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TITLE page:

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Short running title:

Particulate and non-particulate mutagenicity in an urban environment

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ABSTRACT

Commonly the atmospheric pollution research is focussed on particulate indicators especially when mutagenicity was studied. On the other hand the volatile and semi-volatile compounds no adsorbed on to the particles can be genotoxic and mutagenic. Moreover some mutagenic compounds, such as polycyclic aromatic hydrocarbons, are present both in the particulate and in the gas-phase in according to chemical conditions. This work is focussed on the assessing of the total mutagenicity shifting the gas-phase and

particulate phase, during two seasons, in Turin. Two sampling sessions are conducted for total particulate matter and gas-phase pollutants. Moreover meteorological and usual air pollution monitoring data were collected at the same sampling station. The Salmonella assay using the strains TA98 and YG1021 was conducted on each organic extract. The mean level of total suspended particles, PM10 and PM2.5 were 73.63 ± 26.94, 42.85±26.75 and 31.55±26.35 μg/m^3. The observed mutagenicity was PM induced YG1021 > PM induced TA98 > PM induced TA98+S9 >> non-particle induced YG1021 > non-particle induced TA98 > non-particle induced TA98+S9. The multivariate regression is significant when we consider air pollution and meteorological indicators and chemical conditions as predictors.

HIGHLIGHTS

Both chemicals and meteo-chemical parameters can influence the mutagenicity of air pollution.

The gas phase and particulate phase mutagenicity can be different and affected by season.

The gas phase accounted for only 1% of the observed mutagenicity.

The particulate mutagenicity is approximately 5-fold higher during winter.

The contribution of the nitro-derived compounds seems to be crucial.

1. Introduction

Air pollution is one of the most important worldwide health concerns (WHO-Europe, 2013). Particularly in the last 10 years, in both the US and Europe, new directives and regulations supporting more restrictive pollution limits were published (Krzyzanowski, 2008). However, the early effects of air pollution cannot be avoided, especially for the urban population (EEA, 2012). A recent Eurobarometer survey showed that European citizens are deeply concerned about the impact of air pollution and that more than 70% of the European population is worried that air pollution and air quality is worsening over time(EU, 2013). The decision to designate 2013 as the Year of Air reflects both the economic seriousness of the problem but also the impacts on humans. Approximately 3 % of cardiopulmonary and 5 % of lung cancer deaths are attributable to particulate matter (PM) pollution worldwide (HEI, 2013), while the disease burden related specifically to PM2.5 pollution accounts for approximately 3.1% of the global disability-adjusted life years

(Lim et al., 2012).

The total suspended particulate (TSP) air pollution is widespread and consists of a mixture of solid and liquid particles suspended in the air. The physical and chemical characteristics of TSP vary by site. Common chemical constituents of PM include sulphates, nitrates, ammonium and other inorganic ions, but also include organic carbon, crustal material, particle-bound water, metals, aromatic hydrocarbons such as polycyclic hydrocarbons and their nitrated, oxidised, sulphated forms (Claxton et al., 2004; Breysse et al., 2013). Especially in urban polluted locations, the secondary particulates formed from precursor gases are the prevalent toxic agents. Particle accumulation and coagulation reactions in the atmosphere produce a fine fraction of particulate matter (PM2.5) that often constitutes more than fifty percent of the TSP (Dimitriou and Kassomenos, 2013). The emitted chemicals, the dispersion conditions, and physical parameters such as humidity and temperature (Zhang et al., 2012) can all influence particle formation.

A large number of studies provide evidence of a correlation between both for short term and long-term exposure to PM pollution and health effects such as morbidity and mortality from cardiovascular and respiratory diseases, as well as from lung cancer (Krzyzanowski, 2008). At the end of 2013, outdoor air pollution and its major component, outdoor particulate matter were classified as carcinogenic for humans (1 Group) (Loomis et al., 2013).

Many mutagenic and genotoxic compounds are present in air pollution, and the effects are widely known and reviewed (de Kok et al., 2006; Claxton and Woodall, 2007a; Valavanidis et al., 2008; DeMarini, 2013). The finest air pollution fractions, PM10 (particles with a diameter of less than 10 µm) and PM2.5 (particles with a diameter of less than 2.5 µm) show greater genotoxicity (Claxton et al., 2004) , while the ultrafine particles (particles having a diameter of less than 0.1 µm) are the subject of in-depth analyses (Hoek et al., 2010; Kovats et al., 2013). The studies conducted using *in vivo* and *in vitro* models show the induction of mutations and genotoxic effects. However, non-genotoxic effects also occur and various studies focused on the epigenetic effects of the ambient particles (Ji and Hershey, 2012).

Among the typical air pollution chemicals, Polyciclic Aromatic Hydrocarbons PAHs have a relevant role in air pollution toxicity. These compounds are reactive in the atmosphere and primarily form oxidised products, the most notable being oxy-derivatives (mostly quinones) and nitrated compounds (Kim et al., 2013). Some

of these compounds, such as benzo(a)pyrene and 6-ditrochrysene and the 7,12-dimethylbenz(a)anthracene, are also present in primary emissions. Benzo(a)pyrene is the reference compound for the carcinogenic relative potency factor, while others previously cite PAHs as having a carcinogenic factor of 10 and 64, respectively. Also among the secondary PAHs are compounds with high carcinogenic relative potency factors such as benz(j)aceanthrylene (60) and 1,6-dinitropyrene (10) (ATSDR, 1995). The historic list of 16 USEPA priority PAHs is an important source of information, but was developed when knowledge of the relative toxicity of PAH congeners was more limited than at present. As such, it is useful as reference for monitoring but limited for the assessment of human health risks attributable to air PAH mixture exposition (Yang et al., 2007).

Vapor–particle partitioning of mutagens can be quantified using the gas–particle-partitioning coefficient for each compound. This coefficient is influenced by both the adsorption and absorption processes and is strongly temperature dependent (Albinet et al., 2008). Moreover, volatile and semi-volatile organic compounds associated with particulate matter can be influenced by heterogeneous photochemical reactions in the atmosphere (Fraser et al., 2000; Xie et al., 2013). Our typical samplings were conducted using standard methods that are affected by relevant limits (Liu et al., 2007; Forbes et al., 2012).

The aim of this work is to assess the mutagenicity of particulate and not-particulate air pollution and to determine the effects of seasonality and the contribution of nitro-compounds to the mutagenic effects in an urban environment.

2. Materials and methods

2.1 Sampling

Sampling was performed from 20 November to 22 December 2009 for the winter period and from 4 May to 4 June 2010 for the spring period at a meteorological–chemical station of the Environmental Protection Regional Agency (Piedmont A.R.P.A.) located at Torino, in the northwest of the Padana Plain, Italy. The sampling site, called Lingotto, was located outdoors in a small green area within an enclosed zone classified as urban background (ARPA Piemonte, 2010). Turin has 872,367 inhabitants and a population density of approximately 7,000 inhabitants per km^2; thus, the pressure on the territory that is associated with human

activity is very high (ISTAT, 2012). Moreover, the climate and topographical characteristics of the area contribute to critical air pollution (Poncino et al., 2009; Eeftens et al., 2012). The Total Suspended Particles (TSP) were collected on glass micro-fibre filters (Type Fiberfilm T60A20, 150 mm, SKC, 863 Valley View Road Eighty Four, PA 15330, USA) and micro-pollutants were collected in Polyurethane Foam (PUF) Sorbent Tubes (SKC, 226-131 Valley View Road Eighty Four, PA 15330, USA) using an AirFlowPuf Sampler and conforming with the US EPA methods TO-4A, TO-9A, TO13A, ASTM D-6209 and ISO-12884, ISO-16362 (Analitica Strumenti Samplers, via degli Abeti 144 61100 Pesaro, Italy).

The TSP were collected on glass fibre filters, and the polyurethane foam (PUF) cartridge was placed in series after the glass fibre filter. The volatile compounds, which were not trapped on the filter, were retained in the PUF cartridge.

The sampling flow was electronically controlled to be 250 L/min. Each sampling duration was controlled by a timer that was accurate to ± 15 min over a 48-hour sampling period. The exact flow rate was calculated daily and corrected for variations in atmospheric pressure and actual differential pressure across the filter. The filters were conditioned for 48 h and were weighed using an analytical balance (± 10 μg) before and after sampling to calculate the mass of the TSP trapped on the filter. The procedures were conducted according to the European Committee for Standardization. Additionally, , the PUF had been pre-cleaned by 24 h Soxhlet extractions using acetone.

2.2 Extraction and mutagenicity assays

Each sample was extracted with acetone in a Soxhlet apparatus for a minimum of 80 cycles. The samples were dried in a Rotavapor instrument, and suspended in dimethyl sulfoxide (DMSO) to obtain an equivalent concentration of 0.1 m^3 of sampled air per μl of solution. The mutagenicity assay was conducted as previously described (Maron and Ames, 1983; Traversi et al., 2009). Defined amounts of organic extract were tested to generate a dose–response curve (2, 5, 10, 20, 30 air equivalent m^3 for the TSP extracts and 10, 20,30, 50, 100 air equivalent m^3 for the PUF extracts). The slope of the dose–response curve (revertants/m^3) was calculated by the least squares linear regression beginning at the first linear portion of the dose–response curve (Traversi et al., 2011). All experiments were performed in triplicate using at least

three doses. The results are expressed as net revertants per cubic metre (rev/m^3) (the total revertants minus the spontaneous revertants) and were calculated using the dose–response curve (Cassoni et al., 2004; Claxton et al., 2004). The mutagenic activity of the airborne particulate extracts was determined using the Salmonella typhimurium strain TA98, with and without S9 mix, as well as the YG1021 strain. YG1021 is a 'classical' nitroreductase-overproducing strain obtained by cloning the nitroreductase gene of S. typhimurium TA1538 into the pBR322 vector and introducing the recombinant plasmid into TA98 (Josephy et al., 1997). YG1021 has a nitrofurazone reductase activity more than 50 times higher than the original TA98 strain, permitting the efficient detection of mutagenic nitroarenes. The mean number of spontaneous revertants, obtained during a 10 bioassay series, one every two samplings, was 16 ± 4 for TA98, 21 ± 1 for TA98+S9 and 23 ± 5 for YG1021. The genotype of each tester strain was routinely confirmed. In each assay session, positive and negative controls were included. Moreover, the known mutagen 2-nitrofluorene (1 µg/plate) was tested in each assay as a positive control for the strains TA98 and YG1021 while amminofluorene (1 µg/plate) was used as a positive control for the TA98 strain in presence of S9 mix.

2.3 Chemicals and inhalable particles data

Chemical data and inhalable particles data (PM10 and PM2.5) were extracted from a specialised database provided by the Regional System for the real-time monitoring of Air Quality, AriaWeb (ARPA Piemonte, 2014). The data were obtained for the same day as our sampling and for the same sampling station. For example, the NOx data represent a monthly mean of hourly data collected using the standard monitoring method EN 14211:2005 (2008/50/EC, annex VI, section B). All the adopted methods conform to the directive and were validated before being published in the AriaWeb database (ARPA Piemonte, 2014).

2.4 Statistics

The seasons were designated as winter for the first sampling session (November and December) and as spring for the second sampling session (May and June). The statistical analyses were performed using the

SPSS Package, version 21.0. In particular we applied: (1) a log transformation of non-normally distributed data, (2) the Spearman rank-order correlation coefficient to assess relationships between variables, (3) a Wilcoxon-Mann- Whitney test to compare means. The mean differences and correlations were considered significant if $p < 0.05$.

3. Results

3.1 Gravimetric analysis

The descriptive analysis of the collected data is shown in **Table 1.** The gravimetric data showed that, on average, meanly the TSP proportion in the samples was 58% PM10 and 43% PM2.5. Moreover, during the high pollution period in winter, these proportions increased up to 70% and 61%, with a PM2.5/PM10 ratio equal to 0.87. Moreover, **Figure 1** highlights the marked seasonal differences for all three particulate indicators, with the mean comparison between winter and spring means for TSP, PM10 and PM2.5 being significant ($p<0.01$). The mean reduction in TSP in the spring with respect to winter was 30%, with mean reductions of 68% for PM10 and 82% for PM2.5. The mean temperature differences between sampling seasons was significantly different by ($p<0.01$) with the mean winter temperature being 2.95 ± 4.09 °C and the mean spring temperature being 16.55 ± 4.20 °C. However neither the average humidity nor the wind speed were significantly different, with 0.73% humidity during the winter vs. 0.64% spring ($p>0.05$) and ,an average wind speed in both seasons of approximately 10 m/s (**Table 1**).

3.2 Mutagenicity

The mutagenicity tests show a significantly elevated of net revertants per unit of exposure (air equivalent m^3) respect to the negative control. An elevated number of net revertants (250) was recorded at the highest test doses for the winter TSP extracts in the YG1021 strain, while the mutagenicity of the PUF extracts was markedly lower (**Table 1)**. The PUF extracts contribute only about 2% to the total mutagenicity. As **figure 2** also shows, the mutagenicity of the samples, expressed as net revertants, from higher to the lower was PM induced YG1021 > PM induced TA98 > PM induced TA98+S9 >> non-particle induced YG1021 > non-particle induced TA98 > non-particle induced TA98+S9. Moreover, the seasonal

trend is clearly evident and significant only for particulate-induced mutagenicity (YG1021 $p<0.01$; TA98 $p<0.01$; TA98+S9 $p<0.01$). The mutagenicity of the spring TSP samples is less than 10% of the mutagenicity recorded for the winter samples in all the strains.

The higher mutagenicity of the winter particles was confirmed also adjusting the data for particles mass unit (Figure 3), highlighting the worse quality of the particles- in terms of mutagen presence - and not only the higher level of aero-dispersed pollution for each volume unit.

Among the chemicals variability we observed a not so great changeability during the year for PAHs and metals, observing a difference due to seasonality. More variability is instead observable for NO_x and ozone, however also in this case the levels are clearly affected by seasonality (with highest value recoding in winter with the ozone exception)(table 1). Table 2 showed the correlations between variables. Only the variables for which at least one correlation with mutagenicity is significant was included, the not particles induced mutagenicity was however included for its experimental origin, favoring the mutagenicity results comparison.

As presented, the mutagenicity attributed to the non-particle phase was not influenced by the environmental temperature or wind speed and, furthermore, does not correlate with the mutagenicity of the particle phase. Additionally, the chemical parameters did not correlate with the minimal mutagenic effects of the non-particle phase (table 2).

In contrast, the temperature and wind speed significantly inversely correlated with the TSP levels and to mutagenicity of this mixture. The TSP level correlated with mutagenicity and, in particular, this correlation showed a higher Spearman's rho for TA98 strain, with and without the addition of the S9 mixture. The results of the mutagenicity assays conducted using the TSP extracts all correlate with each other (**Table 2**). Among the chemical parameters, the TSP mutagenicity correlates with the presence of nitrogen oxides and, in particular, this relationship is more marked for the nitrogen monoxides. The ozone levels inversely and significantly correlate with the TSP mutagenicity. The cadmium and nickel levels significantly correlate with direct mutagenicity (i.e., without the introduction of the metabolic activation). The TSP mutagenicity correlates to the concentration of the finest fraction of the particulate matter and, in particular, there is a better correlation with the PM10 fraction in the TA98 strain with and without metabolic activation. A

217 significant correlation is not observed between benzo(a)pyrene or benzo(a)antracene and mutagenicity but
218 there was a high correlation between PAHs and with metals (0.929 $p<0.01$) due probably mainly to the
219 same seasonality.
220 Among the meteo climatic variables the temperature showed the high influence to the particulate pollution
221 and associated mutagenicity, moreover this physical parameter is not significantly correlated to the wind
222 that also showed an influence on the particulate pollution dispersion but not on the NO_x and ozone levels.
223 The humidity during the sampling showed a quite constant level so in this study we can't observe an
224 influence on the pollution level.
225 The NO_x, in particular NO, among the chemicals correlated with particulate pollution and associated
226 mutagenicity, moreover with PAHs, cadmium and nickel. This result was similar to those previously
227 observed in other studies (Du Four et al., 2004; Du Four et al., 2005).
228
229 **4. Discussion**
230 In our study, the inhalable fraction and the high-risk inhalable fraction represented a very high proportion
231 of the TSP, highlighting a human health hazard comparable to that estimated for urban sites. The observed
232 pollution levels are significantly higher than both the WHO guidelines (Krzyzanowski, 2008) and the EU
233 regulations 2008/50/CE. In addition, critical particle concentrations are present particularly during the
234 winter and especially for PM2.5. Recently, the IARC classified outdoor pollution and particulate matter, as
235 its major component, as carcinogenic for humans (Loomis et al., 2013). Consequently, reducing air pollution
236 and particle matter to the lowest amount possible is becoming a marked priority.
237 Particulate matter clearly contributed to the overall mutagenicity (**Figure 2**). This observation confirmed
238 the evidence of other studies where PM total air toxicity and genotoxicity was higher than the gas phase
239 fraction. In particular, PM1 was responsible for approximately 80% of the observed effects at various
240 sampling localities (Novak et al., 2014), and the fine particles generally showed higher mutagenicity
241 (Claxton et al., 2004; Claxton and Woodall, 2007b; Lemos et al., 2012). The gas phase mutagenicity was very
242 low and often indeterminable, with the exception of particular sampling sites such as industrial sites (Du
243 Four et al., 2005) and exhaust emissions from gasoline- and diesel-powered passengers cars (Pohjola et al.,

244 2003a; Pohjola et al., 2003b). The contribution of the gas phase with respect to the particulate phase seems
245 to be higher during summer and related to the major PAHs content (Du Four et al., 2004; Kennedy et al.,
246 2010). In the present study, the contribution of the gas phase with respect to the particulate phase is
247 relative; in summer the particulate phase mutagenicity is reduced while the gas phase mutagenicity
248 remains quite constant. It is supposable that this level of mutagenicity is not imputable to climatic or
249 chemical stress condition and it indicates probably a background mutagenicity level hardly to avoid.
250 The benzo(a)pyrene concentration was higher during the winter than summer and higher than the WHO
251 guide line value of 0.12 ng/m^3 (Krzyzanowski, 2008; WHO-Europe, 2013). As widely observed, the PAHs are
252 generally higher in the gas phase (Lemos et al., 2012), however, this fraction is less genotoxic and
253 mutagenic, and thus PAH concentration explains only a small part of air pollution toxicity. Moreover, PAHs
254 can react with nitrogen oxides, generating more genotoxic and mutagenic compounds (Albinet et al., 2008).
255 The contribution of the nitro-derivate compounds to the overall mutagenicity, as assessed by comparing
256 the number of Salmonella YG1021 net revertants to the strain without the modified nitro-reductase
257 activity, was marked. The ratio of the net revertants observed in the TA98 and YG1021 strains is
258 approximately 1:2, during both summer and winter. This observation is widely confirmed by other studies
259 (Ramos de Rainho et al.; Traversi et al., 2009). Moreover the direct mutagens action is higher than indirect
260 mutagens as highlighted by the ratio of the net revertants observed in the TA98 and TA98+S9 that is
261 approximately of 1:1.7.
262 Air pollution and its major components have a marked seasonality, and the toxic content in the gas phase
263 and particulate phase can vary based upon the meteo-climatic conditions (Albinet et al., 2008). In
264 particular, more nitro-derived compounds can be present in the particulates during winter, thus enhancing
265 the genotoxic and mutagenic properties.
266
267 **5. Conclusions**
268 By combining data on meteo-climatic conditions, various air pollution indicators and mutagenicity assays
269 we produced an evaluation of particulate and non-particulate air pollution in Turin during different season..
270 We present the following results:

- In the present study, the mutagenicity of the gas phase sampled by PUF method is practically negligible with respect to the mutagenicity of the particulate phase. The gas phase accounted for only 1% of the observed mutagenicity.
- The mutagenicity of the non-particulate phase remained constant during the summer and winter, while the particulate mutagenicity is approximately 5-fold higher during winter when the finest fraction of the PM increases.
- The contribution of the nitro-derived compounds seems to be crucial in Turin, in both winter and summer.
- Both chemicals (such as NOx, metals and PAHs) and meteo-chemical parameters (such as temperature, wind speed and humidity) can influence the mutagenicity of particulate matter. Moreover, the total mutagenicity recorded in winter most likely results from the combination of not only additive but also synergistic effects among the components of the air pollution, conducing both to higher particulate level and to a higher content of mutagens in each unit particulate mass;
- Although PUF sampling is a common approach used in gas phase studies, there were relevant uncertainties regarding the applicability to biological *in vitro* models. A crucial point is the necessary extraction procedure between the sampling and the in vitro test. It is not presumably able to avoid a partial loss of the volatile and semi-volatile compounds.This more research is necessary to understand this problem.

Finally, the biological assays are relevant tools for the evaluation of the environmental and human health impact of air pollution.

6. Conflict of interest statement

The authors have nothing to declare. Funding source: this study was co-funded by the University of Turin (local funds ex-60% 2012) and the Piedmont Region (Italy) in the field of health projects.

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8 List of abbreviations:

PAHs	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
TSP	Total Suspended Particles
PM	Particulate matter
PM10	Particulate matter with an aerodynamic diameter < 10 µm
PM2.5	Particulate matter with an aerodynamic diameter < 2.5 µm

Table legends:

Table 1 -Descriptive analysis on 20 total measurements for each parameter are showed median and first and third quartiles .

Table 2 -Spearman's correlation between the mutagenicity, gravimetric, chemical and meteorological variables [1] rho = -0.436, p=0.054

Figure legends:

Figure 1 - Mean and standard deviation of TSP, PM10 and PM2.5 levels recorded during the winter and spring sampling sessions.

Figure 2 - Total mutagenicity, subdivided into gas phase and particulate phase, recorded for the winter and summer samples with metabolically different strains.

Figure 3 - Net revertants expressed as unit mass of total suspended particulate for the different strain and the different seasons.

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