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**The North-western Italy air quality monitoring network: Improving experience of PM<sub>2.5</sub> assessment with mutagenicity assay**

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(Article begins on next page)

# Environmental Research

## The North-western Italy air quality monitoring network: improving experience of PM2.5 assessment with mutagenicity assay.

--Manuscript Draft--

<b>Manuscript Number:</b>	ER-20-4274R1
<b>Article Type:</b>	Research paper
<b>Section/Category:</b>	Environmental Chemistry and Toxicology
<b>Keywords:</b>	PM2.5, Salmonella assay, nitro-compounds, PAH, biomonitoring
<b>Corresponding Author:</b>	Tiziana Schiliro' University of Torino Torino, ITALY
<b>First Author:</b>	Daniele MARANGON, Dr
<b>Order of Authors:</b>	Daniele MARANGON, Dr Deborah TRAVERSI, Prof Anna Maria D'AGOSTINO, Dr Marta GEA, Dr Marco FONTANA, Dr Tiziana Schiliro'
<b>Abstract:</b>	<p>The finest fraction of Particulate Matter (PM2.5) carries a large number of pollutants, some of which are assessed as genotoxic, such as some Polycyclic Aromatic Hydrocarbons (PAHs). In many countries, PM2.5 in combination with some PAHs are monitored to assess the concentrations of pollutants, while the air quality is rarely assessed by means of biological assays. Epidemiological studies have demonstrated a significant correlation between these two pollutants and human adverse effects, in particular on the respiratory system. Nevertheless, other air pollutants can induce a biological effect and the cumulative effect of the PM2.5 complex mixture may not be easily deduced by PM2.5 and PAH levels. This study aimed to combine the legislative monitoring of PM2.5 with the study of its mutagenicity. During a full year, daily air samples were collected in nine sites of the North-western Italy air quality monitoring network (Piedmont Region) and PM2.5 and PAH concentrations were assessed. Monthly pooled organic extracts were tested with the Salmonella assay using TA98 and TA100 strains, with and without metabolic activation (<math>\pm</math>S9), and using TA98NR and YG1021 strains. In all sites, a positive response was observed for TA98 and TA100 especially without S9. A significant mutagenic seasonal variation was detected, with higher mutagenicity in winter and lower responses in summer (average total mutagenicity ratio 27:1). The response of TA98NR and YG1021 compared with TA98 suggested a significant contribution of nitro-compounds to the mutagenicity. No significant differences were found between urban background and rural sites denoting the spread of pollution. A mutagenicity increase, 1.28 Total Mutagenicity Factor/20 m<sup>3</sup>, was observed for each PM2.5 <math>\mu</math>g increment. PAH levels and corresponding Toxic Equivalent Factors were highly correlated to mutagenicity results. This work confirms that complex environmental mixtures can be appropriately assessed through the implementation of physical-chemical analyzes with bioassays able to evaluate synergistic and antagonistic effects, especially for highest and lowest pollution settings.</p>
<b>Response to Reviewers:</b>	<p>Answer to Reviewer 1</p> <p>We revised our paper following the reviewer comments and we included a detailed response indicating how each comment is addressed in the revised manuscript (in red) detailing the line numbers.</p> <p>The North-western Italy air quality monitoring network: improving experience of PM2.5 assessment with mutagenicity assay.</p> <p>In the study the authors analyzed the mutagenicity of PM2.5 in the North-western region of Italy. The authors show that mutagenicity could be an important factor to</p>

consider when monitoring PM2.5. The study is important, some interesting results were obtained, but there are some areas that need major improvement as it is explained ahead.

In terms of format, the use of English language needs a detailed revision by a professional editor.

The English language was reviewed by a professional service (revision certificate in attachment).

The following comments are organized by section:

#### Introduction

Page 4 - Line 12. Is B(a)p Benzo(a)pyrene? It has not been defined.  
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Page 4 - Line 29. Volumes are "few". Do you mean are small/low? However, the real problem is that the concentration/mass of the pollutant is small, and if the volume of air collected is small, the mass of the PM2.5 mass is insufficient for speciation.  
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Page 5 - Line 44. This sentence has to be improved. Moreover, the AQHI is defined latter in the text.

We thank the reviewer for the suggestion, we agree the sentence is not very clear, so we deleted this sentence and we modified the following sentence. Lines: 117 - 119.

#### Results

Page 11. Figure 2 show a clear seasonal trend in mutagenic activity of PM2.5. However, there is no explanation of potential reasons of this seasonal trend. Why would the season affect this characteristic of PM2.5?

We added some comments on the factors involved on the higher mutagenicity during winter season. Lines: 274– 280.

"The results showed a typical seasonal trend with the highest effect in winter and autumn and no mutagenic activity in July and August. This result can be explained considering that the air pollution levels were higher in the cold seasons than in the warm seasons. In fact, autumn and winter were characterized by the highest emissions of air pollutants (especially due to household heating) and by meteorological conditions that promoted condensation processes and limited the dispersion of air pollutants."

Page 12 Line 33-34. What is exactly the "quality" of PM2.5?

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Yes, the PM sources are a relevant factor however a significant correlation between PM2.5 mass and PM2.5 mutagenicity is generally observed (Cassoni et al. 2004), because the higher is the quantity of PM2.5 mass the higher is the quantity of

pollutants that can be adsorbed on it. There are particular situations when low PM2.5 mass corresponds to relatively high mutagenicity but they are not so frequent. This is probably due to a uniformity of the emission in urban environments of the involved sites, all placed in the Padana Plain. However, as well as showed on the figure 3B the total mutagenicity associated to each mass unit (PM2.5 mg) varied markedly.

#### Answer to Reviewer 2

We revised our paper following the reviewer comments and we included a detailed response indicating how each comment is addressed in the revised manuscript (in red) detailing the line numbers.

The manuscript addresses the problem of measuring a contaminant and concluding a consequence for which no obvious direct mechanism has been shown. Its potential should be characterized to better understand which health effects can be predicted by PM2,5 (or other particle loads), and which effects cannot be associated with this parameter. Therefore, the point is well taken, and the methods are appropriate to address this problem; the topic should be investigated in further studies.

The authors choose mutagenicity which is a well characterized criterion. However, I cannot follow the authors in some conclusions. On the one hand they find a Spearman correlation between mutagenicity and PM2.5 but a lack of corresponding variations in PAH concentrations. Spearmans correlation is a rank correlation analysis; it is not suitable to investigate dose response effects, which the authors later deduce from their plot of TMF with PM2.5 values. In this regard the data show the relevance (or lack of relevance for PAH) of mutagenicity and air pollution, but some conclusions appear to be exaggerated.

We performed a linear regression model to improve the analysis of the relationship between PAH levels and mutagenicity. Lines: 204-205; 322-326, Figure 4, 568 – 572. The paragraph “conclusion” was also improved. Lines: 363-397.

A few more points may be added:

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The introduction was shortened and modified as suggested by the Reviewer. Lines: 68-137.

Methods: The authors correctly used S9 supplied bacteria. Since PM2,5 is inhaled and the lung microsomal system differs both quantitatively and qualitatively from liver microsomes, why did they use liver S9 homogenates?

We agree with the reviewer, the lung microsomal system differs from liver microsomes. However, it is a common practice to use the liver microsomes to simulate the pollutants transformation at a systemic level not only for the lung. The pollutants transported by the PM2.5 can be diffused to the human body by the blood circulatory system.

For PAH analysis no method is described, nor is a reference given. Thus I cannot see, whether nitroaromatic compounds may have been found in this analysis if present or not.

No, nitroaromatic compounds were not included in the analysis. In this study only the concentrations of B(a)P, benzo(a)anthracene, indeno(1,2,3-cd)pyrene and benzo(b,j,k)fluoranthene in PM2.5 samples were quantified. The PAH analysis was carried out according to the European standard EN 15549. Lines: 191 - 195.

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Page 10 -- the association between temperature and PM2.5 values is not necessarily causal, and chemical interactions are temperature-dependent, but not necessarily faster. And usually, binding of molecules to surfaces is decreased with increasing temperatures which doesn't fit with the data. Additionally, there is a high probability of confounding by season.

Maybe the result discussed at page 10 was not clearly discussed. In this study a negative association was found between temperature and PM2.5 levels, therefore in cold months higher PM2.5 levels were detected than in warm months. We

hypothesized a higher interaction among the primary pollutants in the atmosphere during the cold months, when the dispersion of pollutants is lower (due to meteorological conditions), the sources of pollutants are higher (due to household heating) and the temperature is low so the pollutants are adsorbed on the PM surface interacting each other. We improved the discussion (lines: 242 - 249).

On page 10, lines 51ff likely sources for individual PAH are listed; however, I don't find industrial sources which (I assume) should play a major role in northwestern Italy, as should be household heating.

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In their tables the authors use a different parameter in each Table.

The tables were designed to summarize the main results. Since numerous parameters were investigated in this study, the tables reported the different parameters studied (PM2.5, PAHs and Mutagenicity). We checked all tables and figures and we improved the table 3 and figure 4 adding or modifying the units.

In Table 2 the BaP<sub>eq</sub> values are given. For July and August these values appear to be VERY low since BaP is caused to a considerable degree by traffic exhausts, and is rather stable even in summer heat. Alternatively, the winter values may be higher than expected, but a summer winter difference of up to 600fold should be explained. It also should show in a higher difference for revertants in S9 supplied tests.

As reported in the discussion, the PAHs concentrations are higher in cold months than in warm months. This is probably due to the climatic conditions which facilitate the dispersion of the pollutants and help PAHs degradation in spring and summer with respect to autumn and winter. It seems strange but the PAH concentrations are really so different comparing summer and winter, as reported in some Report of the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte)

(<http://www.cittametropolitana.torino.it/cms/ambiente/qualita-aria/dati-qualita-aria/relazioni-annuali>). Therefore, the BaP<sub>eq</sub> is really so different when comparing summer and winter months.



*Department of Public Health and Pediatrics, University of Torino, Italy*

*Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), Italy*

July 28<sup>th</sup>, 2020

Dear Editor,

We are sending the manuscript "***The North-western Italy air quality monitoring network: improving experience of PM2.5 assessment with mutagenicity assay.***" by Daniele MARANGON, Deborah TRAVERSI, Anna Maria D'AGOSTINO, Marta GEA, Marco FONTANA, Tiziana SCHILIRÒ on *ENVIRONMENTAL RESEARCH*.

The current manuscript deals with the biological-chemical characterization of PM2.5 in 9 different sites in North-West of Italy of the Piedmont air quality monitoring network, every day for a full year.

Monthly pooled organic extracts were tested with the *Salmonella* assay using TA98 and TA100 strains, with and without metabolic activation ( $\pm$ S9), and using TA98NR and YG1021 strains. In all sites, a positive response was observed for TA98 and TA100 especially without S9. A significant mutagenic seasonal variation was detected, with higher mutagenicity in winter and lower responses in summer (average total mutagenicity ratio 27:1). The response of TA98NR and YG1021 compared with TA98 suggested a significant contribution of nitro-compounds to the mutagenicity. No significant differences were found between urban background and rural sites denoting the spread of pollution. A mutagenicity increase, 1.28 Total Mutagenicity Factor/20 m<sup>3</sup>, was observed for each PM2.5  $\mu$ g increment. PAH levels and corresponding Toxic Equivalent Factors were highly correlated to mutagenicity results. This work confirms that complex environmental mixtures can be appropriately assessed through the implementation of physical-chemical analyzes with bioassays able to evaluate synergistic and antagonistic effects, especially for highest and lowest pollution settings.

All of the authors have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal. All authors are aware of and accept responsibility for the manuscript. All figures and tables were produced by the authors. Lastly, all authors declare no conflicting interests.

Hoping that the manuscript may fulfil the scientific standards of *ENVIRONMENTAL RESEARCH*, our best regards.

*Tiziana Schilirò and Co-authors*



*Department of Public Health and Pediatrics, University of Torino, Italy*

*Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), Italy*

November 19<sup>th</sup>, 2020

Dear Editor,

please find enclosed the revised manuscript "The North-western Italy air quality monitoring network: improving experience of PM<sub>2.5</sub> assessment with mutagenicity assay" by Daniele Marangon, Deborah Traversi, Anna Maria D'Agostino, Marta Gea, Marco Fontana, Tiziana Schilirò.

We have answered to the Reviewers' comments and relevant changes have been written in red all over the enclosed text.

Finally, our responses to each Reviewers' comment have been reported as follows, written in red.

Best regards,

Tiziana Schilirò and Co-authors.

We revised our paper following the reviewer comments and we included a detailed response indicating how each comment is addressed in the revised manuscript (in red) detailing the line numbers.

The North-western Italy air quality monitoring network: improving experience of PM2.5 assessment with mutagenicity assay.

In the study the authors analyzed the mutagenicity of PM2.5 in the North-western region of Italy. The authors show that mutagenicity could be an important factor to consider when monitoring PM2.5. The study is important, some interesting results were obtained, but there are some areas that need major improvement as it is explained ahead.

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This document certifies that the manuscript

**The northwestern Italy air quality monitoring network: improving the PM2.5 assessment with a mutagenicity assay.**

prepared by the authors

**Daniele MARANGON, Deborah TRAVERSI, Anna Maria D'AGOSTINO, Marta GEA,  
Marco FONTANA, Tiziana SCHILIRÒ**

was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.

This certificate was issued on **November 13, 2020** and may be verified on the [AJE website](#) using the verification code **31A3-E938-7973-5C79-AAA2**.



Neither the research content nor the authors' intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page at [aje.com/certificate](#). If you have any questions or concerns about this edited document, please contact AJE at [support@aje.com](mailto:support@aje.com).



**The North-western Italy air quality monitoring network: improving experience of PM2.5 assessment with mutagenicity assay.**

Daniele MARANGON <sup>a</sup>, Deborah TRAVERSI <sup>b</sup>, Anna Maria D'AGOSTINO <sup>a</sup>, Marta GEA <sup>b</sup>, Marco FONTANA <sup>a</sup>, Tiziana SCHILIRÒ <sup>b\*</sup>

<sup>a</sup> Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

<sup>b</sup> Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

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Tiziana Schilirò

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Submitted to *ENVIRONMENTAL RESEARCH*

**Highlights (max 5, max 85 characters, including spaces, per bullet point)**

1. PM2.5 mutagenicity was higher in winter and in urban sites even if diffused.
2. A rate of 1.28 mutagenicity increase was observed for each  $\mu\text{g}$  of PM2.5 increment.
3. From 36 to 54% of the PM2.5 mutagenicity was due to nitro-compounds.
4. PM2.5 mass and mutagenicity correlation is significant only from 10 to 25  $\mu\text{g}/\text{m}^3$ .
5. Risk assessment and health policies should be supported by mutagenicity assays.

1 **The North-western Italy air quality monitoring network: improving experience of PM2.5**  
2 **assessment with mutagenicity assay.**

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7 4 Daniele MARANGON <sup>a</sup>, Deborah TRAVERSI <sup>b</sup>, Anna Maria D'AGOSTINO <sup>a</sup>, Marta GEA <sup>b</sup>,  
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27 **Abstract**

28 The finest fraction of Particulate Matter (PM<sub>2.5</sub>) carries a large number of pollutants, some of  
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30 many countries, PM<sub>2.5</sub> in combination with some PAHs are monitored to assess the  
31 concentrations of pollutants, while the air quality is rarely assessed by means of biological  
32 assays. Epidemiological studies have demonstrated a significant correlation between these two  
33 pollutants and human adverse effects, in particular on the respiratory system. Nevertheless,  
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51 synergistic and antagonistic effects, especially for highest and lowest pollution settings.

53 **Keywords:** PM2.5, *Salmonella* assay, nitro-compounds, PAH, biomonitoring.

54 **Abbreviations:**

55 ARPA Piemonte Regional Agency for Environmental Protection of Piedmont

56 B(a)P benzo(a)pyrene

57 BaP<sub>eq</sub> benzo(a)pyrene-equivalent

58 DMSO dimethyl sulfoxide

59 IARC International Agency for Research on Cancer

60 MAMSL m above mean sea level

61 MR mutagenicity ratio

62 PAH Polycyclic Aromatic Hydrocarbon

63 PM Particulate Matter

64 TEF Toxic Equivalent Factor

65 TMF Total Mutagenicity Factor

66 WHO World Health Organization

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## 68 **1. Introduction**

69 **The International Agency for Research on Cancer (IARC) has classified outdoor air pollution**

70 **as carcinogenic to humans (group 1). One of the most important air pollutants is particulate**

71 **matter (PM), and the toxic and genotoxic effects of PM are mainly attributed to PM<sub>2.5</sub>**

72 **(aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ), as this fraction is composed of numerous fine and ultrafine**

73 **particles per unit mass with a large surface, on which mutagenic and genotoxic pollutants can**

74 **be adsorbed (Balakrishnan et al., 2015; IARC, 2016; Rainho et al., 2014). PM<sub>2.5</sub> is widely**

75 **studied because of its harmful effects on human health, such as lung cancer, cardiovascular**

76 **diseases and respiratory disorders, as a result of the high deposition rate in respiratory organs.**

77 **The World Health Organization (WHO) has set guidelines for PM<sub>2.5</sub> in the atmosphere at 10**

78  **$\mu\text{g}/\text{m}^3$  for the annual average concentration and at 25  $\mu\text{g}/\text{m}^3$  for the daily average (WHO, 2006);**



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79 therefore, numerous countries monitor PM<sub>2.5</sub> levels to assess environmental air quality. In all  
80 Italian regions, monitoring PM<sub>2.5</sub>, as well as monitoring benzo(a)pyrene (B(a)P) and metals  
81 (As, Cd, and Ni), is carried out following current Italian and European regulations (Italian  
82 Legislative Decree 155/2010 and European Commission Directive 2008/50/EC). Moreover, to  
83 better characterize PM<sub>2.5</sub> quality, additional chemical analyses are recommended. For  
84 example, the carcinogenic potency of air samples can be estimated by Polycyclic Aromatic  
85 Hydrocarbon (PAH) levels, including the Toxic Equivalent Factor (TEF). Total TEF is assessed  
86 by multiplying the concentration of an individual PAH in the air with its TEF (Samburova et  
87 al., 2017). Most of the total TEF is determined by the finest PM fractions showing that  
88 genotoxic PAH levels are inversely proportional to the particle size (Pehnc and Jakovljević,  
89 2018). However, since PM<sub>2.5</sub> is often collected by low-volume air samplers, chemical analysis  
90 is limited because the air volumes, which can be sampled, are also small, and they may not be  
91 large enough to collect a sufficient PM<sub>2.5</sub> mass for speciation, especially for nano-pollutants.  
92 Moreover, since a wide range of carcinogenic substances (known and not well known) can be  
93 contained in PM<sub>2.5</sub> samples, it is currently impossible to measure all of them. There are both  
94 analytical limits and cost-benefit limits.

95 PM<sub>2.5</sub> and PAH levels are important parameters for assessing air quality; however, the  
96 cumulative effect of the complex PM<sub>2.5</sub> mixture may not be easily deduced by PM<sub>2.5</sub> and PAH  
97 concentrations; therefore, the application of some effect-based monitoring tools (e.g., *in vitro*  
98 assays) could have a key role in improving assessments of the biological effects of PM<sub>2.5</sub> and  
99 PAHs in the context of the different monitoring programmes (Schilirò et al., 2016). In fact,  
100 biological monitoring approaches through effect-based tools allow the detection of cumulative  
101 effects and are useful for bridging the gap between chemical contamination and biological  
102 effects (Wernersson et al., 2015). Among the different biological monitoring approaches, the  
103 use of *in vitro* assays has become increasingly important since *in vivo* methods are characterized

104 by high costs and technical complexity. Moreover, the National Academy of Sciences has noted  
105 the need to eliminate the use of animals in future studies (NRC, 2007).

106 A useful *in vitro* assay to assess mutagenicity is the *Salmonella* reverse mutation assay  
107 (Ames test), which was first used in 1977 and has been applied for 40 years (Mortelmans, 2019;  
108 Tokiwa et al., 1977). The traditional Ames test uses *Salmonella typhimurium* strains TA98 (in  
109 which frameshift mutations are detected) and TA100 (in which base substitution mutations are  
110 detected), but it cannot highlight the mutagenic activity of particular compounds; therefore,  
111 modified Ames tests have been developed to improve the assessment of PM<sub>2.5</sub> mutagenicity.  
112 Specifically, from the TA98 strains, two different strains were derived (YG1021 and TA98NR  
113 strains) that differ in their nitro-reductase expression (Josephy et al., 1997). The Ames test **has**  
114 **become** a requirement for the investigation or regulatory approval of many types of chemicals,  
115 **and it has been applied to characterize the mutagenicity of air in more than 250 studies, leading**  
116 **to a remarkable amount of information.** Although the test has been used for 46 years, it is still  
117 widely applied. **For example, using experimental systems that simulate atmospheric pollution,**  
118 **the Ames test was recently correlated to the air quality health index that is used for atmospheric**  
119 **pollution** (Zavala et al., 2018), and the Ames test showed the crucial role of aromatic  
120 hydrocarbon and NO<sub>x</sub> mixture photooxidation (Riedel et al., 2018).

121 Many studies have shown a positive association between PM mutagenicity measured with  
122 the Ames test and the concentrations of PAHs and their nitro, amino and/or hydroxylamine  
123 derivatives. Moreover, a positive association between PM mutagenicity and polar compounds,  
124 such as aromatic amines and aromatic ketones, has also been found (Alves et al., 2016; Claxton  
125 et al., 2004; Traversi et al., 2011). However, the ability of PM<sub>2.5</sub> and PAH levels to predict the  
126 mutagenic effect of polluted air is still controversial. Therefore, to comprehensively evaluate  
127 the harmful effects of substances associated with PM<sub>2.5</sub>, a mutagenic assay, such as the Ames  
128 test, seems to be needed and has recently been supported for risk assessments (Klapacz and  
129 Gollapudi, 2019) and regulatory decision making (Heflich et al., 2019).

130 Monitoring is particularly important for critical areas such as northwestern Italy since it is a  
131 plain surrounded by mountains where climate and topography promote severe air pollution  
132 episodes (Wallace et al., 2010; EEA, 2019; Traversi et al., 2009).

133 In this study, PM<sub>2.5</sub> was collected daily during a full year at nine different sites of the  
134 northwestern Italian air quality monitoring network (Piedmont Region), and both the PM<sub>2.5</sub>  
135 and PAH concentrations (including associated TEFs) and mutagenicity of the PM<sub>2.5</sub> organic  
136 extract were evaluated to integrate the results of legislatively required monitoring with  
137 biological monitoring.

## 139 **2. Materials and methods**

### 140 ***2.1 Sampling sites, collection, and extraction***

141 The PM<sub>2.5</sub> samples were collected from nine monitoring stations located in the Piedmont  
142 Region, northwest of the Italian Padana Plain. The stations are part of the air quality monitoring  
143 network run by the Regional Agency for Environmental Protection of Piedmont (ARPA  
144 Piemonte). The sampling sites included a traffic site (Settimo Torinese), five urban background  
145 sites (Torino, Novara, Alessandria, Borgosesia and Cuneo), two rural background sites  
146 (Vinchio and Domodossola), and a hill site located far from human settlements as a reference  
147 site with low air pollution levels (Dernice) (Figure 1).

148 PM<sub>2.5</sub> was sampled daily from January to December 2016 utilizing low-volume air samplers  
149 (sampling flow 2.3 m<sup>3</sup>/h). PM<sub>2.5</sub> was collected on quartz-fibre filters ( $\varnothing = 47$  mm, Sartorius,  
150 Goettingen, Germany). The amount of PM collected was determined using the gravimetric  
151 method by double weighing (complying with the EN 12341 norm). The exposed filters were  
152 stored at -20 °C until extraction. The filters collected daily were pooled together to obtain  
153 monthly samples that were subsequently extracted with acetone-hexane 1:1 using a Soxhlet  
154 apparatus for at least 80 cycles. The solvent was evaporated with a rotary evaporator, and the

155 extracts were diluted with dimethyl sulfoxide (DMSO, Carlo Erba reagents, I) to obtain a  
156 concentration of 200 m<sup>3</sup>/mL.

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## 158 **2.2 Salmonella reverse mutation assay (Ames Test)**

159 The mutagenicity assay was performed on every organic extract according to Maron and  
160 Ames (1983). The mutagenic activity of the PM<sub>2.5</sub> extracts was determined using the *S.*  
161 *typhimurium* frameshift strain TA98 and the base-substitution strain TA100, both with and  
162 without Aroclor-induced rat liver homogenate activation (S9). In addition, *S. typhimurium*  
163 TA98NR and YG1021 strains were used to evaluate the nitro compound contribution to the  
164 overall mutagenicity. YG1021 is a nitroreductase-overproducing strain obtained by cloning the  
165 nitroreductase gene of *S. typhimurium* TA1538 into the pBR322 vector and introducing the  
166 recombinant plasmid into TA98. YG1021 has a nitrofurazone reductase activity more than 50  
167 times higher than that of the original TA98 strain, permitting the efficient detection of  
168 mutagenic nitroarenes, while TA98NR lacks nitroreductase; therefore, the response obtained  
169 from these two strains permits efficient detection of mutagenic nitroarenes when compared with  
170 the reference TA98 strain.

171 Four doses of PM<sub>2.5</sub> organic extracts were tested in triplicate to obtain a dose-response curve  
172 (2.5, 5, 10, and 20 m<sup>3</sup>/plate). Positive controls were 4-nitroquinoline 1-oxide (0.5 µg/plate,  
173 TA98), methyl methanesulfonate (0.25 µg/plate, TA100), 2-aminoanthracene (2 µg/plate,  
174 TA98+S9, TA100+S9) and 2-nitrofluorene (1 µg/plate, TA98NR, YG1021). Negative controls  
175 were obtained by exposing each strain to DMSO (100 µL/plate). After 48 h of incubation, the  
176 number of revertant colonies was measured by an automatic colony counter (Synoptics Protos,  
177 UK). A microscopic observation of the background lawn density was used to evaluate the  
178 toxicity. The results are expressed as net revertants (total revertants minus spontaneous  
179 revertants)/m<sup>3</sup> and were calculated by a dose-response curve (rev/m<sup>3</sup>, i.e., the slope of the  
180 regression line) (Buschini et al., 2001; Cassoni et al., 2004). The mutagenicity ratio (MR)/20

181 m<sup>3</sup> sampled air, which is equivalent to the average breathed air by an adult in one day, was  
182 calculated by dividing the number of net revertants on the sample plates by the spontaneous  
183 revertants. To assess the overall mutagenicity of PM<sub>2.5</sub>, the Total Mutagenicity Factor (TMF)  
184 was calculated by adding the MRs of strains TA98 and TA100 with and without S9. A positive  
185 mutagenic response was defined when there was at least a twofold increase in revertants over  
186 the negative control (MR=1) and a dose-response effect over the range tested.

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### 188 **2.3 PAH determinations**

189 The determinations of PAHs were carried out by ARPA Piemonte on monthly pooled filters  
190 sampled at the same sites used for biological analysis.

191 In addition to the determination of B(a)P, as required by Italian law (Italian Legislative  
192 Decree 155/2010), the following PAHs were also determined in this study: benzo(a)anthracene,  
193 indeno(1,2,3-cd)pyrene and benzo(b,j,k)fluoranthene. Samples were extracted with acetone-  
194 hexane under sonication in a water bath, and PAHs were quantified using GC/MS analysis  
195 according to the European standard EN 15549.

196 The TEF approach was applied to convert PAH concentrations into B(a)P-equivalent  
197 (BaP<sub>eq</sub>) toxicity (Nisbet and LaGoy, 1992; Reeves et al., 2001).

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### 199 **2.4 Statistical analyses**

200 The autumn-winter mean for each parameter was calculated taking into account the  
201 monthly values from October to March, and the spring-summer mean was calculated  
202 considering the months from April to September. The t-test was used to compare the differences  
203 between the two periods for every site. The Spearman test was used to calculate correlations  
204 and the relationship between PM<sub>2.5</sub> levels and mutagenicity was evaluated through a linear  
205 regression model. Differences among sites were tested using ANOVA with Tukey's *post hoc*  
206 test (normally distributed data) and the Kruskal-Wallis test (not normally distributed data).

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207 Differences were considered significant when  $p < 0.05$ . Statistical analyses were performed  
208 using the IBM SPSS Statistics package, version 24.0.

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### 210 3. Results and Discussion

#### 211 3.1 Gravimetric analysis

212 Airborne PM<sub>2.5</sub> concentrations showed a similar seasonal trend at almost every site  
213 considered in the study, with higher levels in the autumn-winter season than in the spring-  
214 summer season (t-test  $p < 0.05$ ). No significant differences were found between seasons only  
215 at the reference site Dernice (Table 1); moreover, at this site, the annual mean PM<sub>2.5</sub> levels  
216 were lower than those at the other sites (ANOVA with Tukey's *post hoc*  $p < 0.05$ ).

217 The data showed that the annual mean PM<sub>2.5</sub> was always below the Italian limit level of  
218 25  $\mu\text{g}/\text{m}^3$  (Italian Legislative Decree 155/2010, European Commission Directive 2008/50/EC),  
219 except for at the traffic site Settimo Torinese. On the other hand, Dernice is a unique site where  
220 the annual mean PM<sub>2.5</sub> was below the WHO guideline level (WHO, 2006) (Table 1).  
221 Moreover, the daily PM<sub>2.5</sub> mean concentration recorded at all stations ranged between 5.7 and  
222 64.9  $\mu\text{g}/\text{m}^3$ , and for numerous days during the year, the daily PM<sub>2.5</sub> mean concentration was  
223 higher than the WHO guideline value (25  $\mu\text{g}/\text{m}^3$ ); thus, almost all the mean concentrations  
224 related to the autumn-winter season were above 25  $\mu\text{g}/\text{m}^3$  (Table 1), except for those in cities  
225 placed at more than 500 m above mean sea level (MAMSL) (Figure 1). These results  
226 demonstrated that although the different sites were characterized by different emission levels  
227 (e.g., rural sites and urban sites), especially in winter, most showed PM<sub>2.5</sub> levels above the  
228 WHO guideline value, posing a potential risk to human health. These levels were probably due  
229 to the climatic and topographic conditions of the Piedmont Region, which is located on the  
230 Padana Plain, an area surrounded by mountains and where such conditions reduce emission  
231 pollutant dispersion and promote severe air pollution episodes. These findings are in agreement  
232 with those in the literature describing measurements taken at different locations on the Padana

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233 Plain where winter meteorological conditions in addition to thermal inversion prevent the  
234 dispersion of pollutants, causing high PM levels (Bocchi et al. 2016; Bonetta et al., 2009; Feretti  
235 et al., 2019; Gilli et al., 2007). Moreover, since the sites located at high altitudes, such as  
236 Dernice, Borgosesia, Domodossola and Cuneo (altitudes ranging from 295 to 580 MAMSL),  
237 showed lower PM<sub>2.5</sub> levels than those at low altitude sites, our results demonstrated that  
238 pollutant dispersion seems to be more frequent at higher altitudes, especially on the Padana  
239 Plain.

240 PM<sub>2.5</sub> monthly mean concentrations were inversely correlated with measured  
241 temperature (Spearman rho -0.783 - -0.888; p < 0.01), except for at the Dernice site, where PM  
242 concentrations were mostly constant over the year. **The increase in PM<sub>2.5</sub> levels in the cold  
243 months, characterized by the lowest temperature, can be explained considering that in  
244 comparison to in the warm months, in the cold months, a stronger chemical interaction among  
245 the primary pollutants can occur in the atmosphere. This interaction is due to lower pollutant  
246 dispersion (due to meteorological conditions) and higher pollutant sources (due to household  
247 heating) during cold months than during warm months. Moreover, the cold temperatures in the  
248 cold months increase the binding of molecules to surfaces, so the pollutants are stably adsorbed  
249 on the PM surface and can interact with each other.**

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### 251 3.2 *PAH levels*

252 The recorded values for total PAHs (sum of the 4 compounds), TEFs, and B(a)P are  
253 summarized in Table 2. Total PAH concentrations showed the same trend as that of the PM<sub>2.5</sub>  
254 levels. In particular, PAH concentrations were high in the winter season, while from April to  
255 September, PAH values were low and uniform at every site. The decrease in PAH levels in  
256 spring and summer was probably due to the climatic conditions that facilitated the dispersion  
257 of pollutants and to the high solar radiation, which helped PAH degradation. The mean PAH

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258 levels measured in this study were similar to those reported in recent studies in Europe (Dvorská  
259 et al., 2011).

260 The most abundant compound measured at every site was benzo(b,j,k)fluoranthene  
261 (46.1% - 57.5%), followed by indeno(1,2,3-cd)pyrene (16.9% - 23.5%), B(a)P (8.6% - 21.2%)  
262 and benzo(a)anthracene (10.3% - 15.8%). According to the literature, burning of fossil fuels  
263 was probably the prominent source of atmospheric PAHs investigated:  
264 benzo(b,j,k)fluoranthene is primarily found in gasoline exhaust, indeno(1,2,3-cd)pyrene is  
265 mainly produced by the incomplete combustion of biomass, B(a)P is primarily found in  
266 gasoline, and benzo(a)anthracene is mainly found in diesel exhaust (Chang et al., 2019). **In the  
267 study area, the burning of fossil fuels was attributed to not only household heating and vehicular  
268 traffic but also other pollution sources, such as combustion processes of industrial plants.**

269 The traffic site of Settimo Torinese and the rural site of Domodossola exceeded the annual  
270 limit of 1 ng/m<sup>3</sup> in European and Italian laws (European Commission Directive 2004/107/EC;  
271 Italian Legislative Decree 155/2010) for B(a)P (Group 1 IARC).

### 273 **3.1 Salmonella reverse mutation assay (Ames test)**

274 The mutagenic effect of the PM<sub>2.5</sub> organic extracts is illustrated in Figure 2. **The results  
275 showed a typical seasonal trend with the highest effect in winter and autumn and no mutagenic  
276 activity in July and August. This result can be explained considering that the air pollution levels  
277 were higher in the cold seasons than in the warm seasons. In fact, autumn and winter were  
278 characterized by the highest emissions of air pollutants (especially due to household heating)  
279 and by meteorological conditions that promoted condensation processes and limited the  
280 dispersion of air pollutants.** As reported in the literature (Cassoni et al., 2004), an enhanced  
281 response of the strains without S9 was observed at every site, denoting a higher presence of  
282 substances that can act directly on DNA. In comparison to the other sites, the traffic station  
283 (Settimo Torinese) showed higher mutagenic values, particularly in January and December,



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284 while the reference site with low air pollution (Dernice) always showed low mutagenic values,  
285 even in the winter season. At the other rural and urban background stations, there were no  
286 significant differences, denoting a spread of PM<sub>2.5</sub> pollution over the whole region.

287 Figure 3A shows the results obtained in the cold period (November-January), expressed  
288 as the TMF of the 20 m<sup>3</sup> air equivalent, obtained by adding the MRs of the strains TA98 and  
289 TA100 with and without S9. Significant differences between the stations were found with the  
290 non-parametric Kruskal-Wallis H test (Dernice *vs* all the other stations, *p* <0.05); nevertheless,  
291 no significant differences were detected among the other background sites (both urban and  
292 rural). This emphasizes again that the PM pollution issue does not exclusively affect urban areas  
293 but entire regions such as the Piedmont, whereas critical areas can also be identified at a low  
294 altitude, such as Alessandria, where PM emissions were lower than those in the Torino  
295 metropolitan area.

296 No significant differences among the stations were observed when evaluating the overall  
297 mutagenicity based on the amount of PM<sub>2.5</sub> analysed (Figure 3B). A positive response was  
298 also found when analysing samples of the reference site, even if lower than that at the other  
299 sites (Figure 3).

300 To compare the results of traditional monitoring with biological monitoring, correlations  
301 between PM<sub>2.5</sub> levels and mutagenic effects were tested. The correlations between the PM<sub>2.5</sub>  
302 levels and the calculated revertants for every strain were statistically significant throughout the  
303 studied period, except for those at the reference site (Table 3). This result suggests that PM<sub>2.5</sub>  
304 level can also be a good parameter to estimate its mutagenic potential. The PM<sub>2.5</sub> levels showed  
305 a high correlation with the mutagenicity of critical sites such as Settimo Torinese, highlighting  
306 the presence of more mutagenic compounds on PM<sub>2.5</sub>. However, it is important to note that  
307 the PM<sub>2.5</sub> levels were usually not correlated with the mutagenicity at the reference site  
308 (Dernice), and the significance of the correlation decreased at sites with low PM<sub>2.5</sub> levels.  
309 Therefore, the mutagenic compound contribution to the mass seemed to decrease from

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310 urban/critical sites to the reference site (Table 3), suggesting that the mutagenicity at sites  
311 characterized by low PM<sub>2.5</sub> levels may mainly depend on the quality (type and chemical nature  
312 of the particles and of the particle-transported pollutants) and not on the quantity of the PM<sub>2.5</sub>  
313 mass. From these results, it seems that traditional monitoring is an approach that is usually  
314 suitable for the assessment of air quality; however, biological monitoring can be a  
315 complementary approach that is particularly important for the correct assessment of air quality  
316 at sites characterized by low PM<sub>2.5</sub> levels and unusual, poorly understood pollutants.

317 The relationship between airborne PM<sub>2.5</sub> concentrations and total mutagenicity was  
318 further evaluated by comparing all the data obtained at all the sites throughout the year (Figure  
319 4). A significant relationship was observed, and the samples with PM<sub>2.5</sub> concentrations below  
320 the annual limit of 10 µg/m<sup>3</sup> suggested by the WHO (WHO, 2006) showed negative or weakly  
321 positive mutagenic results, demonstrating that PM<sub>2.5</sub> levels can be a good parameter to estimate  
322 air mutagenic effects and that the WHO limit is adequate. However, the linear regression model  
323 between mass and mutagenicity disappeared at PM<sub>2.5</sub> levels below 10 µg/m<sup>3</sup>. On the other  
324 hand, biological monitoring may be a useful complementary approach. Moreover, the presence  
325 of a mutagenic effect induced by low PM<sub>2.5</sub> levels suggested that a reduction in PM<sub>2.5</sub> mass  
326 did not seem to be sufficient to reduce its toxicity (Schilirò et al., 2015).

327 PAH concentrations, as one of the other parameters generally assessed in traditional  
328 monitoring programmes, were correlated with PM<sub>2.5</sub> levels. The PAH concentrations  
329 statistically correlated with the total mutagenicity (TMF/20 m<sup>3</sup>) considering all the data  
330 obtained at all the sites throughout the year (Spearman's rho = 0.876 ÷ 0.915; p < 0.01). Taking  
331 into account only the autumn and winter seasons, the correlations were usually not significant  
332 (Table S.1, supplementary materials); for example, the correlation was not significant in Torino  
333 (Spearman's rho = 0.696; p > 0.05) and Domodossola (Spearman's rho = 0.200; p > 0.05) but  
334 highly significant at sites with extreme pollution levels, very high in Settimo Torinese  
335 (Spearman's rho = 0.945; p < 0.01) and very low in Dernice (Spearman's rho = 0.943; p < 0.01).

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336 Since only four PAHs were quantified in this study, the lack of correlation could have been due  
337 to the presence of other PAHs or pollutants with a mutagenic effect that was not quantified.  
338 This observation emphasizes that the analytical determination of a few chemical compounds is  
339 not always enough to characterize the mutagenicity of a complex environmental mixture such  
340 as PM, as has been confirmed by other recent European studies (Bocchi et al., 2017; Bonetta et  
341 al., 2019; Velali et al., 2019).

342 Finally, in this study, the nitro compound contribution to the overall mutagenicity was  
343 evaluated by comparing the results obtained with the TA98, TA98NR, and YG1021 strains.  
344 The quantification of the mutagenicity linked to the amplified nitroreductase activity was  
345 calculated as  $[\text{YG1021 net revertants}/\text{m}^3/\text{TA98 net revertants}/\text{m}^3]$ , while the residual  
346 mutagenicity linked to the nitroreductase deficiency was quantified as follows:  $\{1 - [(\text{TA98 net}$   
347  $\text{revertants}/\text{m}^3 - \text{TA98 NR net revertants}/\text{m}^3)/\text{TA98 net revertants}/\text{m}^3]\}$  (Traversi et al., 2009).  
348 At every site, the YG1021 strain showed the highest response, followed by TA98 and TA98NR.  
349 Table S.2 (supplementary materials) summarizes the amplified and residual mutagenicity  
350 during the winter period. The values for the amplified mutagenicity ranged from 1.41 for  
351 Borgosesia to 2.32 for Dernice, denoting a high contribution of the nitro compounds to the total  
352 mutagenicity measured in every site (Rainho et al., 2014; Traversi et al., 2011). The amplified  
353 mutagenicity due to the nitro-compound contribution did not explain the higher mutagenicity  
354 observed in the critical area than in the other areas; moreover, the residual mutagenicity seemed  
355 to be constant (from 0.46 for Domodossola to 0.64 for Vinchio) (Table S.2, supplementary  
356 materials). Nonetheless, at some sites (Torino, Domodossola and Dernice), the nitro compound  
357 contribution to mutagenicity was remarkable regardless of the PM<sub>2.5</sub> or PAH concentrations.  
358 These results further confirmed that it is useful, where possible, to monitor air quality using  
359 biological monitoring. Moreover, this result suggests that it is convenient to test samples with  
360 different *S. typhimurium* strains to investigate different biological effects that are induced by  
361 different chemicals (Landkocz, et al., 2017; Lemos et al., 2012).

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#### 363 4. Conclusion

364 This study confirmed that PM<sub>2.5</sub> pollution is a critical issue to address to protect human  
365 health, especially in northwestern Italy, an area characterized by climatic and topographic  
366 conditions that prevent pollutant dispersion and promote severe air pollution episodes. In this  
367 area, PM<sub>2.5</sub> levels exceed the WHO guidelines except for some background sites, which are  
368 generally located at a higher altitude, suggesting that the air pollution phenomenon is attenuated  
369 by altitude.

370 Our results suggest that PM<sub>2.5</sub> concentration is a good parameter to estimate air quality  
371 because of the correlations between PM<sub>2.5</sub> levels and mutagenic effects; however, since this  
372 correlation was generally not significant **at the site with the lowest pollution levels, the results**  
373 **also suggested that** biological monitoring can be a complementary approach for the correct  
374 assessment of air quality at sites characterized by low PM levels or unusual pollutant sources.  
375 Moreover, considering **that the correlations between PAH levels and mutagenic effects were**  
376 **not always significant**, our results demonstrate that the analytical determination of a few  
377 chemical compounds is not enough to characterize the mutagenicity of a complex  
378 environmental mixture.

379 Finally, this study shows that it is useful to test air samples with different *S. typhimurium*  
380 strains to consider different biological effects induced by different pollutants; in particular, **for**  
381 **the assessment of** the potential risk associated with PM<sub>2.5</sub>, it seems important to also evaluate  
382 the mutagenic effect due to nitro compounds, highlighting the necessity of a strength assessment  
383 for reactive nitrogen molecules in the air.

384 Our study confirms the usefulness of combining biological tests with regulatory  
385 assessments, especially for air quality monitoring networks; **moreover, our study suggests that**  
386 the use of *in vitro* models to assess biological effects induced by environmental matrices might  
387 be a valid tool for supporting environmental risk assessment and public health policies.

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388 The application of a combination of tests is scientifically desirable; however, taking into  
389 account a large number of assays and the associated costs (in terms of both technical resources  
390 and time), the obtained results seem to indicate the *Salmonella* assay is a good, cost-efficient  
391 evaluation tool for PM2.5 mutagenicity assessment. The use of simplified models such as  
392 bacteria instead of human cells and the *in vitro* application of high levels of the organic extract  
393 are often critical factors for the extrapolation of data for risk assessment purposes. Human  
394 exposure and genotoxic effect induction are not described by the *Salmonella* assay alone. **On  
395 the other hand, this assay represents a simulation of the biological action induced by a complex  
396 mixture of pollutants such as PM2.5, so it has been considered for use in preventing effects due  
397 to chronic exposure and a long latency period.**

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556 **Figure captions:**

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558 **Figure 1.** Sampling sites in the study area. Cartographic coordinate system ED1950 (UTM)

559 Zone 32. MAMSL= m above mean sea level.

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561 **Figure 2.** Mutagenicity expressed as number of net revertants/m<sup>3</sup> of air sampled – Strains TA98

562 and TA100 with (+S9) and without metabolic activation.

563

564 **Figure 3.** Total mutagenicity measured in the sites during autumn-winter months expressed as

565 A: TMF/20 m<sup>3</sup> of air sampled, B: TMF/mg of PM2.5. Boxes represent the 25-75<sup>th</sup> percentile;

566 outer lines represent the 10-90<sup>th</sup> percentile.

567

568 **Figure 4.** Linear regression between PM2.5 (monthly values, µg/m<sup>3</sup>) and mutagenicity

569 (monthly values, TMF/20 m<sup>3</sup>), all data (108 pairing). The green area corresponds to mass

570 concentration levels ranging between the WHO guideline value (WHO, 2006) and the limit

571 value of the Italian law (Italian Legislative Decree 155/2010). The regression shows R = 0.837

572 and is statistically significant (Anova p=0.000).

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582 **Table captions:**

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584 **Table 1.** Annual means of PM2.5 ( $\mu\text{g}/\text{m}^3$ ) in the sites and comparison between means of PM2.5  
585 ( $\mu\text{g}/\text{m}^3$ ) measured during autumn-winter months and means of PM2.5 ( $\mu\text{g}/\text{m}^3$ ) measured  
586 during spring-summer months in the sites– t-test.

587

588 **Table 2.** Total PAHs (monthly values,  $\text{ng}/\text{m}^3$ ), TEFs (monthly values, BaP<sub>eq</sub>) and B(a)P  
589 (annual means,  $\text{ng}/\text{m}^3$ ) measured in the sites during the 2016.

590

591 **Table 3.** Correlations between PM2.5 (monthly values,  $\mu\text{g}/\text{m}^3$ ) and mutagenicity (monthly  
592 values,  $\text{rev}/\text{m}^3$ ) measured in the sites during the 2016 – Spearman’s rho (\*\*p < 0.01, \*p  
593 < 0.01, \*p < 0.05).

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1 **The North-western Italy air quality monitoring network: improving experience of PM2.5**  
2 **assessment with mutagenicity assay.**

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27 **Abstract**

28 The finest fraction of Particulate Matter (PM<sub>2.5</sub>) carries a large number of pollutants, some of  
29 which are assessed as genotoxic, such as some Polycyclic Aromatic Hydrocarbons (PAHs). In  
30 many countries, PM<sub>2.5</sub> in combination with some PAHs are monitored to assess the  
31 concentrations of pollutants, while the air quality is rarely assessed by means of biological  
32 assays. Epidemiological studies have demonstrated a significant correlation between these two  
33 pollutants and human adverse effects, in particular on the respiratory system. Nevertheless,  
34 other air pollutants can induce a biological effect and the cumulative effect of the PM<sub>2.5</sub>  
35 complex mixture may not be easily deduced by PM<sub>2.5</sub> and PAH levels.

36 This study aimed to combine the legislative monitoring of PM<sub>2.5</sub> with the study of its  
37 mutagenicity. During a full year, daily air samples were collected in nine sites of the North-  
38 western Italy air quality monitoring network (Piedmont Region) and PM<sub>2.5</sub> and PAH  
39 concentrations were assessed. Monthly pooled organic extracts were tested with the *Salmonella*  
40 assay using TA98 and TA100 strains, with and without metabolic activation ( $\pm$ S9), and using  
41 TA98NR and YG1021 strains. In all sites, a positive response was observed for TA98 and  
42 TA100 especially without S9. A significant mutagenic seasonal variation was detected, with  
43 higher mutagenicity in winter and lower responses in summer (average total mutagenicity ratio  
44 27:1). The response of TA98NR and YG1021 compared with TA98 suggested a significant  
45 contribution of nitro-compounds to the mutagenicity. No significant differences were found  
46 between urban background and rural sites denoting the spread of pollution. A mutagenicity  
47 increase, 1.28 Total Mutagenicity Factor/20 m<sup>3</sup>, was observed for each PM<sub>2.5</sub>  $\mu$ g increment.  
48 PAH levels and corresponding Toxic Equivalent Factors were highly correlated to mutagenicity  
49 results. This work confirms that complex environmental mixtures can be appropriately assessed  
50 through the implementation of physical-chemical analyzes with bioassays able to evaluate  
51 synergistic and antagonistic effects, especially for highest and lowest pollution settings.

53 **Keywords:** PM2.5, *Salmonella* assay, nitro-compounds, PAH, biomonitoring.

54 **Abbreviations:**

55 ARPA Piemonte Regional Agency for Environmental Protection of Piedmont

56 B(a)P benzo(a)pyrene

57 BaP<sub>eq</sub> benzo(a)pyrene-equivalent

58 DMSO dimethyl sulfoxide

59 IARC International Agency for Research on Cancer

60 MAMSL m above mean sea level

61 MR mutagenicity ratio

62 PAH Polycyclic Aromatic Hydrocarbon

63 PM Particulate Matter

64 TEF Toxic Equivalent Factor

65 TMF Total Mutagenicity Factor

66 WHO World Health Organization

67

## 68 **1. Introduction**

69 The International Agency for Research on Cancer (IARC) has classified outdoor air pollution  
70 as carcinogenic to humans (group 1). One of the most important air pollutants is particulate  
71 matter (PM), and the toxic and genotoxic effects of PM are mainly attributed to PM<sub>2.5</sub>  
72 (aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ), as this fraction is composed of numerous fine and ultrafine  
73 particles per unit mass with a large surface, on which mutagenic and genotoxic pollutants can  
74 be adsorbed (Balakrishnan et al., 2015; IARC, 2016; Rainho et al., 2014). PM<sub>2.5</sub> is widely  
75 studied because of its harmful effects on human health, such as lung cancer, cardiovascular  
76 diseases and respiratory disorders, as a result of the high deposition rate in respiratory organs.

77 The World Health Organization (WHO) has set guidelines for PM<sub>2.5</sub> in the atmosphere at 10  
78  $\mu\text{g}/\text{m}^3$  for the annual average concentration and at 25  $\mu\text{g}/\text{m}^3$  for the daily average (WHO, 2006);



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79 therefore, numerous countries monitor PM<sub>2.5</sub> levels to assess environmental air quality. In all  
80 Italian regions, monitoring PM<sub>2.5</sub>, as well as monitoring benzo(a)pyrene (B(a)P) and metals  
81 (As, Cd, and Ni), is carried out following current Italian and European regulations (Italian  
82 Legislative Decree 155/2010 and European Commission Directive 2008/50/EC). Moreover, to  
83 better characterize PM<sub>2.5</sub> quality, additional chemical analyses are recommended. For  
84 example, the carcinogenic potency of air samples can be estimated by Polycyclic Aromatic  
85 Hydrocarbon (PAH) levels, including the Toxic Equivalent Factor (TEF). Total TEF is assessed  
86 by multiplying the concentration of an individual PAH in the air with its TEF (Samburova et  
87 al., 2017). Most of the total TEF is determined by the finest PM fractions showing that  
88 genotoxic PAH levels are inversely proportional to the particle size (Pehnek and Jakovljević,  
89 2018). However, since PM<sub>2.5</sub> is often collected by low-volume air samplers, chemical analysis  
90 is limited because the air volumes, which can be sampled, are also small, and they may not be  
91 large enough to collect a sufficient PM<sub>2.5</sub> mass for speciation, especially for nano-pollutants.  
92 Moreover, since a wide range of carcinogenic substances (known and not well known) can be  
93 contained in PM<sub>2.5</sub> samples, it is currently impossible to measure all of them. There are both  
94 analytical limits and cost-benefit limits.

95 PM<sub>2.5</sub> and PAH levels are important parameters for assessing air quality; however, the  
96 cumulative effect of the complex PM<sub>2.5</sub> mixture may not be easily deduced by PM<sub>2.5</sub> and PAH  
97 concentrations; therefore, the application of some effect-based monitoring tools (e.g., *in vitro*  
98 assays) could have a key role in improving assessments of the biological effects of PM<sub>2.5</sub> and  
99 PAHs in the context of the different monitoring programmes (Schilirò et al., 2016). In fact,  
100 biological monitoring approaches through effect-based tools allow the detection of cumulative  
101 effects and are useful for bridging the gap between chemical contamination and biological  
102 effects (Wernersson et al., 2015). Among the different biological monitoring approaches, the  
103 use of *in vitro* assays has become increasingly important since *in vivo* methods are characterized

104 by high costs and technical complexity. Moreover, the National Academy of Sciences has noted  
105 the need to eliminate the use of animals in future studies (NRC, 2007).

106 A useful *in vitro* assay to assess mutagenicity is the *Salmonella* reverse mutation assay  
107 (Ames test), which was first used in 1977 and has been applied for 40 years (Mortelmans, 2019;  
108 Tokiwa et al., 1977). The traditional Ames test uses *Salmonella typhimurium* strains TA98 (in  
109 which frameshift mutations are detected) and TA100 (in which base substitution mutations are  
110 detected), but it cannot highlight the mutagenic activity of particular compounds; therefore,  
111 modified Ames tests have been developed to improve the assessment of PM<sub>2.5</sub> mutagenicity.  
112 Specifically, from the TA98 strains, two different strains were derived (YG1021 and TA98NR  
113 strains) that differ in their nitro-reductase expression (Josephy et al., 1997). The Ames test has  
114 become a requirement for the investigation or regulatory approval of many types of chemicals,  
115 and it has been applied to characterize the mutagenicity of air in more than 250 studies, leading  
116 to a remarkable amount of information. Although the test has been used for 46 years, it is still  
117 widely applied. For example, using experimental systems that simulate atmospheric pollution,  
118 the Ames test was recently correlated to the air quality health index that is used for atmospheric  
119 pollution (Zavala et al., 2018), and the Ames test showed the crucial role of aromatic  
120 hydrocarbon and NO<sub>x</sub> mixture photooxidation (Riedel et al., 2018).

121 Many studies have shown a positive association between PM mutagenicity measured with  
122 the Ames test and the concentrations of PAHs and their nitro, amino and/or hydroxylamine  
123 derivatives. Moreover, a positive association between PM mutagenicity and polar compounds,  
124 such as aromatic amines and aromatic ketones, has also been found (Alves et al., 2016; Claxton  
125 et al., 2004; Traversi et al., 2011). However, the ability of PM<sub>2.5</sub> and PAH levels to predict the  
126 mutagenic effect of polluted air is still controversial. Therefore, to comprehensively evaluate  
127 the harmful effects of substances associated with PM<sub>2.5</sub>, a mutagenic assay, such as the Ames  
128 test, seems to be needed and has recently been supported for risk assessments (Klapacz and  
129 Gollapudi, 2019) and regulatory decision making (Heflich et al., 2019).

130 Monitoring is particularly important for critical areas such as northwestern Italy since it is a  
131 plain surrounded by mountains where climate and topography promote severe air pollution  
132 episodes (Wallace et al., 2010; EEA, 2019; Traversi et al., 2009).

133 In this study, PM<sub>2.5</sub> was collected daily during a full year at nine different sites of the  
134 northwestern Italian air quality monitoring network (Piedmont Region), and both the PM<sub>2.5</sub>  
135 and PAH concentrations (including associated TEFs) and mutagenicity of the PM<sub>2.5</sub> organic  
136 extract were evaluated to integrate the results of legislatively required monitoring with  
137 biological monitoring.

138

## 139 **2. Materials and methods**

### 140 ***2.1 Sampling sites, collection, and extraction***

141 The PM<sub>2.5</sub> samples were collected from nine monitoring stations located in the Piedmont  
142 Region, northwest of the Italian Padana Plain. The stations are part of the air quality monitoring  
143 network run by the Regional Agency for Environmental Protection of Piedmont (ARPA  
144 Piemonte). The sampling sites included a traffic site (Settimo Torinese), five urban background  
145 sites (Torino, Novara, Alessandria, Borgosesia and Cuneo), two rural background sites  
146 (Vinchio and Domodossola), and a hill site located far from human settlements as a reference  
147 site with low air pollution levels (Dernice) (Figure 1).

148 PM<sub>2.5</sub> was sampled daily from January to December 2016 utilizing low-volume air samplers  
149 (sampling flow 2.3 m<sup>3</sup>/h). PM<sub>2.5</sub> was collected on quartz-fibre filters ( $\varnothing = 47$  mm, Sartorius,  
150 Goettingen, Germany). The amount of PM collected was determined using the gravimetric  
151 method by double weighing (complying with the EN 12341 norm). The exposed filters were  
152 stored at -20 °C until extraction. The filters collected daily were pooled together to obtain  
153 monthly samples that were subsequently extracted with acetone-hexane 1:1 using a Soxhlet  
154 apparatus for at least 80 cycles. The solvent was evaporated with a rotary evaporator, and the

155 extracts were diluted with dimethyl sulfoxide (DMSO, Carlo Erba reagents, I) to obtain a  
156 concentration of 200 m<sup>3</sup>/mL.

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## 158 **2.2 Salmonella reverse mutation assay (Ames Test)**

159 The mutagenicity assay was performed on every organic extract according to Maron and  
160 Ames (1983). The mutagenic activity of the PM<sub>2.5</sub> extracts was determined using the *S.*  
161 *typhimurium* frameshift strain TA98 and the base-substitution strain TA100, both with and  
162 without Aroclor-induced rat liver homogenate activation (S9). In addition, *S. typhimurium*  
163 TA98NR and YG1021 strains were used to evaluate the nitro compound contribution to the  
164 overall mutagenicity. YG1021 is a nitroreductase-overproducing strain obtained by cloning the  
165 nitroreductase gene of *S. typhimurium* TA1538 into the pBR322 vector and introducing the  
166 recombinant plasmid into TA98. YG1021 has a nitrofurazone reductase activity more than 50  
167 times higher than that of the original TA98 strain, permitting the efficient detection of  
168 mutagenic nitroarenes, while TA98NR lacks nitroreductase; therefore, the response obtained  
169 from these two strains permits efficient detection of mutagenic nitroarenes when compared with  
170 the reference TA98 strain.

171 Four doses of PM<sub>2.5</sub> organic extracts were tested in triplicate to obtain a dose-response curve  
172 (2.5, 5, 10, and 20 m<sup>3</sup>/plate). Positive controls were 4-nitroquinoline 1-oxide (0.5 µg/plate,  
173 TA98), methyl methanesulfonate (0.25 µg/plate, TA100), 2-aminoanthracene (2 µg/plate,  
174 TA98+S9, TA100+S9) and 2-nitrofluorene (1 µg/plate, TA98NR, YG1021). Negative controls  
175 were obtained by exposing each strain to DMSO (100 µL/plate). After 48 h of incubation, the  
176 number of revertant colonies was measured by an automatic colony counter (Synoptics Protos,  
177 UK). A microscopic observation of the background lawn density was used to evaluate the  
178 toxicity. The results are expressed as net revertants (total revertants minus spontaneous  
179 revertants)/m<sup>3</sup> and were calculated by a dose-response curve (rev/m<sup>3</sup>, i.e., the slope of the  
180 regression line) (Buschini et al., 2001; Cassoni et al., 2004). The mutagenicity ratio (MR)/20

181 m<sup>3</sup> sampled air, which is equivalent to the average breathed air by an adult in one day, was  
182 calculated by dividing the number of net revertants on the sample plates by the spontaneous  
183 revertants. To assess the overall mutagenicity of PM<sub>2.5</sub>, the Total Mutagenicity Factor (TMF)  
184 was calculated by adding the MRs of strains TA98 and TA100 with and without S9. A positive  
185 mutagenic response was defined when there was at least a twofold increase in revertants over  
186 the negative control (MR=1) and a dose-response effect over the range tested.

187

### 188 **2.3 PAH determinations**

189 The determinations of PAHs were carried out by ARPA Piemonte on monthly pooled filters  
190 sampled at the same sites used for biological analysis.

191 In addition to the determination of B(a)P, as required by Italian law (Italian Legislative  
192 Decree 155/2010), the following PAHs were also determined in this study: benzo(a)anthracene,  
193 indeno(1,2,3-cd)pyrene and benzo(b,j,k)fluoranthene. Samples were extracted with acetone-  
194 hexane under sonication in a water bath, and PAHs were quantified using GC/MS analysis  
195 according to the European standard EN 15549.

196 The TEF approach was applied to convert PAH concentrations into B(a)P-equivalent  
197 (BaP<sub>eq</sub>) toxicity (Nisbet and LaGoy, 1992; Reeves et al., 2001).

198

### 199 **2.4 Statistical analyses**

200 The autumn-winter mean for each parameter was calculated taking into account the  
201 monthly values from October to March, and the spring-summer mean was calculated  
202 considering the months from April to September. The t-test was used to compare the differences  
203 between the two periods for every site. The Spearman test was used to calculate correlations  
204 and the relationship between PM<sub>2.5</sub> levels and mutagenicity was evaluated through a linear  
205 regression model. Differences among sites were tested using ANOVA with Tukey's *post hoc*  
206 test (normally distributed data) and the Kruskal-Wallis test (not normally distributed data).

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207 Differences were considered significant when  $p < 0.05$ . Statistical analyses were performed  
208 using the IBM SPSS Statistics package, version 24.0.

209

### 210 3. Results and Discussion

#### 211 3.1 Gravimetric analysis

212 Airborne PM<sub>2.5</sub> concentrations showed a similar seasonal trend at almost every site  
213 considered in the study, with higher levels in the autumn-winter season than in the spring-  
214 summer season (t-test  $p < 0.05$ ). No significant differences were found between seasons only  
215 at the reference site Dernice (Table 1); moreover, at this site, the annual mean PM<sub>2.5</sub> levels  
216 were lower than those at the other sites (ANOVA with Tukey's *post hoc*  $p < 0.05$ ).

217 The data showed that the annual mean PM<sub>2.5</sub> was always below the Italian limit level of  
218 25  $\mu\text{g}/\text{m}^3$  (Italian Legislative Decree 155/2010, European Commission Directive 2008/50/EC),  
219 except for at the traffic site Settimo Torinese. On the other hand, Dernice is a unique site where  
220 the annual mean PM<sub>2.5</sub> was below the WHO guideline level (WHO, 2006) (Table 1).  
221 Moreover, the daily PM<sub>2.5</sub> mean concentration recorded at all stations ranged between 5.7 and  
222 64.9  $\mu\text{g}/\text{m}^3$ , and for numerous days during the year, the daily PM<sub>2.5</sub> mean concentration was  
223 higher than the WHO guideline value (25  $\mu\text{g}/\text{m}^3$ ); thus, almost all the mean concentrations  
224 related to the autumn-winter season were above 25  $\mu\text{g}/\text{m}^3$  (Table 1), except for those in cities  
225 placed at more than 500 m above mean sea level (MAMSL) (Figure 1). These results  
226 demonstrated that although the different sites were characterized by different emission levels  
227 (e.g., rural sites and urban sites), especially in winter, most showed PM<sub>2.5</sub> levels above the  
228 WHO guideline value, posing a potential risk to human health. These levels were probably due  
229 to the climatic and topographic conditions of the Piedmont Region, which is located on the  
230 Padana Plain, an area surrounded by mountains and where such conditions reduce emission  
231 pollutant dispersion and promote severe air pollution episodes. These findings are in agreement  
232 with those in the literature describing measurements taken at different locations on the Padana

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233 Plain where winter meteorological conditions in addition to thermal inversion prevent the  
234 dispersion of pollutants, causing high PM levels (Bocchi et al. 2016; Bonetta et al., 2009; Feretti  
235 et al., 2019; Gilli et al., 2007). Moreover, since the sites located at high altitudes, such as  
236 Dernice, Borgosesia, Domodossola and Cuneo (altitudes ranging from 295 to 580 MAMSL),  
237 showed lower PM<sub>2.5</sub> levels than those at low altitude sites, our results demonstrated that  
238 pollutant dispersion seems to be more frequent at higher altitudes, especially on the Padana  
239 Plain.

240 PM<sub>2.5</sub> monthly mean concentrations were inversely correlated with measured  
241 temperature (Spearman rho -0.783 - -0.888;  $p < 0.01$ ), except for at the Dernice site, where PM  
242 concentrations were mostly constant over the year. The increase in PM<sub>2.5</sub> levels in the cold  
243 months, characterized by the lowest temperature, can be explained considering that in  
244 comparison to in the warm months, in the cold months, a stronger chemical interaction among  
245 the primary pollutants can occur in the atmosphere. This interaction is due to lower pollutant  
246 dispersion (due to meteorological conditions) and higher pollutant sources (due to household  
247 heating) during cold months than during warm months. Moreover, the cold temperatures in the  
248 cold months increase the binding of molecules to surfaces, so the pollutants are stably adsorbed  
249 on the PM surface and can interact with each other.

250

### 251 3.2 *PAH levels*

252 The recorded values for total PAHs (sum of the 4 compounds), TEFs, and B(a)P are  
253 summarized in Table 2. Total PAH concentrations showed the same trend as that of the PM<sub>2.5</sub>  
254 levels. In particular, PAH concentrations were high in the winter season, while from April to  
255 September, PAH values were low and uniform at every site. The decrease in PAH levels in  
256 spring and summer was probably due to the climatic conditions that facilitated the dispersion  
257 of pollutants and to the high solar radiation, which helped PAH degradation. The mean PAH

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258 levels measured in this study were similar to those reported in recent studies in Europe (Dvorská  
259 et al., 2011).

260 The most abundant compound measured at every site was benzo(b,j,k)fluoranthene  
261 (46.1% - 57.5%), followed by indeno(1,2,3-cd)pyrene (16.9% - 23.5%), B(a)P (8.6% - 21.2%)  
262 and benzo(a)anthracene (10.3% - 15.8%). According to the literature, burning of fossil fuels  
263 was probably the prominent source of atmospheric PAHs investigated:  
264 benzo(b,j,k)fluoranthene is primarily found in gasoline exhaust, indeno(1,2,3-cd)pyrene is  
265 mainly produced by the incomplete combustion of biomass, B(a)P is primarily found in  
266 gasoline, and benzo(a)anthracene is mainly found in diesel exhaust (Chang et al., 2019). In the  
267 study area, the burning of fossil fuels was attributed to not only household heating and vehicular  
268 traffic but also other pollution sources, such as combustion processes of industrial plants.

269 The traffic site of Settimo Torinese and the rural site of Domodossola exceeded the annual  
270 limit of 1 ng/m<sup>3</sup> in European and Italian laws (European Commission Directive 2004/107/EC;  
271 Italian Legislative Decree 155/2010) for B(a)P (Group 1 IARC).

### 272 273 **3.1 Salmonella reverse mutation assay (Ames test)**

274 The mutagenic effect of the PM<sub>2.5</sub> organic extracts is illustrated in Figure 2. The results  
275 showed a typical seasonal trend with the highest effect in winter and autumn and no mutagenic  
276 activity in July and August. This result can be explained considering that the air pollution levels  
277 were higher in the cold seasons than in the warm seasons. In fact, autumn and winter were  
278 characterized by the highest emissions of air pollutants (especially due to household heating)  
279 and by meteorological conditions that promoted condensation processes and limited the  
280 dispersion of air pollutants. As reported in the literature (Cassoni et al., 2004), an enhanced  
281 response of the strains without S9 was observed at every site, denoting a higher presence of  
282 substances that can act directly on DNA. In comparison to the other sites, the traffic station  
283 (Settimo Torinese) showed higher mutagenic values, particularly in January and December,



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284 while the reference site with low air pollution (Dernice) always showed low mutagenic values,  
285 even in the winter season. At the other rural and urban background stations, there were no  
286 significant differences, denoting a spread of PM<sub>2.5</sub> pollution over the whole region.

287 Figure 3A shows the results obtained in the cold period (November-January), expressed  
288 as the TMF of the 20 m<sup>3</sup> air equivalent, obtained by adding the MRs of the strains TA98 and  
289 TA100 with and without S9. Significant differences between the stations were found with the  
290 non-parametric Kruskal-Wallis H test (Dernice *vs* all the other stations, *p* <0.05); nevertheless,  
291 no significant differences were detected among the other background sites (both urban and  
292 rural). This emphasizes again that the PM pollution issue does not exclusively affect urban areas  
293 but entire regions such as the Piedmont, whereas critical areas can also be identified at a low  
294 altitude, such as Alessandria, where PM emissions were lower than those in the Torino  
295 metropolitan area.

296 No significant differences among the stations were observed when evaluating the overall  
297 mutagenicity based on the amount of PM<sub>2.5</sub> analysed (Figure 3B). A positive response was  
298 also found when analysing samples of the reference site, even if lower than that at the other  
299 sites (Figure 3).

300 To compare the results of traditional monitoring with biological monitoring, correlations  
301 between PM<sub>2.5</sub> levels and mutagenic effects were tested. The correlations between the PM<sub>2.5</sub>  
302 levels and the calculated revertants for every strain were statistically significant throughout the  
303 studied period, except for those at the reference site (Table 3). This result suggests that PM<sub>2.5</sub>  
304 level can also be a good parameter to estimate its mutagenic potential. The PM<sub>2.5</sub> levels showed  
305 a high correlation with the mutagenicity of critical sites such as Settimo Torinese, highlighting  
306 the presence of more mutagenic compounds on PM<sub>2.5</sub>. However, it is important to note that  
307 the PM<sub>2.5</sub> levels were usually not correlated with the mutagenicity at the reference site  
308 (Dernice), and the significance of the correlation decreased at sites with low PM<sub>2.5</sub> levels.  
309 Therefore, the mutagenic compound contribution to the mass seemed to decrease from

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310 urban/critical sites to the reference site (Table 3), suggesting that the mutagenicity at sites  
311 characterized by low PM<sub>2.5</sub> levels may mainly depend on the quality (type and chemical nature  
312 of the particles and of the particle-transported pollutants) and not on the quantity of the PM<sub>2.5</sub>  
313 mass. From these results, it seems that traditional monitoring is an approach that is usually  
314 suitable for the assessment of air quality; however, biological monitoring can be a  
315 complementary approach that is particularly important for the correct assessment of air quality  
316 at sites characterized by low PM<sub>2.5</sub> levels and unusual, poorly understood pollutants.

317         The relationship between airborne PM<sub>2.5</sub> concentrations and total mutagenicity was  
318 further evaluated by comparing all the data obtained at all the sites throughout the year (Figure  
319 4). A significant relationship was observed, and the samples with PM<sub>2.5</sub> concentrations below  
320 the annual limit of 10 µg/m<sup>3</sup> suggested by the WHO (WHO, 2006) showed negative or weakly  
321 positive mutagenic results, demonstrating that PM<sub>2.5</sub> levels can be a good parameter to estimate  
322 air mutagenic effects and that the WHO limit is adequate. However, the linear regression model  
323 between mass and mutagenicity disappeared at PM<sub>2.5</sub> levels below 10 µg/m<sup>3</sup>. On the other  
324 hand, biological monitoring may be a useful complementary approach. Moreover, the presence  
325 of a mutagenic effect induced by low PM<sub>2.5</sub> levels suggested that a reduction in PM<sub>2.5</sub> mass  
326 did not seem to be sufficient to reduce its toxicity (Schilirò et al., 2015).

327         PAH concentrations, as one of the other parameters generally assessed in traditional  
328 monitoring programmes, were correlated with PM<sub>2.5</sub> levels. The PAH concentrations  
329 statistically correlated with the total mutagenicity (TMF/20 m<sup>3</sup>) considering all the data  
330 obtained at all the sites throughout the year (Spearman's rho = 0.876 ÷ 0.915; p < 0.01). Taking  
331 into account only the autumn and winter seasons, the correlations were usually not significant  
332 (Table S.1, supplementary materials); for example, the correlation was not significant in Torino  
333 (Spearman's rho = 0.696; p > 0.05) and Domodossola (Spearman's rho = 0.200; p > 0.05) but  
334 highly significant at sites with extreme pollution levels, very high in Settimo Torinese  
335 (Spearman's rho = 0.945; p < 0.01) and very low in Dernice (Spearman's rho = 0.943; p < 0.01).

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336 Since only four PAHs were quantified in this study, the lack of correlation could have been due  
337 to the presence of other PAHs or pollutants with a mutagenic effect that was not quantified.  
338 This observation emphasizes that the analytical determination of a few chemical compounds is  
339 not always enough to characterize the mutagenicity of a complex environmental mixture such  
340 as PM, as has been confirmed by other recent European studies (Bocchi et al., 2017; Bonetta et  
341 al., 2019; Velali et al., 2019).

342 Finally, in this study, the nitro compound contribution to the overall mutagenicity was  
343 evaluated by comparing the results obtained with the TA98, TA98NR, and YG1021 strains.  
344 The quantification of the mutagenicity linked to the amplified nitroreductase activity was  
345 calculated as  $[\text{YG1021 net revertants/m}^3/\text{TA98 net revertants/m}^3]$ , while the residual  
346 mutagenicity linked to the nitroreductase deficiency was quantified as follows:  $\{1 - [(\text{TA98 net}$   
347  $\text{revertants/m}^3 - \text{TA98 NR net revertants/m}^3)/\text{TA98 net revertants /m}^3]\}$  (Traversi et al., 2009).  
348 At every site, the YG1021 strain showed the highest response, followed by TA98 and TA98NR.  
349 Table S.2 (supplementary materials) summarizes the amplified and residual mutagenicity  
350 during the winter period. The values for the amplified mutagenicity ranged from 1.41 for  
351 Borgosesia to 2.32 for Dernice, denoting a high contribution of the nitro compounds to the total  
352 mutagenicity measured in every site (Rainho et al., 2014; Traversi et al., 2011). The amplified  
353 mutagenicity due to the nitro-compound contribution did not explain the higher mutagenicity  
354 observed in the critical area than in the other areas; moreover, the residual mutagenicity seemed  
355 to be constant (from 0.46 for Domodossola to 0.64 for Vinchio) (Table S.2, supplementary  
356 materials). Nonetheless, at some sites (Torino, Domodossola and Dernice), the nitro compound  
357 contribution to mutagenicity was remarkable regardless of the PM<sub>2.5</sub> or PAH concentrations.  
358 These results further confirmed that it is useful, where possible, to monitor air quality using  
359 biological monitoring. Moreover, this result suggests that it is convenient to test samples with  
360 different *S. typhimurium* strains to investigate different biological effects that are induced by  
361 different chemicals (Landkocz, et al., 2017; Lemos et al., 2012).

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#### 363 4. Conclusion

364 This study confirmed that PM<sub>2.5</sub> pollution is a critical issue to address to protect human  
365 health, especially in northwestern Italy, an area characterized by climatic and topographic  
366 conditions that prevent pollutant dispersion and promote severe air pollution episodes. In this  
367 area, PM<sub>2.5</sub> levels exceed the WHO guidelines except for some background sites, which are  
368 generally located at a higher altitude, suggesting that the air pollution phenomenon is attenuated  
369 by altitude.

370 Our results suggest that PM<sub>2.5</sub> concentration is a good parameter to estimate air quality  
371 because of the correlations between PM<sub>2.5</sub> levels and mutagenic effects; however, since this  
372 correlation was generally not significant at the site with the lowest pollution levels, the results  
373 also suggested that biological monitoring can be a complementary approach for the correct  
374 assessment of air quality at sites characterized by low PM levels or unusual pollutant sources.  
375 Moreover, considering that the correlations between PAH levels and mutagenic effects were  
376 not always significant, our results demonstrate that the analytical determination of a few  
377 chemical compounds is not enough to characterize the mutagenicity of a complex  
378 environmental mixture.

379 Finally, this study shows that it is useful to test air samples with different *S. typhimurium*  
380 strains to consider different biological effects induced by different pollutants; in particular, for  
381 the assessment of the potential risk associated with PM<sub>2.5</sub>, it seems important to also evaluate  
382 the mutagenic effect due to nitro compounds, highlighting the necessity of a strength assessment  
383 for reactive nitrogen molecules in the air.

384 Our study confirms the usefulness of combining biological tests with regulatory  
385 assessments, especially for air quality monitoring networks; moreover, our study suggests that  
386 the use of *in vitro* models to assess biological effects induced by environmental matrices might  
387 be a valid tool for supporting environmental risk assessment and public health policies.

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388 The application of a combination of tests is scientifically desirable; however, taking into  
389 account a large number of assays and the associated costs (in terms of both technical resources  
390 and time), the obtained results seem to indicate the *Salmonella* assay is a good, cost-efficient  
391 evaluation tool for PM2.5 mutagenicity assessment. The use of simplified models such as  
392 bacteria instead of human cells and the *in vitro* application of high levels of the organic extract  
393 are often critical factors for the extrapolation of data for risk assessment purposes. Human  
394 exposure and genotoxic effect induction are not described by the *Salmonella* assay alone. On  
395 the other hand, this assay represents a simulation of the biological action induced by a complex  
396 mixture of pollutants such as PM2.5, so it has been considered for use in preventing effects due  
397 to chronic exposure and a long latency period.

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556 **Figure captions:**

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558 **Figure 1.** Sampling sites in the study area. Cartographic coordinate system ED1950 (UTM)

559 Zone 32. MAMSL= m above mean sea level.

560

561 **Figure 2.** Mutagenicity expressed as number of net revertants/m<sup>3</sup> of air sampled – Strains TA98

562 and TA100 with (+S9) and without metabolic activation.

563

564 **Figure 3.** Total mutagenicity measured in the sites during autumn-winter months expressed as

565 A: TMF/20 m<sup>3</sup> of air sampled, B: TMF/mg of PM2.5. Boxes represent the 25-75<sup>th</sup> percentile;

566 outer lines represent the 10-90<sup>th</sup> percentile.

567

568 **Figure 4.** Linear regression between PM2.5 (monthly values, µg/m<sup>3</sup>) and mutagenicity

569 (monthly values, TMF/20 m<sup>3</sup>), all data (108 pairing). The green area corresponds to mass

570 concentration levels ranging between the WHO guideline value (WHO, 2006) and the limit

571 value of the Italian law (Italian Legislative Decree 155/2010). The regression shows R = 0.837

572 and is statistically significant (Anova p=0.000).

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582 **Table captions:**

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584 **Table 1.** Annual means of PM2.5 ( $\mu\text{g}/\text{m}^3$ ) in the sites and comparison between means of PM2.5  
585 ( $\mu\text{g}/\text{m}^3$ ) measured during autumn-winter months and means of PM2.5 ( $\mu\text{g}/\text{m}^3$ ) measured  
586 during spring-summer months in the sites– t-test.

587

588 **Table 2.** Total PAHs (monthly values,  $\text{ng}/\text{m}^3$ ), TEFs (monthly values, BaP<sub>eq</sub>) and B(a)P  
589 (annual means,  $\text{ng}/\text{m}^3$ ) measured in the sites during the 2016.

590

591 **Table 3.** Correlations between PM2.5 (monthly values,  $\mu\text{g}/\text{m}^3$ ) and mutagenicity (monthly  
592 values,  $\text{rev}/\text{m}^3$ ) measured in the sites during the 2016 – Spearman’s rho (\*\*p < 0.01, \*p  
593 < 0.01, \*p < 0.05).

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**Table 1**

<i>Sampling Site</i>	<b>Annual (<math>\mu\text{g}/\text{m}^3</math>)</b>				<b>Autumn-Winter (<math>\mu\text{g}/\text{m}^3</math>)</b>		<b>Spring-Summer (<math>\mu\text{g}/\text{m}^3</math>)</b>		<i>T-test</i>
	<i>Min</i>	<i>Max</i>	<i>Mean</i>	<i>St. Dev.</i>	<i>Mean</i>	<i>St. Dev.</i>	<i>Mean</i>	<i>St. Dev.</i>	
Settimo Torinese	8.9	64.9	26.0	18.7	40.1	16.6	11.8	2.6	p < 0.01
Torino	9.9	55.2	22.8	14.5	33.5	13.6	12.0	1.9	p < 0.01
Novara	7.1	37.3	18.6	11.1	27.7	8.2	9.5	2.7	p < 0.01
Alessandria	9.2	47.7	21.2	13.2	30.2	13.5	12.1	2.3	p < 0.05
Borgosesia	6.7	32.7	14.5	8.9	20.8	8.8	8.2	1.2	p < 0.05
Cuneo	9.2	28.9	16.2	7.1	21.9	5.5	10.5	1.5	p < 0.01
Vinchio	8.5	36.9	19.2	10.3	26.6	9.9	11.7	2.1	p < 0.05
Domodossola	4.5	43.3	15.3	12.4	23.6	12.9	6.9	2.2	p < 0.05
Dernice	5.7	12.2	9.2	1.8	9.5	1.9	8.9	1.8	p > 0.05

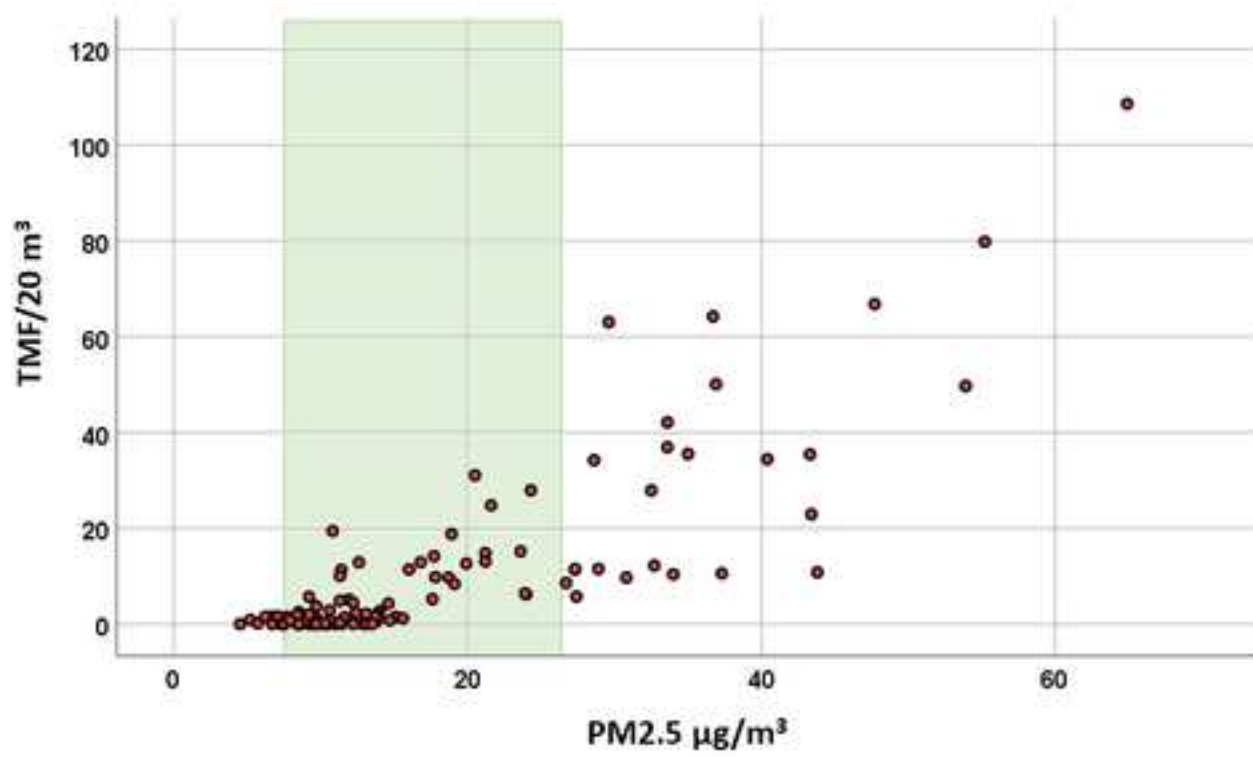
Table 2

<b>ΣPAHs (ng/m<sup>3</sup>)-TEF (BaPeq)</b>									
<b>Month</b>	<b>SETTIMO TORINESE</b>	<b>TORINO</b>	<b>NOVARA</b>	<b>ALESSANDRIA</b>	<b>BORGOSIESIA</b>	<b>CUNEO</b>	<b>VINCHIO</b>	<b>DOMODOSSOLA</b>	<b>DERNICE</b>
Jan	17.97-4.88	10.55-2.69	7.49-1.98	11.83-3.06	15.2-4.17	4.20-1.08	7.25-1.78	25.2-7.20	1.60-0.31
Feb	8.97-2.28	6.07-1.49	4.14-1.06	4.17-1.07	7.51-1.98	3.95-0.96	4.76-1.21	10.91-3.16	0.72-0.15
Mar	3.46-0.72	2.13-0.43	1.26-0.28	1.97-0.52	4.41-1.08	1.19-0.27	1.78-0.42	4.39-1.09	0.22-0.02
Apr	1.09-0.27	0.69-0.15	0.67-0.15	0.69-0.15	1.34-0.28	0.60-0.14	0.90-0.15	2.07-0.43	0.41-0.03
May	0.51-0.13	0.44-0.04	0.12-0.01	0.13-0.01	0.54-0.14	0.24-0.02	0.10-0	0.75-0.15	0.12-0.01
Jun	0.26-0.02	0.18-0.01	0.16-0.01	0.16-0.01	0.18-0.01	0.20-0.02	0.18-0.01	0.20-0.02	0.14-0.01
Jul	0.12-0.01	0.13-0.01	0.25-0.02	0.24-0.02	0.29-0.03	0.15-0.01	0.30-0.03	0.25-0.03	0.23-0.02
Aug	0.15-0.01	0.15	0.13-0.01	0.12-0.01	0.16-0.01	0.12-0.01	0.14-0.01	0.13-0.01	0.12-0.01
Sep	0.31-0.03	0.26-0.02	0.24-0.02	0.17-0.01	0.36-0.03	0.19-0.02	0.18-0.01	0.23-0.02	0.12-0.01
Oct	5.36-1.35	2.27-0.54	1.59-0.31	1.10-0.27	2.53-0.20	1.03-0.27	1.31-0.28	6.24-1.60	0.29-0.02
Nov	7.82-2.19	5.34-1.43	4.42-1.07	2.66-0.76	7.88-2.18	2.54-0.66	4.00-0.95	13.3-3.64	0.80-0.15
Dec	21.98-6.09	18.5-4.79	8.58-2.16	9.32-2.40	14.74-4.23	3.67-0.93	6.53-1.62	26.05-7.36	1.06-0.17
<b>annual mean b(a)p (ng/m<sup>3</sup>)</b>	<b>1.13</b>	<b>0.72</b>	<b>0.43</b>	<b>0.52</b>	<b>0.90</b>	<b>0.27</b>	<b>0.39</b>	<b>1.58</b>	<b>0.04</b>

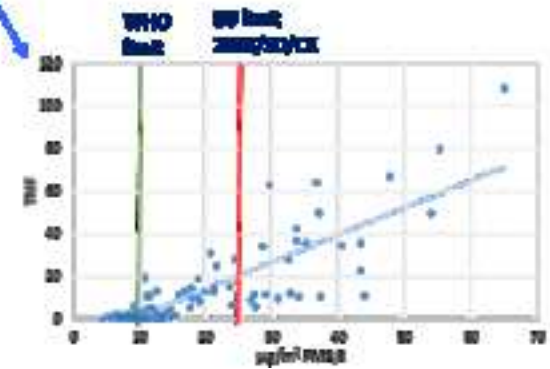
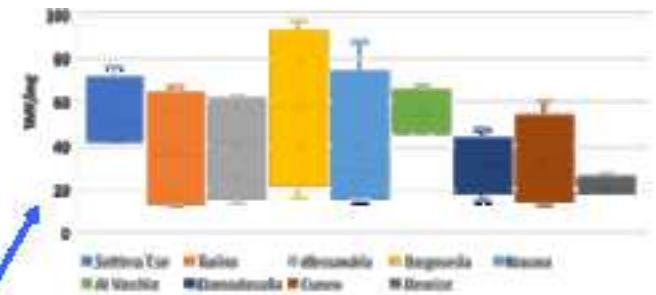
Table 3

Spearman's rho	PM2.5 ( $\mu\text{g}/\text{m}^3$ )								
	Settimo Torinese	Torino	Novara	Alessandria	Borgosesia	Cuneo	Vinchio	Domodossola	Dernice
<b>TA98 (net rev/m<sup>3</sup>)</b>	0.977***	0.862***	0.761**	0.916***	0.794**	0.726**	0.951***	0.636*	0.618*
<b>TA98+S9 (net rev/m<sup>3</sup>)</b>	0.920***	0.835***	0.819**	0.881***	0.709**	0.715**	0.917***	0.871***	0.360
<b>TA100 (net rev/m<sup>3</sup>)</b>	0.972***	0.818***	0.709**	0.885***	0.732**	0.592*	0.915***	0.832***	0.526
<b>TA100+S9 (net rev/m<sup>3</sup>)</b>	0.989***	0.851***	0.772**	0.929***	0.865***	0.794**	0.937***	0.857***	0.464





**PM2.5 in 9 sites, sampled every day for a full year.**





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**The North-western Italy air quality monitoring network: improving experience of PM<sub>2.5</sub> assessment with mutagenicity assay.**

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.