INVOLVEMENT OF NITRO-COMPOUNDS IN THE MUTAGENICITY OF URBAN PM2.5 AND PM10 IN TURIN

TITLE PAGE:

NITRO-COMPOUND INVOLVEMENT IN THE MUTAGENICITY OF URBAN PM2.5 AND PM10 IN TURIN

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ABSTRACT:

Fine particles can be active carriers of toxic compounds into the alveoli of lungs. Among these compounds are numerous mutagens and carcinogens. The direct acting mutagenicity per unit mass of fine particulate matter (PM) is significantly higher than those of coarse particles, especially in urban areas. In this study, the mutagenic properties of urban PM2.5 and PM10 were evaluated, and the role of nitro-compounds was estimated. PM2.5 and PM10 samplings, NO_x and some PAHs determinations were performed daily in 2007 in Turin following a consolidated in vitro test – the *Salmonella* assay – that was conducted with PM2.5 and PM10 organic extracts. The mutagenic properties were assessed for each month of sampling with *S. typhimurium* TA98- and TA98-derived strains: a nitroreductase-less mutant strain (TA98NR) and an added nitroreductase-producing plasmid strain (YG1021). The annual measured mean levels of PM2.5 and PM10 were 34±20 and 48±18 μg/m^3. The PM2.5/PM10 ratio ranged from 0.36 to 0.89. The *Salmonella* assay showed higher mutagenicity in autumn/winter (20±15 TA98NR, 54±39 TA98, 173±161 YG1021 net revertens/m^3) with respect to spring/summer (2±2 TA98NR, 7±8 TA98, 24±27 YG1021 net revertens/m^3)($p<0.01$). There are also statistically significant seasonal differences in the gravimetric analysis data. The ratio between the TA98 net revertants per PM2.5 μg is 6.5 times greater than per PM10 μg. Moreover, the bioassay results showed an amplified response in the YG1021 strain and a reduced response in the TA98NR strain. The net revertant ratio TA98NR/YG1021 is equal to 11±4 for PM2.5 and 13±6 for PM10 organic extracts ($p<0.01$). There is a significant correlation with NO_x and PAHs concentrations. These findings can describe the relevant role of nitro compounds, and they underline the priority in prevention improvements to reduce nitrated molecule air pollution.

INTRODUCTION:

Atmospheric pollution has significant effects on human health and, more generally, on a population's quality of life [58]. Epidemiological studies have clearly associated negative health effects, especially respiratory diseases, with exposure to air pollution [1]. Also, the relationship between lung cancer and air pollution is widely debated [2], and various studies have discussed the significance of this kind of correlation [3,4]. Borderline results are justified by the complexity of the factors involved in this environmental problem. First of all, the physiological effects of air pollution on lung function have only been studied since the 1990s by epidemiologic studies, and difficulties arise from the heterogeneity of air pollution (gaseous phase and particle phase), the long-term appearance of the main effects, and the heterogeneity of genetic and other environmental factors in the involved populations [5]. However, numerous experimental studies conducted in vitro and in vivo have shown evidence of mutagenic and genotoxic effects [6]. These effects range from a simple puntiform mutation [7] to epigenetic effects [8]. In vitro toxicology evaluations have reported significant negative effects attributable to the particulate fraction in relation to the dose of exposure [9]. However, air pollution is the result of continuous interactions between gaseous and particulate phases. The equilibrium depends on various factors, including environmental temperature, primary compound emission sources, and dispersion ratio [10]. Among the more genotoxic and mutagenic compounds, we can distinguish nitro compounds, such as nitro-polycyclic aromathic hydrocarbons (nitro-PAHs). These chemicals can be both directly emitted into the atmosphere and produced by the interaction between PAHs and NOx. The presence of these two groups of chemicals is common in both outdoor [11] and indoor environments [12], and their interactions have been observed in the

combustion rooms of diesel motors [13]. Environmentally higher levels of PAHs and NOx with a low dispersion rate entail relevant formations of nitro-derived compounds [10,14,15]. The particulate phase or gas phase form of specific nitro-PAHs depends on molecular weight, temperature and pollution entity [14,16]. Fine particles present a higher number of particles per volume unit, and they have major superficial extension. They may be active carriers of toxic compounds into the lung alveoli [17]. These toxic compounds may also cause DNA damage and complex genotoxic effects. Particulate matter shows a higher toxicity indirectly proportional to the aerodynamic diameter of the particles. Thus, fine particles seem to be more genotoxic than coarse particles [18]. Although the effects of particle sizes below 10 µm (PM10) were investigated in the initial studies, even smaller particles have recently been regarded as questionable, and research has been conducted on the health effects of minute particulate matter below 2.5 µm (PM2.5). Ultrafine particles have been sporadically measured [19,20].

Air pollution levels are tightly linked to climate and topography [21]. Air pollution episodes can be particularly troublesome if the affected city is located in a valley surrounded by mountains, as in the Meuse Valley in Belgium [22] and the Valley of Mexico [23], but they are also a concern in the Padana Plain, where Torino is located [24]. In this study, we analyse the NOx levels and the particulate pollution in Torino's metropolitan area over the course of a full year before the new European Regulation (2008/50/CE) and its Italian introduction (D. Lgs. 155, 30/07/2010) were promulgated. The mutagenic properties of urban PM2.5 and PM10 were evaluated while focusing on the role of nitro-compounds by a biological approach and related NO_x and PAHs levels that are able to influence the presence of nitro-compounds in the air [25].

MATERIALS & METHODS:

PM10 and PM2.5 sampling

Sampling was performed from January 2007 to December 2007 at two meteorological–chemical stations of the Environmental Protection Regional Agency (Piedmont A.R.P.A.) located at Torino. Torino is placed in the northwest of the Padana Plain (Italy). The first sampling site (station 1), Lingotto, is located outdoors in a small green area within an enclosure zone classified as urban background. The second sampling site was located in the centre of the city (station 2) in a traffic-regulated street, and it is classified as a traffic station [26]. Turin has a population density of 7,000 inhabitants per km^2, so the pressure correlated to human activity on the territory is very high [56]. Moreover, the climate and topography characteristics of the area contribute to critical air pollution [27]. PM10 (PM passing through a size-selective inlet with a 50% efficiency cut-off at a 10 µm aerodynamic diameter) was sampled on glass microfibre filters (Type A/E, 8 in×10 in, Gelman Sciences, Michigan, USA) with a Sierra Andersen High Volume Sampler 1200/VFC (Andersen Samplers, Atlanta, Georgia, USA) using a flow of 1,160 L/min. The sample duration was controlled by a timer accurate to ±15 min over a 24-h sample period. The PM2.5 filters were glass micro-fibre filters (Type A/E 47 mm ⌀, Gelman Sciences, Michigan, USA), and the PM2.5 sampler was a PM2.5 MicroVol 1100 Low Volume Air Sampler using a flow of approximately 32 L/min. This sampler is certified in compliance with EN-14907 norm requirements. The exact flow was calculated daily and was corrected for variations in atmospheric pressure and actual differential pressure across the filter. The filters were conditioned for 48 hours and were weighted with an analytical balance (±10 µg) before and after sampling to calculate the PM

mass concentration. The procedures were conducted according to the European Committee for Standardization [55], as previously described [28].

Extractions and biological assays

Daily filters were pooled to obtain one monthly sample in each of the three cities. Extractions of each pooled sample were carried out with a Soxhlet apparatus for at least 80 cycles with acetone. Subsequent evaporation was induced by a Rotavapor instrument, and the re-suspension of the sample was performed with dimethyl-3-sulfoxide (DMSO) to obtain an equivalent concentration of 0.1 m^3/µl. The mutagenicity assay was executed according to [29]. Definite concentrations of PM2.5 organic extract were tested to generate a dose-response curve (20, 50, 100 µl of the DMSO 0.1 m^3/µl suspension). The slope of the dose-response curve (revertants/m^3) was calculated by the least squares linear regression from the first linear portion of the dose-response curve [30]. All experiments were done in triplicate with at least three doses. The results were expressed as total revertants minus spontaneous revertants to obtain net revertants per cubic metre (rev/m^3), and they were calculated by the dose-response curve [31-33]. The mutagenic activity of airborne particulate extracts was studied using *S. typhimurium* strains TA98, TA98NR and YG1021. YG1021 is a 'classical' nitroreductase-overproducing strain that is obtained by cloning the nitroreductase gene of *S. typhimurium* TA1538 into pBR 322 and introducing the recombinant plasmid into TA98 [34]. YG1021 has a nitrofurazone reductase activity more than 50 times higher than the original TA98 strain, permitting an efficient detection of mutagenic nitroarenes. TA98NR lacks of 'classical' nitroreductase, and thus, the bioassays describe a reduced mutagenicity proportionally to the nitroarene amounts [35,36]. The spontaneous revertants obtained during 12 bioassay sessions, one for each sampling month,

137 ranged from 13 to 20 (18±2) for TA98, from 12 to 20 (16±3) for TA98NR, and from 19 to 29
138 (25±2) for YG1021. The genotype of each tester strain was routinely confirmed, and in each
139 assay session, positive and negative controls were included. 2-nitrofluorene (1 μg/plate) was
140 tested in each assay as a known mutagen positive control.
141
142 **NOx and PAHs data**
143 The NOx and PAHs data were extracted from a specialised database by the Regional System
144 for the real time monitoring of Air Quality, AriaWeb (Regione Piemonte, 2007). The data are
145 referred to the same day of PM samplings and to the same sampling station, and for NOx
146 they represent a monthly mean of hourly data collected with the standard monitoring
147 method EN 14211:2005 (2008/50/EC, annex VI, section B), while for PAHs they represent a
148 monthly mean of a daily data collected on the PM2.5 for the station 1 and on the PM10 for
149 the station 2. The determined PAHs are benzo(a)pyrene (b(a)p), benz(a)anthracene (b(a)a),
150 benzo(b)fluoranthene, benzo(j)fluoranthene and benzo(k)fluoranthene (benzo(bjk)f). The
151 adopted method consisted of extraction in toluene under sonication in a water bath
152 followed by HRGC/LRMS analysis using the internal method UT2.M128 R01 2002 (Pereira et
153 al., 2001). The benzo(b)fluoranthene, benzo(j)fluoranthene and benzo(k)fluoranthene as
154 expressed as the sum of the three compounds by the synthetic term b(b,j,k)f.
155
156 **Statistics**
157 The seasons were designated as winter/autumn (January–March and October–December),
158 and spring/summer (April–June and July–September). Statistical analyses were performed
159 using the SPSS Package, version 17.0. In particular, 1) a log transformation of non-normally
160 distributed data was carried out, 2) the Spearman rank order correlation coefficient was

used to assess relationships between variables, 3) a Wilcoxon test was used to compare means, and 4) ANOVA was used for multivariate analysis in which we assumed an equal variance while using Tukey as post-hoc multiple comparisons. The mean differences and correlations were considered significant at $p<0.05$.

RESULTS and DISCUSSION:

A description of the statistics of the measured variables for each sampling station is detailed in Table 1. The particulate matter levels exceeded the yearly WHO Air Quality Guideline values [37] for 7 and 6 months for PM2.5 and PM10, respectively. The annual measured mean levels of PM2.5 and PM10 were 34±20 and 48±18 µg/m^3. The PM10 annual mean was 2.4 times higher than the levels proposed as safe for human health. This ratio for PM2.5 was 3.4, which is more troubling. Station 1 showed higher pollution as expected because of its zone characteristics [26]. The PM2.5/PM10 ratio ranged from 0.36 to 0.89, and it was higher in winter/autumn than in spring/summer. This ratio is affected by the different sampling sites in terms of traffic pressure, but it is comparable to those reported in the literature [38]. Also, the nitrogen dioxide yearly mean is higher than the reference level, but the ratio is much less (1.2). The guideline of hourly value was never observed (Table 1). B[a]P is taken as a marker of the PAH mixture [60]. Only for the benzo(a)pyrene (classified as IARC group 1 compound) there is an air guideline evaluation, the corresponding concentrations for lifetime exposure producing excess lifetime cancer risks of 1/10000, 1/100000 and 1/1000000 are approximately 1.2, 0.12 and 0.012 ng/m^3, respectively [62]. The figure 1 showed data lower than the highest risk corresponding evaluation. Moreover other PAHs are classified by the IARC and the benzo(a)anthracene, benzo(b)fluoranthene,

benzo(k)fluoranthene and benzo(j)fluoranthene are classified as 2B group. At the moment it isn't defined a real exhaustive risk evaluation on the PAHs air pollution [61].

The mutagenicity is much higher for the PM2.5 organic extracts and with the YG1021 strain, especially in the first months of the year. The biological effects observed in the samples with the strain TA98NR were limited (Table 1, Figures 1 and 3). The trend of mutagenicity and NOx are shown in Figure 1/A for the first sampling station and Figure 1/B for the second sampling station. The measured PAHs showed a similar level distribution during the year. Both figures highlight a marked seasonal trend for every variable. As shown in Table 2, all the pollutants, the nitrogen oxides, PAHs and particulate matter, are highly correlated with mutagenicity with the exception of the correlation between benzo(a)anthracene and YG1021 mutagenicity that is significant at the $p<0.05$ level. The lower value of the Spearman rho, observed between YG1021 mutagenicity and PAHs on the PM2.5 organic extract, seems to indicate other responsibility than maternal PAHs. Recently data nitro-PAHs were definitely encounter as environmental genotoxic/mutagenic hazards confirming that environmental aromatic nitration reactions lead to a relevant increase in genotoxicity and mutagenicity properties [39]. The interactions of nitro-aromatic compounds with DNA and the resulting mutagenicity have been characterized extensively and reviewed for a wide variety of monocyclic, polycyclic, and heterocyclic nitroaromatic compounds [40]. These compounds produce transitions, transversions, and frameshift mutations in gene coding sequences moreover oxidation and reduction products of nitroaromatic compounds can damage DNA. Structural and spectroscopic studies have found that the position of the nitro group on the aromatic ring and the presence of other functional groups can influence the mutagenicity and carcinogenicity potency of these chemicals [41]. The NO levels are characterized by a more emphatic seasonal trend as with the mutagenicity (Figure 1). The

208 *Salmonella* assay showed higher mutagenicity in autumn/winter (20±15 TA98NR, 54±39
209 TA98, 173±161 YG1021 net revertants/m^3) with respect to spring/summer (2±2 TA98NR,
210 7±8 TA98, 24±27 YG1021 net revertants/m^3) (T-test; $p<0.01$). The mean levels analysis is
211 shown in the Figure 2, and they are subdivided by particulate fraction and season. There are
212 also statistically significant seasonal differences in the gravimetric analysis both for PM2.5
213 and PM10 levels ($p<0.01$). The ratio between the TA98 net revertants per PM2.5 µg and per
214 PM10 µg is meanly equal to 6.5. Moreover, the bioassay results show an amplified response
215 in the YG1021 strain and a reduced response in the TA98NR strain. The determined PAH
216 levels were included on the figure 3A. The PAH levels in the PM2.5 mixtures are as equal as
217 in the PM10. Furthermore is widely supported in literature that carcinogenic compounds
218 were for the most part present in the fine particles [42]. In particular the b(a)p is often
219 higher in the PM2.5 than in the PM10, this happened for example on February (1.03 *vs* 0.93
220 ng/m^3), March (0.28 *vs* 0.27 ng/m^3) and December (2.25 *vs* 2.22 ng/m^3). This supports the
221 evidence of the higher mutagenicity properties of the PM2.5 organic extract. Firstly we have
222 with a 2.5 µm cut-off during the sampling the collection of an higher number of fine
223 particles, than the total particle surface is more extensive and suitable for the adhesion of
224 the organic pollutants [59]. Successively these characteristic influence the removability
225 during the organic extraction protocol both in terms of solvent-extractable organic fraction
226 [43,44] than semi-volatile mass [45], at the end also little differences in the amount of
227 potent mutagens such as 1-nitropiyrene, 1.3-dinitropyrene, 1.8-dinitropyrene are able to
228 produce a great increase of the more sensible *Salmonella* strain revertants [40]. This
229 increase is not proportional to the increase of the single pollutant, there are probably
230 synergic action that produces a biological response amplification [46]. The mutagenicity
231 data are shown by season and particulate fraction in Figure 3. Although the results show an

appraisable mutagenic activity for the PM organic extract, the higher mutagenicity observed per PM2.5 μg in winter for the YG1021 strain is 6 times lower than the mutagenicity observed with the same strain in urban areas of South America [47]. The ratio between TA98NR/YG1021 net revertants is equal to 11±4 for PM2.5 and 13±6 for PM10 organic extracts ($p<0.01$), while the ratio between the mutagenicity recorded with the PM2.5 and PM10 organic extract in winter with the YG1021 strain is 5.75. These results highlight the critical role of urban fine and ultra-fine PM pollution, and they show the predominant role of bacterial nitroreductase on induced mutagenicity. The high ratio between YG1021 and TA98NR mutagenicity recorded with the PM2.5 organic extract in winter/autumn of 8.9 (Figure 3B) suggests a reduction in photodecay of nitro compounds during the autumn/winter that promoted the accumulation of these kinds of pollutants.

CONCLUSION:

The north of Italy is an area of widespread air pollution [48]. The weak dispersion rate observed during winter due to the conformation of the territory represents a relevant factor [49]. Various air pollution indicators are above the WHO guidelines, especially for fine particulate matter, or PM2.5. Also, the exposure concentration obligation value for PM2.5 of 20 μg/m^3 defined by the European Directive 2008/50/EC, and that is to be met by 2015, was clearly exceeded, and it is difficult to identify improvement safety margins. The pollutant emission reductions are obtainable by action, focusing on the winter/autumn seasons, which involve, for example, heating systems and traffic regulation. Various improvements are in progress on these crucial points. First, centralised district heating is being introduced that covers almost all the urban area, reducing the difficulty of controllable emission points. Second, the limited green traffic zone has been expanded [57].

Today, the mutagenicity recorded with the *Salmonella* assay associated with pollution is relevant especially during the winter or in industrial areas, as previously described [47,50] and it is mainly due to the fine fraction of the particulate pollution [51,52]. The reduction of NO_2 functional groups and the generation of more reactive intermediates is fundamental to the production of DNA mutations.

In this study the relationship among the mutagenic results for YG1021:TA98:TA98NR was quantified as approximately 9:2.7:1 for PM2.5 and 7.7:2.8:1 for PM10 ($p<0.001$). Other previous published PM2.5 data showed comparable ratios [53]. These findings further highlight the important role nitro compounds play, and they underline the necessity of primary prevention improvement to reduce nitrated molecule air pollution. In our work without nitroreductase activity, the genotoxicity was limited. Today, the known sources of these kinds of compounds are various combustion processes, especially in diesel motors, where certain nitro-PAHs are formed in DPFs; however, other compounds are reduced, and nitration is not a general trend [13]. A mutagenic/carcinogenic activities is linked mainly to o-nitroanisole, 1-nitropyrene, 4-nitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, 6-nitrochrysene, and nitrofen [60]. Moreover, concentrations of nitro-PAHs and other nitro compounds produced from gas-phase reactions are generally correlated to NO_x pollution [25], and our work with the obtained mutagenicity data highlight this finding. Despite their critical role in the genotoxic properties of fine particulate pollution, there is currently no monitoring procedure for these kinds of pollutants. On the other hands known mutagens accounted for only 20% of the total mutagenicity of the fine particulate extracts [52,54]. New control and monitoring strategies are auspicious in the new directives in order to evaluate nitro-compounds but also with a biologic approach mutagenic/genotoxic properties of the airborne mixtures.

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TABLES:

Table 1 – Descriptive analysis of the collected data at the two sampling stations.

Table 2 – Correlation between the collected pollution indicators (µg/m^3 for NOx and ng/m^3 for PAHs) and mutagenicity (net revertants/m^3). All Spearman's rho correlations are highly significant ($p<0.01$) with the exception of data labelled with *, in this case the correlation is significant at $p<0.05$ level.

FIGURE LEGENDS

Figure 1 - Trend of the mutagenicity evaluated with the three *Salmonella* strains and NOx values with their standard deviations. **A**: data collected at station 1, the particulate indicator is PM2.5; **B**: data collected at station 1, the particulate indicator is PM10.

Figure 2 - Seasonal gravimetric analysis for PM2.5 (station 1) and PM10 (station 2). Data are divided by particles cut-off and by season. Cold period (autumn-winter) is set against hot period (spring-summer). The squares indicate the mean value while the up and down lines the standard deviations.

Figure 3 - Seasonal analysis of the data for each strain for PM2.5 (station 1) and PM10 (station 2). Cold period (autumn-winter) is set against hot period (spring-summer). **(A) PAHs data** are divided by particles cut-off, by season and by each determined compounds The circles indicate the mean value while the up and down lines the standard deviations. **(B)** Mutagenicity data are divided by particles cut-off, by season and by each Salmonella strain. The rhombus indicate the mean value while the up and down lines the standard deviations.

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