	VI
URBAN SITE	4,561	0,561	1,86	1,635	0,11	0,333
RURAL SITE	2,582	0,557	1,466	2,094	0,061	0,285

Graphic 5.7: values cancer risk assessment during the different monitoring campaigns

CHAPTER 6

Discussion and conclusions

6.1 Cell Transformation Assays

Even if in most areas the air quality has improved substantially over the past decades, the air pollution still causes several problems that can impact the quality of life and even life expectation of the population resident in polluted areas. It has been estimated that the cost to society of air pollution in Europe, including damages to agriculture and buildings, is about € 23 billion per year.

While several harmful pollutants, such as sulphur dioxide, lead, nitrogen dioxide, carbon monoxide and benzene have decreased sensibly as a consequence of the EU air quality policy (EU, 2008), fine particulates ($PM_{2.5}$, PM_1) and ozone, in particular, continue to present significant health risks.

$PM_{2.5}$, measured at the urban background locations in larger cities, is considered the best indicator to assess the exposure of general population. The average exposure indicator (AEI) is determined as a 3-year running annual mean of $PM_{2.5}$ concentrations and should not exceed 20 $\mu g/m^3$ $PM_{2.5}$ according to the Clean Air Policy Package (EU, 2013). These limits are often exceeded in European cities mainly due to road traffic emissions, particularly from diesel vehicles (EEA, 2013).

Po valley is considered one of the most polluted areas in Europe due to particular climate conditions and geographical location. The average exposure in the area has been calculated to be 21-23 $\mu g/m^3$ $PM_{2.5}$, ranging 14-28 $\mu g/m^3$.

Modelling the human exposure in *in vitro* system is always challenging. When designing the study, we took into account the need of simulating a realistic human exposure, the level of concentration that gave an acceptable rate of toxicity. The most recent calculation of the recommended Long-Term Exposure Values for Inhalation was reported by USA-EPA (Exposure Factors Handbook: 2011 Edition). The lowest value was calculated for 1-3 months old children (mean 3.5 m^3/day, 95th percentile 5.8 m^3/day), the highest value was referred to 16-21 years old adults (mean 16.3 m^3/day, 95th percentile 24.6 m^3/day) (US-EPA, 2011). Before then, it was common practice to consider the inhalation values ranging from 21 to 23 m^3/ day in adults (Snyder *et al*., 1975; US-EPA, 1989) and derive the exposure for infant and children by simple extrapolation from these values.

Based on the available information at the time we planned the experiment, 12 m^3 represent the highest exposure for residents in the Po Valley in 12 h/ day of outside activity. The lowest experimental concentration was representative of the infant exposure that we calculated as being 0.9 m^3/day. This calculation was performed as the worst exposure scenario (the most polluted site, in the most polluted season). For this reason and to make the comparison possible, we use the same volumetric concentration for all the samples, even if the amount of PM was different. The highest experimental concentration was also the maximum tolerated dose, based on the preliminary results on cell clonal efficiency.

The evaluation of the toxicity and carcinogenicity of complex mixtures by alternative methods to animal bioassay could help in

the prediction of risk linked to carcinogenic compounds present in the environmental sample (Colacci *et al*., 2014).

The BALB/c 3T3 and Bhas 42 CTAs were used to highlight the cytotoxicity and transforming properties of airborne samples collected ($PM_{2.5}$ and PM_1) during different seasons at the urban site.

None of the organic and inorganic extracts of $PM_{2.5}$ and PM_1 induced a significant increase in the average number of transformed foci/plate or in the transformation frequency of BALB/c 3T3 A31-1-1 cells, except for the organic extract of $PM_{2.5}$ collected during the I autumn campaign (2011) (**Figure 5.3**).

The results obtained by BALB/c 3T3 cell transformation showed a dose-related toxicity for all the organic samples of PM_1 and $PM_{2.5}$, whereas the aqueous extracts (PM_1 and $PM_{2.5}$) did not induce any cytotoxic effects. The cytotoxicity induced by the samples varies in relation to the sampling period, showing seasonal effects (**Graphic 5.1**). These effects are probably related to the high levels of $PM_{2.5}$ and PM_1 ($\mu g/m^3$) and to the higher concentration of PAHs in the winter-autumn particulate (**Graphic 5.2**).

The toxicological effects of airborne particulate have been correlated with the total amount of PAHs or modified PAHs measured in the extracts or in the sampled air (Colacci *et al*., 2007; De Martinis *et al*., 1999; Zhao *et al.*, 2003; McDonald *et al.*, 2004; Sharma *et al.*, 2007; Kang *et al.*, 2010). Some PAHs and nitro-PAHs induce transformation in BALB/c 3T3 cells or in Bhas 42 cells, which originates from the oncogenic transfection of 3T3 cells (Asada *et al.*, 2005; OECD, 2007; Sakai *et al.*, 2010).

In 2010, IARC revised the classification of PAHs for carcinogenicity, taking into account the epidemiological evidence and results from bioassay and mechanistic *in vitro* studies that were available at the time (IARC 2010). From studies on binary mixtures of PAHs characterized by different carcinogenic potencies, it has been hypothesized that PAHs exhibit an additive behaviour at high doses, which became more than additive at low and very low doses. The effect at higher doses could be due to metabolic saturation (IARC 2010).

It was previously demonstrated that the *in vitro* cell transformation does not depend only on the content of well known carcinogens, such as PAHs, but also on other components in the complex mixtures (Ohmori et al., 2013). The chemical characterization of the PM samples collected in this study showed that the concentrations of well known carcinogens, such as several PAHs, were generally low.

Two different campaigns (III autumn 2012 and V summer 2013) were evaluated by the Bhas 42 CTA:

- in the initiation assay the organic extracts of $PM_{2.5}$ and PM_1 collected during the III autumn campaign 2012 induced a significant increase in the average number of transformed foci/plate (**Figure 5.17**) whereas the organic extracts of $PM_{2.5}$ and PM_1 collected during the V campaign (summer 2013) did not show any initiating effect (**Figure 5.19**);
- in the promotion assay all the analyzed organic extracts showed promoting effect (**Figure 5.18** ; **Figure 5.20).**

These results apparently did not agree with those obtained in the BALB/c 3T3 CTA, where the analyzed PM extracts were not able

to enhance cell transformation. The results obtained in the two different CTAs (BALB/c 3T3 and Bhas 42) are not easy to compare, since the two protocols differ in relevant parameters such as cell number at seeding, treatment schedule, the maximum concentration of particulate that can be delivered to cells and also in term of statistical analysis.

The different characteristics of the cell lines constitute another relevant aspect that should be taken into account in the evaluation of the experimental results. The Bhas 42 cell line was established by the transfection with a plasmid pBR322 containing the v.Ha-*ras* oncogene into the BALB/c 3T3 A31-1-1 cell line and is presumed to be initiated toward transformation by the introduced *ras* sequences (Sasaki *et al*., 1988; Sasaki *et al*., 1990). So, the Bhas 42 cell line is commonly considered to be an *initiated cell line* having progressed to a certain extent along the multi-step carcinogenesis process (Bozic, *et al*., 2010). This means that these cells advanced beyond a "normal" condition toward a more atypical pathological state. This attribute makes Bhas 42 cells highly sensitive to carcinogenic stimuli and accounts for the short latency period of expression of the transformed focus phenotype (Sasaki et al., 2015; DRAFT Guidance Document, 2015). Actually, the ras gene is a major component in the signal transduction pathways and its activation is an important factor in the malignant growth (Zenonos *et al*., 2013).

The cell transformation assays are considered one of the most appropriate approaches to predict the carcinogenic potential of chemicals by using alternative *in vitro* methods. The CTAs have been validated for individual chemicals, but could be successfully used to analyze complex mixtures and environmental contaminants

(Vasseur *et al*., 2012; Vanparys et al., 2011; Corvi *et al.,* 2012). The combined use of both BALB/c 3T3 and Bhas 42 CTA could help in enhancing the accuracy of the evaluation of the carcinogenic potential, leading to the reduction of the occurrence of false negatives.

Results from a transcriptomics-based study performed by treating BALB/c 3T3 with $PM_{2.5}$ organic extracts supported the seasonal effect on the toxic behavior of PM extracts: Moreover, the analysis of the KEGG's pathways suggested the induction of early events in the multistep process leading to cellular transformation and cancer (Vaccari *et al*, 2015).

The concentration of pollutants in the mixtures and the time of exposure were probably sufficient to highlight toxic effects, but not to lead to the growth of fully malignant foci in the 3T3 model. The winter extracts, which are characterized by higher concentration of pollutants, were able to induce transformation in the more sensitive Bhas 42 CTA.

6.2 Risk assessment

The risk estimation of environmental exposures is still a critical issue. The composition profile of the environmental mixtures of PAHs and NPAHs associated with airborne PM is deeply affected by vehicular traffic, antropogenic activities and meteorological conditions. The large number of chemicals which are contained in PM extracts varies between the source of the sample and the sampling seasons. Not all components in the complex mixture may be equally identified and characterized. Individual chemicals present in the mixture may induce cancer by more than one mechanism of action. The mechanisms operate with different dose–response relationships and interactions between several environmental carcinogens co-present in a complex mixture at low doses are difficult to predict. All these factors contribute to the difficulties in obtaining reliable cancer risk estimates with relevance for humans.

The approach used in this study is based on the WHO recommendations for the quantitative assessment of cancer risk by inhaled PAHs (WHO/IPCS, 1998). It takes into account the micropollutants concentrations which are measured in the environmental mixtures and the potency equivalence factors which correlate the carcinogenic potential of each PAH or Nitro-PAH (NPAH) to that of the reference compound B(a)P.

As expected for airborne samples collected in the Po valley area, the samples collected during winter presented micropollutants concentrations higher than that determined in summer. Moreover, the amounts of PAHs were significantly higher in the urban samples.

In the directive 2004/107/EC (Fourth Daughter Directive), the EU set a target value for polycyclic aromatic hydrocarbons (PAH) for the protection of human health. The target is defined in terms of concentration of benzo(a)pyrene (BaP) which is used as a marker substance for PAHs. The B(a)P annual mean value may not exceed 1 nanogram per cubic metre (ng/m^3). The B(a)P concentrations measured in summer (2012 and 2013) and autumn 2013 are 2 orders of magnitude lower than to the target value . None of the daily mean concentrations of B(a)P measured during the project exceeded the limit value (**Table 5.5**).

The application of the UR cancer risk to the transformed value of B(a)P equivalents in the winter–autumn campaigns leads to estimate an increase in the cancer risk similar to that defined in the literature (1×10^{-4} for exposure to 1 ng/m^3) (WHO 2000; CCNT, 1991). The calculated cancer risk was about one order of magnitude lower in the summer campaigns (**Table 5.9**).

The prediction of excess of deaths for cancer diseases due to the inhalation exposure did not raise specific concern and strengthened the results obtained in the investigation carried out with biological approaches.

In conclusion, the proposed approach, based on the integration of the data derived from *in vitro* testing and cancer risk assessment, could represent a reliable model for investigating environmental mixtures and predicting their effects on toxicological relevant endpoints.

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Papers

1. A. Colacci, M. Vaccari, M. G. Mascolo, F. Rotondo, E. Morandi, D. Quercioli, S. Perdichizzi, C. Zanzi, **S. Serra**, P. Angelini, S. Grilli, F. Zinoni. *Alternative testing methods for predicting health risk from environmental exposures* (2014). Sustainability vol. 6, 5265-5283; doi:10.3390/su6085265.

2. M. Vaccari, M. G. Mascolo, F. Rotondo, E. Morandi, D. Quercioli, S. Perdichizzi, C. Zanzi, **S**. **Serra**, V. Poluzzi, P. Angelini, S. Grilli, A. Colacci. *Identification of pathway-based toxicity in the BALB/c 3T3 cell model (2015)*. Toxicology *in vitro*, Vol 29, 1240-1253.

Abstracts and oral presentations

1. *Cell transformation assay, gene expression profiles and cancer risk assessment: an integrate approach to predict environmental mixtures carcinogenicity*.
   M. Vaccari, M.G. Mascolo, E. Morandi, S. Perdichizzi, F. Rotondo, A. Guerrini, D. Quercioli, **S. Serra**, C. Zanzi, P. Angelini, S. Grilli,. A. Colacci. ESTIV 2014. 18 th Congress of the European Society of Toxicology *in vitro*, Egmond aan Zee, The Netherlands, June 10-13 2014, Poster N. 7.16 Abstracts book p. 118.

2. *Alternative testing methods for predicting health risk from environmental exposures.* Colacci A., Vaccari M. G., Mascolo M.G., Rotondo F., Morandi E., Quercioli D., Perdichizzi S, Zanzi C., **Serra S**., Angelini P., Grilli S., Zinoni F. 14th CIRIAF National Congress. Energy, Environment and Sustainable Development. Italy, Perugia, April 4-5, 2014.

3. *Pathway-based approach to highlight key events in in vitro cell transformation.*
   Monica Vaccari, Stefania Perdichizzi, Maria Grazia Mascolo, Francesca Rotondo, Cristina Zanzi, **Stefania Serra**, Sandro Grilli, Raffaella Corvi, Annamaria Colacci. EEMGS 2015 European Environmental Mutagenesis and Genomics Society 44th Annual Meeting 23-26 August 2015, Prague Czech Republic.