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

Environmental Research

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--Manuscript Draft--

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Corresponding Author:	Tiziana Schiliro' University of Torino Torino, ITALY
First Author:	Daniele MARANGON, Dr
Order of Authors:	Daniele MARANGON, Dr
	Deborah TRAVERSI, Prof
	Anna Maria D'AGOSTINO, Dr
	Marta GEA, Dr
	Marco FONTANA, Dr
	Tiziana Schiliro'
Abstract:	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 (±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 m3, was observed for each PM2.5 µg 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.
Response to Reviewers:	Answer to Reviewer 1 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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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.

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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 is very long and lacks a strong focus; it should either emphasize the importance of combining functional assays like the Ames test with exposure measurements, or the general toxicity of air pollution in Northern Italy.
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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. As suggested by the reviewer, we improved the discussion. Lines: 266 – 268. 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 BaPeq 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 BaPeq 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 28th, 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 (±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^3, was observed for each PM2.5 µg 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 19th, 2020

Dear Editor,

please find enclosed the revised 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.

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.

Answer to Reviewer 1 **Submission ER-20-4274**

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

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Editing Certificate

This document certifies that the manuscript

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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.

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

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

[a] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

[b] Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

***Corresponding author**:
Tiziana Schilirò
tiziana.schiliro@unito.it
Department of Public Health and Pediatrics,
University of Torino,
Piazza Polonia 94, 10126 Torino, Italy.
Phone: +39 011 6705822

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 µg 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 µg/m^3.
5. Risk assessment and health policies should be supported by mutagenicity assays.

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

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*__Corresponding author__:
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tiziana.schiliro@unito.it
Department of Public Health and Pediatrics,
University of Torino,
Piazza Polonia 94, 10126 Torino, Italy.
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Abstract

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 (±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^3, was observed for each PM2.5 µg 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.

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

Abbreviations:

ARPA Piemonte Regional Agency for Environmental Protection of Piedmont

B(a)P benzo(a)pyrene

BaPeq benzo(a)pyrene-equivalent

DMSO dimethyl sulfoxide

IARC International Agency for Research on Cancer

MAMSL m above mean sea level

MR mutagenicity ratio

PAH Polycyclic Aromatic Hydrocarbon

PM Particulate Matter

TEF Toxic Equivalent Factor

TMF Total Mutagenicity Factor

WHO World Health Organization

1. Introduction

The International Agency for Research on Cancer (IARC) has classified outdoor air pollution as carcinogenic to humans (group 1). One of the most important air pollutants is particulate matter (PM), and the toxic and genotoxic effects of PM are mainly attributed to PM2.5 (aerodynamic diameter ≤2.5 μm), as this fraction is composed of numerous fine and ultrafine particles per unit mass with a large surface, on which mutagenic and genotoxic pollutants can be adsorbed (Balakrishnan et al., 2015; IARC, 2016; Rainho et al., 2014). PM2.5 is widely studied because of its harmful effects on human health, such as lung cancer, cardiovascular diseases and respiratory disorders, as a result of the high deposition rate in respiratory organs. The World Health Organization (WHO) has set guidelines for PM2.5 in the atmosphere at 10 $\mu g/m^3$ for the annual average concentration and at 25 $\mu g/m^3$ for the daily average (WHO, 2006);

therefore, numerous countries monitor PM2.5 levels to assess environmental air quality. In all Italian regions, monitoring PM2.5, as well as monitoring benzo(a)pyrene (B(a)P) and metals (As, Cd, and Ni), is carried out following current Italian and European regulations (Italian Legislative Decree 155/2010 and European Commission Directive 2008/50/EC). Moreover, to better characterize PM2.5 quality, additional chemical analyses are recommended. For example, the carcinogenic potency of air samples can be estimated by Polycyclic Aromatic Hydrocarbon (PAH) levels, including the Toxic Equivalent Factor (TEF). Total TEF is assessed by multiplying the concentration of an individual PAH in the air with its TEF (Samburova et al., 2017). Most of the total TEF is determined by the finest PM fractions showing that genotoxic PAH levels are inversely proportional to the particle size (Pehnec and Jakovljević, 2018). However, since PM2.5 is often collected by low-volume air samplers, chemical analysis is limited because the air volumes, which can be sampled, are also small, and they may not be large enough to collect a sufficient PM2.5 mass for speciation, especially for nano-pollutants. Moreover, since a wide range of carcinogenic substances (known and not well known) can be contained in PM2.5 samples, it is currently impossible to measure all of them. There are both analytical limits and cost-benefit limits.

PM2.5 and PAH levels are important parameters for assessing air quality; however, the cumulative effect of the complex PM2.5 mixture may not be easily deduced by PM2.5 and PAH concentrations; therefore, the application of some effect-based monitoring tools (e.g., *in vitro* assays) could have a key role in improving assessments of the biological effects of PM2.5 and PAHs in the context of the different monitoring programmes (Schilirò et al., 2016). In fact, biological monitoring approaches through effect-based tools allow the detection of cumulative effects and are useful for bridging the gap between chemical contamination and biological effects (Wernersson et al., 2015). Among the different biological monitoring approaches, the use of *in vitro* assays has become increasingly important since *in vivo* methods are characterized

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

A useful *in vitro* assay to assess mutagenicity is the *Salmonella* reverse mutation assay (Ames test), which was first used in 1977 and has been applied for 40 years (Mortelmans, 2019; Tokiwa et al., 1977). The traditional Ames test uses *Salmonella typhimurium* strains TA98 (in which frameshift mutations are detected) and TA100 (in which base substitution mutations are detected), but it cannot highlight the mutagenic activity of particular compounds; therefore, modified Ames tests have been developed to improve the assessment of PM2.5 mutagenicity. Specifically, from the TA98 strains, two different strains were derived (YG1021 and TA98NR strains) that differ in their nitro-reductase expression (Josephy et al., 1997). The Ames test has become a requirement for the investigation or regulatory approval of many types of chemicals, and it has been applied to characterize the mutagenicity of air in more than 250 studies, leading to a remarkable amount of information. Although the test has been used for 46 years, it is still widely applied. For example, using experimental systems that simulate atmospheric pollution, the Ames test was recently correlated to the air quality health index that is used for atmospheric pollution (Zavala et al., 2018), and the Ames test showed the crucial role of aromatic hydrocarbon and NOx mixture photooxidation (Riedel et al., 2018).

Many studies have shown a positive association between PM mutagenicity measured with the Ames test and the concentrations of PAHs and their nitro, amino and/or hydroxylamine derivatives. Moreover, a positive association between PM mutagenicity and polar compounds, such as aromatic amines and aromatic ketones, has also been found (Alves et al., 2016; Claxton et al., 2004; Traversi et al., 2011). However, the ability of PM2.5 and PAH levels to predict the mutagenic effect of polluted air is still controversial. Therefore, to comprehensively evaluate the harmful effects of substances associated with PM2.5, a mutagenic assay, such as the Ames test, seems to be needed and has recently been supported for risk assessments (Klapacz and Gollapudi, 2019) and regulatory decision making (Heflich et al., 2019).

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

In this study, PM2.5 was collected daily during a full year at nine different sites of the northwestern Italian air quality monitoring network (Piedmont Region), and both the PM2.5 and PAH concentrations (including associated TEFs) and mutagenicity of the PM2.5 organic extract were evaluated to integrate the results of legislatively required monitoring with biological monitoring.

2. Materials and methods

2.1 Sampling sites, collection, and extraction

The PM2.5 samples were collected from nine monitoring stations located in the Piedmont Region, northwest of the Italian Padana Plain. The stations are part of the air quality monitoring network run by the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte). The sampling sites included a traffic site (Settimo Torinese), five urban background sites (Torino, Novara, Alessandria, Borgosesia and Cuneo), two rural background sites (Vinchio and Domodossola), and a hill site located far from human settlements as a reference site with low air pollution levels (Dernice) (Figure 1).

PM2.5 was sampled daily from January to December 2016 utilizing low-volume air samplers (sampling flow 2.3 m^3/h). PM2.5 was collected on quartz-fibre filters (Ø = 47 mm, Sartorius, Goettingen, Germany). The amount of PM collected was determined using the gravimetric method by double weighing (complying with the EN 12341 norm). The exposed filters were stored at -20 °C until extraction. The filters collected daily were pooled together to obtain monthly samples that were subsequently extracted with acetone-hexane 1:1 using a Soxhlet apparatus for at least 80 cycles. The solvent was evaporated with a rotary evaporator, and the

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

2.2 Salmonella *reverse mutation assay (Ames Test)*

The mutagenicity assay was performed on every organic extract according to Maron and Ames (1983). The mutagenic activity of the PM2.5 extracts was determined using the *S. typhimurium* frameshift strain TA98 and the base-substitution strain TA100, both with and without Aroclor-induced rat liver homogenate activation (S9). In addition, *S. typhimurium* TA98NR and YG1021 strains were used to evaluate the nitro compound contribution to the overall mutagenicity. YG1021 is a nitroreductase-overproducing strain obtained by cloning the nitroreductase gene of *S. typhimurium* TA1538 into the pBR322 vector and introducing the recombinant plasmid into TA98. YG1021 has a nitrofurazone reductase activity more than 50 times higher than that of the original TA98 strain, permitting the efficient detection of mutagenic nitroarenes, while TA98NR lacks nitroreductase; therefore, the response obtained from these two strains permits efficient detection of mutagenic nitroarenes when compared with the reference TA98 strain.

Four doses of PM2.5 organic extracts were tested in triplicate to obtain a dose-response curve (2.5, 5, 10, and 20 m^3/plate). Positive controls were 4-nitroquinoline 1-oxide (0.5 µg/plate, TA98), methyl methanesulfonate (0.25 µg/plate, TA100), 2-aminoanthracene (2 µg/plate, TA98+S9, TA100+S9) and 2-nitrofluorene (1 µg/plate, TA98NR, YG1021). Negative controls were obtained by exposing each strain to DMSO (100 µL/plate). After 48 h of incubation, the number of revertant colonies was measured by an automatic colony counter (Synoptics Protos, UK). A microscopic observation of the background lawn density was used to evaluate the toxicity. The results are expressed as net revertants (total revertants minus spontaneous revertants)/m^3 and were calculated by a dose-response curve (rev/m^3, i.e., the slope of the regression line) (Buschini et al., 2001; Cassoni et al., 2004). The mutagenicity ratio (MR)/20

m^3 sampled air, which is equivalent to the average breathed air by an adult in one day, was calculated by dividing the number of net revertants on the sample plates by the spontaneous revertants. To assess the overall mutagenicity of PM2.5, the Total Mutagenicity Factor (TMF) was calculated by adding the MRs of strains TA98 and TA100 with and without S9. A positive mutagenic response was defined when there was at least a twofold increase in revertants over the negative control (MR=1) and a dose-response effect over the range tested.

2.3 PAH determinations

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

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

The TEF approach was applied to convert PAH concentrations into B(a)P-equivalent (BaPeq) toxicity (Nisbet and LaGoy, 1992; Reeves et al., 2001).

2.4 Statistical analyses

The autumn-winter mean for each parameter was calculated taking into account the monthly values from October to March, and the spring-summer mean was calculated considering the months from April to September. The t-test was used to compare the differences between the two periods for every site. The Spearman test was used to calculate correlations and the relationship between PM2.5 levels and mutagenicity was evaluated though a linear regression model. Differences among sites were tested using ANOVA with Tukey's *post hoc* test (normally distributed data) and the Kruskal-Wallis test (not normally distributed data).

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

3. Results and Discussion

3.1 Gravimetric analysis

Airborne PM2.5 concentrations showed a similar seasonal trend at almost every site considered in the study, with higher levels in the autumn-winter season than in the spring-summer season (t-test $p < 0.05$). No significant differences were found between seasons only at the reference site Dernice (Table 1); moreover, at this site, the annual mean PM2.5 levels were lower than those at the other sites (ANOVA with Tukey's *post hoc* $p < 0.05$).

The data showed that the annual mean PM2.5 was always below the Italian limit level of 25 $\mu g/m^3$ (Italian Legislative Decree 155/2010, European Commission Directive 2008/50/EC), except for at the traffic site Settimo Torinese. On the other hand, Dernice is a unique site where the annual mean PM2.5 was below the WHO guideline level (WHO, 2006) (Table 1). Moreover, the daily PM2.5 mean concentration recorded at all stations ranged between 5.7 and 64.9 $\mu g/m^3$, and for numerous days during the year, the daily PM2.5 mean concentration was higher than the WHO guideline value (25 $\mu g/m^3$); thus, almost all the mean concentrations related to the autumn-winter season were above 25 $\mu g/m^3$ (Table 1), except for those in cities placed at more than 500 m above mean sea level (MAMSL) (Figure 1). These results demonstrated that although the different sites were characterized by different emission levels (e.g., rural sites and urban sites), especially in winter, most showed PM2.5 levels above the WHO guideline value, posing a potential risk to human health. These levels were probably due to the climatic and topographic conditions of the Piedmont Region, which is located on the Padana Plain, an area surrounded by mountains and where such conditions reduce emission pollutant dispersion and promote severe air pollution episodes. These findings are in agreement with those in the literature describing measurements taken at different locations on the Padana

Plain where winter meteorological conditions in addition to thermal inversion prevent the dispersion of pollutants, causing high PM levels (Bocchi et al. 2016; Bonetta et al., 2009; Feretti et al., 2019; Gilli et al., 2007). Moreover, since the sites located at high altitudes, such as Dernice, Borgosesia, Domodossola and Cuneo (altitudes ranging from 295 to 580 MAMSL), showed lower PM2.5 levels than those at low altitude sites, our results demonstrated that pollutant dispersion seems to be more frequent at higher altitudes, especially on the Padana Plain.

PM2.5 monthly mean concentrations were inversely correlated with measured temperature (Spearman rho -0.783 - -0.888; $p < 0.01$), except for at the Dernice site, where PM concentrations were mostly constant over the year. The increase in PM2.5 levels in the cold months, characterized by the lowest temperature, can be explained considering that in comparison to in the warm months, in the cold months, a stronger chemical interaction among the primary pollutants can occur in the atmosphere. This interaction is due to lower pollutant dispersion (due to meteorological conditions) and higher pollutant sources (due to household heating) during cold months than during warm months. Moreover, the cold temperatures in the cold months increase the binding of molecules to surfaces, so the pollutants are stably adsorbed on the PM surface and can interact with each other.

3.2 ***PAH levels***

The recorded values for total PAHs (sum of the 4 compounds), TEFs, and B(a)P are summarized in Table 2. Total PAH concentrations showed the same trend as that of the PM2.5 levels. In particular, PAH concentrations were high in the winter season, while from April to September, PAH values were low and uniform at every site. The decrease in PAH levels in spring and summer was probably due to the climatic conditions that facilitated the dispersion of pollutants and to the high solar radiation, which helped PAH degradation. The mean PAH

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

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

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

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

The mutagenic effect of the PM2.5 organic extracts is illustrated in Figure 2. 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. As reported in the literature (Cassoni et al., 2004), an enhanced response of the strains without S9 was observed at every site, denoting a higher presence of substances that can act directly on DNA. In comparison to the other sites, the traffic station (Settimo Torinese) showed higher mutagenic values, particularly in January and December,

while the reference site with low air pollution (Dernice) always showed low mutagenic values, even in the winter season. At the other rural and urban background stations, there were no significant differences, denoting a spread of PM2.5 pollution over the whole region.

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

No significant differences among the stations were observed when evaluating the overall mutagenicity based on the amount of PM2.5 analysed (Figure 3B). A positive response was also found when analysing samples of the reference site, even if lower than that at the other sites (Figure 3).

To compare the results of traditional monitoring with biological monitoring, correlations between PM2.5 levels and mutagenic effects were tested. The correlations between the PM2.5 levels and the calculated revertants for every strain were statistically significant throughout the studied period, except for those at the reference site (Table 3). This result suggests that PM2.5 level can also be a good parameter to estimate its mutagenic potential. The PM2.5 levels showed a high correlation with the mutagenicity of critical sites such as Settimo Torinese, highlighting the presence of more mutagenic compounds on PM2.5. However, it is important to note that the PM2.5 levels were usually not correlated with the mutagenicity at the reference site (Dernice), and the significance of the correlation decreased at sites with low PM2.5 levels. Therefore, the mutagenic compound contribution to the mass seemed to decrease from

urban/critical sites to the reference site (Table 3), suggesting that the mutagenicity at sites characterized by low PM2.5 levels may mainly depend on the quality (type and chemical nature of the particles and of the particle-transported pollutants) and not on the quantity of the PM2.5 mass. From these results, it seems that traditional monitoring is an approach that is usually suitable for the assessment of air quality; however, biological monitoring can be a complementary approach that is particularly important for the correct assessment of air quality at sites characterized by low PM2.5 levels and unusual, poorly understood pollutants.

The relationship between airborne PM2.5 concentrations and total mutagenicity was further evaluated by comparing all the data obtained at all the sites throughout the year (Figure 4). A significant relationship was observed, and the samples with PM2.5 concentrations below the annual limit of 10 µg/m^3 suggested by the WHO (WHO, 2006) showed negative or weakly positive mutagenic results, demonstrating that PM2.5 levels can be a good parameter to estimate air mutagenic effects and that the WHO limit is adequate. However, the linear regression model between mass and mutagenicity disappeared at PM2.5 levels below 10 µg/m^3. On the other hand, biological monitoring may be a useful complementary approach. Moreover, the presence of a mutagenic effect induced by low PM2.5 levels suggested that a reduction in PM2.5 mass did not seem to be sufficient to reduce its toxicity (Schilirò et al., 2015).

PAH concentrations, as one of the other parameters generally assessed in traditional monitoring programmes, were correlated with PM2.5 levels. The PAH concentrations statistically correlated with the total mutagenicity (TMF/20 m^3) considering all the data obtained at all the sites throughout the year (Spearman's rho = 0.876 ÷ 0.915; $p < 0.01$). Taking into account only the autumn and winter seasons, the correlations were usually not significant (Table S.1, supplementary materials); for example, the correlation was not significant in Torino (Spearman's rho = 0.696; $p > 0.05$) and Domodossola (Spearman's rho = 0.200; $p > 0.05$) but highly significant at sites with extreme pollution levels, very high in Settimo Torinese (Spearman's rho = 0.945; $p < 0.01$) and very low in Dernice (Spearman's rho = 0.943; $p < 0.01$).

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

Finally, in this study, the nitro compound contribution to the overall mutagenicity was evaluated by comparing the results obtained with the TA98, TA98NR, and YG1021 strains. The quantification of the mutagenicity linked to the amplified nitroreductase activity was calculated as [YG1021 net revertants/m^3/TA98 net revertants/m^3], while the residual mutagenicity linked to the nitroreductase deficiency was quantified as follows: {1-[(TA98 net revertants/m^3-TA98 NR net revertants/m3)/TA98 net revertants /m^3]} (Traversi et al., 2009). At every site, the YG1021 strain showed the highest response, followed by TA98 and TA98NR. Table S.2 (supplementary materials) summarizes the amplified and residual mutagenicity during the winter period. The values for the amplified mutagenicity ranged from 1.41 for Borgosesia to 2.32 for Dernice, denoting a high contribution of the nitro compounds to the total mutagenicity measured in every site (Rainho et al., 2014; Traversi et al., 2011). The amplified mutagenicity due to the nitro-compound contribution did not explain the higher mutagenicity observed in the critical area than in the other areas; moreover, the residual mutagenicity seemed to be constant (from 0.46 for Domodossola to 0.64 for Vinchio) (Table S.2, supplementary materials). Nonetheless, at some sites (Torino, Domodossola and Dernice), the nitro compound contribution to mutagenicity was remarkable regardless of the PM2.5 or PAH concentrations. These results further confirmed that it is useful, where possible, to monitor air quality using biological monitoring. Moreover, this result suggests that it is convenient to test samples with different *S. typhimurium* strains to investigate different biological effects that are induced by different chemicals (Landkocz, et al., 2017; Lemos et al., 2012).

4. Conclusion

This study confirmed that PM2.5 pollution is a critical issue to address to protect human health, especially in northwestern Italy, an area characterized by climatic and topographic conditions that prevent pollutant dispersion and promote severe air pollution episodes. In this area, PM2.5 levels exceed the WHO guidelines except for some background sites, which are generally located at a higher altitude, suggesting that the air pollution phenomenon is attenuated by altitude.

Our results suggest that PM2.5 concentration is a good parameter to estimate air quality because of the correlations between PM2.5 levels and mutagenic effects; however, since this correlation was generally not significant at the site with the lowest pollution levels, the results also suggested that biological monitoring can be a complementary approach for the correct assessment of air quality at sites characterized by low PM levels or unusual pollutant sources. Moreover, considering that the correlations between PAH levels and mutagenic effects were not always significant, our results demonstrate that the analytical determination of a few chemical compounds is not enough to characterize the mutagenicity of a complex environmental mixture.

Finally, this study shows that it is useful to test air samples with different *S. typhimurium* strains to consider different biological effects induced by different pollutants; in particular, for the assessment of the potential risk associated with PM2.5, it seems important to also evaluate the mutagenic effect due to nitro compounds, highlighting the necessity of a strength assessment for reactive nitrogen molecules in the air.

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

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

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Figure captions:

Figure 1. Sampling sites in the study area. Cartographic coordinate system ED1950 (UTM) Zone 32. MAMSL= m above mean sea level.

Figure 2. Mutagenicity expressed as number of net revertants/m^3 of air sampled – Strains TA98 and TA100 with (+S9) and without metabolic activation.

Figure 3. Total mutagenicity measured in the sites during autumn-winter months expressed as A: TMF/20 m^3 of air sampled, B: TMF/mg of PM2.5. Boxes represent the 25-75th percentile; outer lines represent the 10-90th percentile.

Figure 4. Linear regression between PM2.5 (monthly values, µg/m^3) and mutagenicity (monthly values, TMF/20 m^3), all data (108 pairing). The green area corresponds to mass concentration levels ranging between the WHO guideline value (WHO, 2006) and the limit value of the Italian law (Italian Legislative Decree 155/2010)**.** The regression shows R = 0.837 and is statistically significant (Anova p=0.000).

Table captions:

Table 1. Annual means of PM2.5 (µg/m^3) in the sites and comparison between means of PM2.5 (µg/m^3) measured during autumn-winter months and means of PM2.5 (µg/m^3) measured during spring-summer months in the sites– t-test.

Table 2. Total PAHs (monthly values, ng/m^3), TEFs (monthly values, BaPeq) and B(a)P (annual means, ng/m^3) measured in the sites during the 2016.

Table 3. Correlations between PM2.5 (monthly values, µg/m^3) and mutagenicity (monthly values, rev/m^3) measured in the sites during the 2016 – Spearman's rho (***p <0.01, **p < 0.01, *p < 0.05).

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

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

[a] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

[b] Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

*__Corresponding author__:

Tiziana Schilirò

tiziana.schiliro@unito.it

Department of Public Health and Pediatrics,

University of Torino,

Piazza Polonia 94, 10126 Torino, Italy.

Phone: +39 011 6705822

Abstract

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 (±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^3, was observed for each PM2.5 µg 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.

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

Abbreviations:

ARPA Piemonte Regional Agency for Environmental Protection of Piedmont

B(a)P benzo(a)pyrene

BaPeq benzo(a)pyrene-equivalent

DMSO dimethyl sulfoxide

IARC International Agency for Research on Cancer

MAMSL m above mean sea level

MR mutagenicity ratio

PAH Polycyclic Aromatic Hydrocarbon

PM Particulate Matter

TEF Toxic Equivalent Factor

TMF Total Mutagenicity Factor

WHO World Health Organization

1. Introduction

The International Agency for Research on Cancer (IARC) has classified outdoor air pollution as carcinogenic to humans (group 1). One of the most important air pollutants is particulate matter (PM), and the toxic and genotoxic effects of PM are mainly attributed to PM2.5 (aerodynamic diameter ≤2.5 μm), as this fraction is composed of numerous fine and ultrafine particles per unit mass with a large surface, on which mutagenic and genotoxic pollutants can be adsorbed (Balakrishnan et al., 2015; IARC, 2016; Rainho et al., 2014). PM2.5 is widely studied because of its harmful effects on human health, such as lung cancer, cardiovascular diseases and respiratory disorders, as a result of the high deposition rate in respiratory organs. The World Health Organization (WHO) has set guidelines for PM2.5 in the atmosphere at 10 $\mu g/m^3$ for the annual average concentration and at 25 $\mu g/m^3$ for the daily average (WHO, 2006);

therefore, numerous countries monitor PM2.5 levels to assess environmental air quality. In all Italian regions, monitoring PM2.5, as well as monitoring benzo(a)pyrene (B(a)P) and metals (As, Cd, and Ni), is carried out following current Italian and European regulations (Italian Legislative Decree 155/2010 and European Commission Directive 2008/50/EC). Moreover, to better characterize PM2.5 quality, additional chemical analyses are recommended. For example, the carcinogenic potency of air samples can be estimated by Polycyclic Aromatic Hydrocarbon (PAH) levels, including the Toxic Equivalent Factor (TEF). Total TEF is assessed by multiplying the concentration of an individual PAH in the air with its TEF (Samburova et al., 2017). Most of the total TEF is determined by the finest PM fractions showing that genotoxic PAH levels are inversely proportional to the particle size (Pehnec and Jakovljević, 2018). However, since PM2.5 is often collected by low-volume air samplers, chemical analysis is limited because the air volumes, which can be sampled, are also small, and they may not be large enough to collect a sufficient PM2.5 mass for speciation, especially for nano-pollutants. Moreover, since a wide range of carcinogenic substances (known and not well known) can be contained in PM2.5 samples, it is currently impossible to measure all of them. There are both analytical limits and cost-benefit limits.

PM2.5 and PAH levels are important parameters for assessing air quality; however, the cumulative effect of the complex PM2.5 mixture may not be easily deduced by PM2.5 and PAH concentrations; therefore, the application of some effect-based monitoring tools (e.g., *in vitro* assays) could have a key role in improving assessments of the biological effects of PM2.5 and PAHs in the context of the different monitoring programmes (Schilirò et al., 2016). In fact, biological monitoring approaches through effect-based tools allow the detection of cumulative effects and are useful for bridging the gap between chemical contamination and biological effects (Wernersson et al., 2015). Among the different biological monitoring approaches, the use of *in vitro* assays has become increasingly important since *in vivo* methods are characterized

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

A useful *in vitro* assay to assess mutagenicity is the *Salmonella* reverse mutation assay (Ames test), which was first used in 1977 and has been applied for 40 years (Mortelmans, 2019; Tokiwa et al., 1977). The traditional Ames test uses *Salmonella typhimurium* strains TA98 (in which frameshift mutations are detected) and TA100 (in which base substitution mutations are detected), but it cannot highlight the mutagenic activity of particular compounds; therefore, modified Ames tests have been developed to improve the assessment of PM2.5 mutagenicity. Specifically, from the TA98 strains, two different strains were derived (YG1021 and TA98NR strains) that differ in their nitro-reductase expression (Josephy et al., 1997). The Ames test has become a requirement for the investigation or regulatory approval of many types of chemicals, and it has been applied to characterize the mutagenicity of air in more than 250 studies, leading to a remarkable amount of information. Although the test has been used for 46 years, it is still widely applied. For example, using experimental systems that simulate atmospheric pollution, the Ames test was recently correlated to the air quality health index that is used for atmospheric pollution (Zavala et al., 2018), and the Ames test showed the crucial role of aromatic hydrocarbon and NOx mixture photooxidation (Riedel et al., 2018).

Many studies have shown a positive association between PM mutagenicity measured with the Ames test and the concentrations of PAHs and their nitro, amino and/or hydroxylamine derivatives. Moreover, a positive association between PM mutagenicity and polar compounds, such as aromatic amines and aromatic ketones, has also been found (Alves et al., 2016; Claxton et al., 2004; Traversi et al., 2011). However, the ability of PM2.5 and PAH levels to predict the mutagenic effect of polluted air is still controversial. Therefore, to comprehensively evaluate the harmful effects of substances associated with PM2.5, a mutagenic assay, such as the Ames test, seems to be needed and has recently been supported for risk assessments (Klapacz and Gollapudi, 2019) and regulatory decision making (Heflich et al., 2019).

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

In this study, PM2.5 was collected daily during a full year at nine different sites of the northwestern Italian air quality monitoring network (Piedmont Region), and both the PM2.5 and PAH concentrations (including associated TEFs) and mutagenicity of the PM2.5 organic extract were evaluated to integrate the results of legislatively required monitoring with biological monitoring.

2. Materials and methods

2.1 Sampling sites, collection, and extraction

The PM2.5 samples were collected from nine monitoring stations located in the Piedmont Region, northwest of the Italian Padana Plain. The stations are part of the air quality monitoring network run by the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte). The sampling sites included a traffic site (Settimo Torinese), five urban background sites (Torino, Novara, Alessandria, Borgosesia and Cuneo), two rural background sites (Vinchio and Domodossola), and a hill site located far from human settlements as a reference site with low air pollution levels (Dernice) (Figure 1).

PM2.5 was sampled daily from January to December 2016 utilizing low-volume air samplers (sampling flow 2.3 m^3/h). PM2.5 was collected on quartz-fibre filters (Ø = 47 mm, Sartorius, Goettingen, Germany). The amount of PM collected was determined using the gravimetric method by double weighing (complying with the EN 12341 norm). The exposed filters were stored at -20 °C until extraction. The filters collected daily were pooled together to obtain monthly samples that were subsequently extracted with acetone-hexane 1:1 using a Soxhlet apparatus for at least 80 cycles. The solvent was evaporated with a rotary evaporator, and the

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

2.2 Salmonella *reverse mutation assay (Ames Test)*

The mutagenicity assay was performed on every organic extract according to Maron and Ames (1983). The mutagenic activity of the PM2.5 extracts was determined using the *S. typhimurium* frameshift strain TA98 and the base-substitution strain TA100, both with and without Aroclor-induced rat liver homogenate activation (S9). In addition, *S. typhimurium* TA98NR and YG1021 strains were used to evaluate the nitro compound contribution to the overall mutagenicity. YG1021 is a nitroreductase-overproducing strain obtained by cloning the nitroreductase gene of *S. typhimurium* TA1538 into the pBR322 vector and introducing the recombinant plasmid into TA98. YG1021 has a nitrofurazone reductase activity more than 50 times higher than that of the original TA98 strain, permitting the efficient detection of mutagenic nitroarenes, while TA98NR lacks nitroreductase; therefore, the response obtained from these two strains permits efficient detection of mutagenic nitroarenes when compared with the reference TA98 strain.

Four doses of PM2.5 organic extracts were tested in triplicate to obtain a dose-response curve (2.5, 5, 10, and 20 m^3/plate). Positive controls were 4-nitroquinoline 1-oxide (0.5 µg/plate, TA98), methyl methanesulfonate (0.25 µg/plate, TA100), 2-aminoanthracene (2 µg/plate, TA98+S9, TA100+S9) and 2-nitrofluorene (1 µg/plate, TA98NR, YG1021). Negative controls were obtained by exposing each strain to DMSO (100 µL/plate). After 48 h of incubation, the number of revertant colonies was measured by an automatic colony counter (Synoptics Protos, UK). A microscopic observation of the background lawn density was used to evaluate the toxicity. The results are expressed as net revertants (total revertants minus spontaneous revertants)/m^3 and were calculated by a dose-response curve (rev/m^3, i.e., the slope of the regression line) (Buschini et al., 2001; Cassoni et al., 2004). The mutagenicity ratio (MR)/20

m^3 sampled air, which is equivalent to the average breathed air by an adult in one day, was calculated by dividing the number of net revertants on the sample plates by the spontaneous revertants. To assess the overall mutagenicity of PM2.5, the Total Mutagenicity Factor (TMF) was calculated by adding the MRs of strains TA98 and TA100 with and without S9. A positive mutagenic response was defined when there was at least a twofold increase in revertants over the negative control (MR=1) and a dose-response effect over the range tested.

2.3 PAH determinations

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

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

The TEF approach was applied to convert PAH concentrations into B(a)P-equivalent (BaPeq) toxicity (Nisbet and LaGoy, 1992; Reeves et al., 2001).

2.4 Statistical analyses

The autumn-winter mean for each parameter was calculated taking into account the monthly values from October to March, and the spring-summer mean was calculated considering the months from April to September. The t-test was used to compare the differences between the two periods for every site. The Spearman test was used to calculate correlations and the relationship between PM2.5 levels and mutagenicity was evaluated though a linear regression model. Differences among sites were tested using ANOVA with Tukey's *post hoc* test (normally distributed data) and the Kruskal-Wallis test (not normally distributed data).

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

3. Results and Discussion

3.1 Gravimetric analysis

Airborne PM2.5 concentrations showed a similar seasonal trend at almost every site considered in the study, with higher levels in the autumn-winter season than in the spring-summer season (t-test $p < 0.05$). No significant differences were found between seasons only at the reference site Dernice (Table 1); moreover, at this site, the annual mean PM2.5 levels were lower than those at the other sites (ANOVA with Tukey's *post hoc* $p < 0.05$).

The data showed that the annual mean PM2.5 was always below the Italian limit level of 25 $\mu g/m^3$ (Italian Legislative Decree 155/2010, European Commission Directive 2008/50/EC), except for at the traffic site Settimo Torinese. On the other hand, Dernice is a unique site where the annual mean PM2.5 was below the WHO guideline level (WHO, 2006) (Table 1). Moreover, the daily PM2.5 mean concentration recorded at all stations ranged between 5.7 and 64.9 $\mu g/m^3$, and for numerous days during the year, the daily PM2.5 mean concentration was higher than the WHO guideline value (25 $\mu g/m^3$); thus, almost all the mean concentrations related to the autumn-winter season were above 25 $\mu g/m^3$ (Table 1), except for those in cities placed at more than 500 m above mean sea level (MAMSL) (Figure 1). These results demonstrated that although the different sites were characterized by different emission levels (e.g., rural sites and urban sites), especially in winter, most showed PM2.5 levels above the WHO guideline value, posing a potential risk to human health. These levels were probably due to the climatic and topographic conditions of the Piedmont Region, which is located on the Padana Plain, an area surrounded by mountains and where such conditions reduce emission pollutant dispersion and promote severe air pollution episodes. These findings are in agreement with those in the literature describing measurements taken at different locations on the Padana

Plain where winter meteorological conditions in addition to thermal inversion prevent the dispersion of pollutants, causing high PM levels (Bocchi et al. 2016; Bonetta et al., 2009; Feretti et al., 2019; Gilli et al., 2007). Moreover, since the sites located at high altitudes, such as Dernice, Borgosesia, Domodossola and Cuneo (altitudes ranging from 295 to 580 MAMSL), showed lower PM2.5 levels than those at low altitude sites, our results demonstrated that pollutant dispersion seems to be more frequent at higher altitudes, especially on the Padana Plain.

PM2.5 monthly mean concentrations were inversely correlated with measured temperature (Spearman rho -0.783 - -0.888; $p < 0.01$), except for at the Dernice site, where PM concentrations were mostly constant over the year. The increase in PM2.5 levels in the cold months, characterized by the lowest temperature, can be explained considering that in comparison to in the warm months, in the cold months, a stronger chemical interaction among the primary pollutants can occur in the atmosphere. This interaction is due to lower pollutant dispersion (due to meteorological conditions) and higher pollutant sources (due to household heating) during cold months than during warm months. Moreover, the cold temperatures in the cold months increase the binding of molecules to surfaces, so the pollutants are stably adsorbed on the PM surface and can interact with each other.

3.2 ***PAH levels***

The recorded values for total PAHs (sum of the 4 compounds), TEFs, and B(a)P are summarized in Table 2. Total PAH concentrations showed the same trend as that of the PM2.5 levels. In particular, PAH concentrations were high in the winter season, while from April to September, PAH values were low and uniform at every site. The decrease in PAH levels in spring and summer was probably due to the climatic conditions that facilitated the dispersion of pollutants and to the high solar radiation, which helped PAH degradation. The mean PAH

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

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

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

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

The mutagenic effect of the PM2.5 organic extracts is illustrated in Figure 2. 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. As reported in the literature (Cassoni et al., 2004), an enhanced response of the strains without S9 was observed at every site, denoting a higher presence of substances that can act directly on DNA. In comparison to the other sites, the traffic station (Settimo Torinese) showed higher mutagenic values, particularly in January and December,

while the reference site with low air pollution (Dernice) always showed low mutagenic values, even in the winter season. At the other rural and urban background stations, there were no significant differences, denoting a spread of PM2.5 pollution over the whole region.

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

No significant differences among the stations were observed when evaluating the overall mutagenicity based on the amount of PM2.5 analysed (Figure 3B). A positive response was also found when analysing samples of the reference site, even if lower than that at the other sites (Figure 3).

To compare the results of traditional monitoring with biological monitoring, correlations between PM2.5 levels and mutagenic effects were tested. The correlations between the PM2.5 levels and the calculated revertants for every strain were statistically significant throughout the studied period, except for those at the reference site (Table 3). This result suggests that PM2.5 level can also be a good parameter to estimate its mutagenic potential. The PM2.5 levels showed a high correlation with the mutagenicity of critical sites such as Settimo Torinese, highlighting the presence of more mutagenic compounds on PM2.5. However, it is important to note that the PM2.5 levels were usually not correlated with the mutagenicity at the reference site (Dernice), and the significance of the correlation decreased at sites with low PM2.5 levels. Therefore, the mutagenic compound contribution to the mass seemed to decrease from

urban/critical sites to the reference site (Table 3), suggesting that the mutagenicity at sites characterized by low PM2.5 levels may mainly depend on the quality (type and chemical nature of the particles and of the particle-transported pollutants) and not on the quantity of the PM2.5 mass. From these results, it seems that traditional monitoring is an approach that is usually suitable for the assessment of air quality; however, biological monitoring can be a complementary approach that is particularly important for the correct assessment of air quality at sites characterized by low PM2.5 levels and unusual, poorly understood pollutants.

The relationship between airborne PM2.5 concentrations and total mutagenicity was further evaluated by comparing all the data obtained at all the sites throughout the year (Figure 4). A significant relationship was observed, and the samples with PM2.5 concentrations below the annual limit of 10 μg/m^3 suggested by the WHO (WHO, 2006) showed negative or weakly positive mutagenic results, demonstrating that PM2.5 levels can be a good parameter to estimate air mutagenic effects and that the WHO limit is adequate. However, the linear regression model between mass and mutagenicity disappeared at PM2.5 levels below 10 μg/m^3. On the other hand, biological monitoring may be a useful complementary approach. Moreover, the presence of a mutagenic effect induced by low PM2.5 levels suggested that a reduction in PM2.5 mass did not seem to be sufficient to reduce its toxicity (Schilirò et al., 2015).

PAH concentrations, as one of the other parameters generally assessed in traditional monitoring programmes, were correlated with PM2.5 levels. The PAH concentrations statistically correlated with the total mutagenicity (TMF/20 m^3) considering all the data obtained at all the sites throughout the year (Spearman's rho = 0.876 ÷ 0.915; $p < 0.01$). Taking into account only the autumn and winter seasons, the correlations were usually not significant (Table S.1, supplementary materials); for example, the correlation was not significant in Torino (Spearman's rho = 0.696; $p > 0.05$) and Domodossola (Spearman's rho = 0.200; $p > 0.05$) but highly significant at sites with extreme pollution levels, very high in Settimo Torinese (Spearman's rho = 0.945; $p < 0.01$) and very low in Dernice (Spearman's rho = 0.943; $p < 0.01$).

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

Finally, in this study, the nitro compound contribution to the overall mutagenicity was evaluated by comparing the results obtained with the TA98, TA98NR, and YG1021 strains. The quantification of the mutagenicity linked to the amplified nitroreductase activity was calculated as [YG1021 net revertants/m^3/TA98 net revertants/m^3], while the residual mutagenicity linked to the nitroreductase deficiency was quantified as follows: {1-[(TA98 net revertants/m^3-TA98 NR net revertants/m3)/TA98 net revertants /m^3]} (Traversi et al., 2009). At every site, the YG1021 strain showed the highest response, followed by TA98 and TA98NR. Table S.2 (supplementary materials) summarizes the amplified and residual mutagenicity during the winter period. The values for the amplified mutagenicity ranged from 1.41 for Borgosesia to 2.32 for Dernice, denoting a high contribution of the nitro compounds to the total mutagenicity measured in every site (Rainho et al., 2014; Traversi et al., 2011). The amplified mutagenicity due to the nitro-compound contribution did not explain the higher mutagenicity observed in the critical area than in the other areas; moreover, the residual mutagenicity seemed to be constant (from 0.46 for Domodossola to 0.64 for Vinchio) (Table S.2, supplementary materials). Nonetheless, at some sites (Torino, Domodossola and Dernice), the nitro compound contribution to mutagenicity was remarkable regardless of the PM2.5 or PAH concentrations. These results further confirmed that it is useful, where possible, to monitor air quality using biological monitoring. Moreover, this result suggests that it is convenient to test samples with different *S. typhimurium* strains to investigate different biological effects that are induced by different chemicals (Landkocz, et al., 2017; Lemos et al., 2012).

4. Conclusion

This study confirmed that PM2.5 pollution is a critical issue to address to protect human health, especially in northwestern Italy, an area characterized by climatic and topographic conditions that prevent pollutant dispersion and promote severe air pollution episodes. In this area, PM2.5 levels exceed the WHO guidelines except for some background sites, which are generally located at a higher altitude, suggesting that the air pollution phenomenon is attenuated by altitude.

Our results suggest that PM2.5 concentration is a good parameter to estimate air quality because of the correlations between PM2.5 levels and mutagenic effects; however, since this correlation was generally not significant at the site with the lowest pollution levels, the results also suggested that biological monitoring can be a complementary approach for the correct assessment of air quality at sites characterized by low PM levels or unusual pollutant sources. Moreover, considering that the correlations between PAH levels and mutagenic effects were not always significant, our results demonstrate that the analytical determination of a few chemical compounds is not enough to characterize the mutagenicity of a complex environmental mixture.

Finally, this study shows that it is useful to test air samples with different *S. typhimurium* strains to consider different biological effects induced by different pollutants; in particular, for the assessment of the potential risk associated with PM2.5, it seems important to also evaluate the mutagenic effect due to nitro compounds, highlighting the necessity of a strength assessment for reactive nitrogen molecules in the air.

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

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

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Figure captions:

Figure 1. Sampling sites in the study area. Cartographic coordinate system ED1950 (UTM) Zone 32. MAMSL= m above mean sea level.

Figure 2. Mutagenicity expressed as number of net revertants/m^3 of air sampled – Strains TA98 and TA100 with (+S9) and without metabolic activation.

Figure 3. Total mutagenicity measured in the sites during autumn-winter months expressed as A: TMF/20 m^3 of air sampled, B: TMF/mg of PM2.5. Boxes represent the 25-75th percentile; outer lines represent the 10-90th percentile.

Figure 4. Linear regression between PM2.5 (monthly values, µg/m^3) and mutagenicity (monthly values, TMF/20 m^3), all data (108 pairing). The green area corresponds to mass concentration levels ranging between the WHO guideline value (WHO, 2006) and the limit value of the Italian law (Italian Legislative Decree 155/2010)**.** The regression shows R = 0.837 and is statistically significant (Anova p=0.000).

Table captions:

Table 1. Annual means of PM2.5 (µg/m^3) in the sites and comparison between means of PM2.5 (µg/m^3) measured during autumn-winter months and means of PM2.5 (µg/m^3) measured during spring-summer months in the sites– t-test.

Table 2. Total PAHs (monthly values, ng/m^3), TEFs (monthly values, BaPeq) and B(a)P (annual means, ng/m^3) measured in the sites during the 2016.

Table 3. Correlations between PM2.5 (monthly values, µg/m^3) and mutagenicity (monthly values, rev/m^3) measured in the sites during the 2016 – Spearman's rho (***p <0.01, **p < 0.01, *p < 0.05).

table 1

Table 1

	Annual (µg/m^3)				**Autumn-Winter** (µg/m^3)		**Spring-Summer** (µg/m^3)		***T-test***
Sampling Site	***Min***	***Max***	***Mean***	***St. Dev.***	***Mean***	***St. Dev.***	***Mean***	***St. Dev.***	
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

Table 2

	∑PAHs (ng/m³)-TEF (BaPeq)								
Month	**SETTIMO TORINESE**	**TORINO**	**NOVARA**	**ALESSANDRIA**	**BORGOSESIA**	**CUNEO**	**VINCHIO**	**DOMODOSSOLA**	**DERNICE**
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
annual mean b(a)p (ng/m³)	**1.13**	**0.72**	**0.43**	**0.52**	**0.90**	**0.27**	**0.39**	**1.58**	**0.04**

table 3

Table 3

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

figure 4

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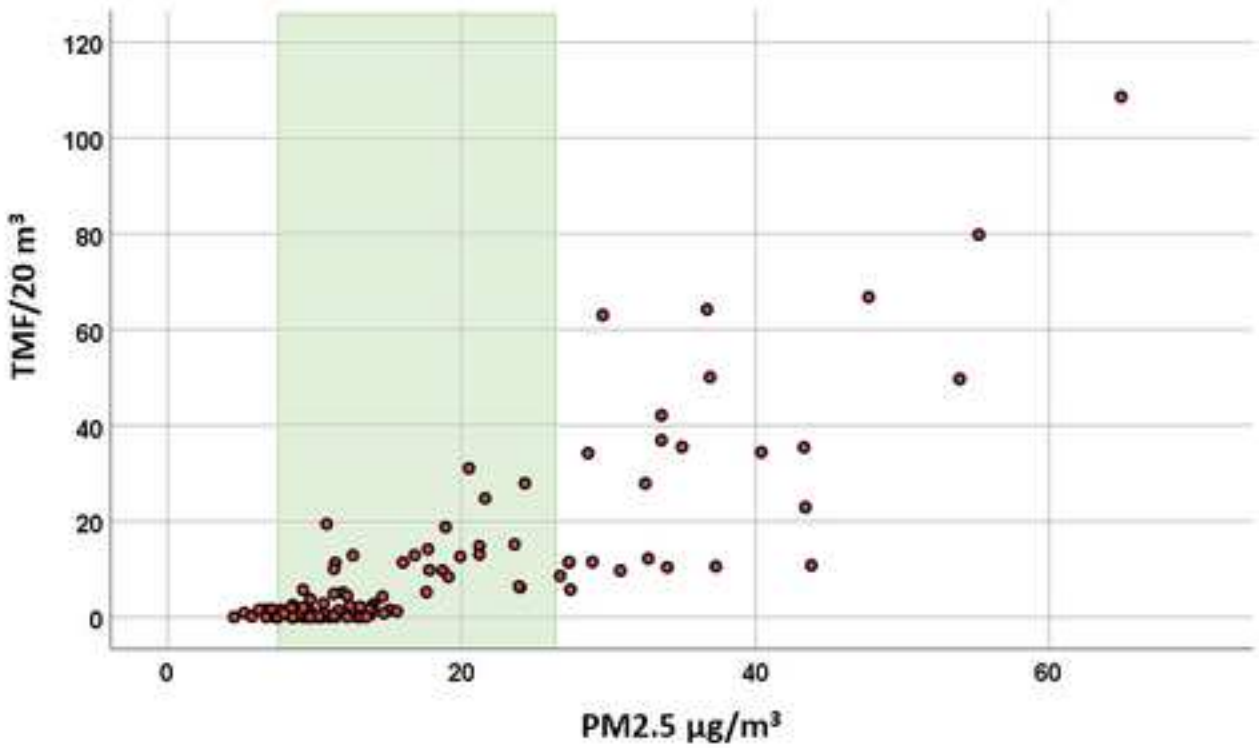

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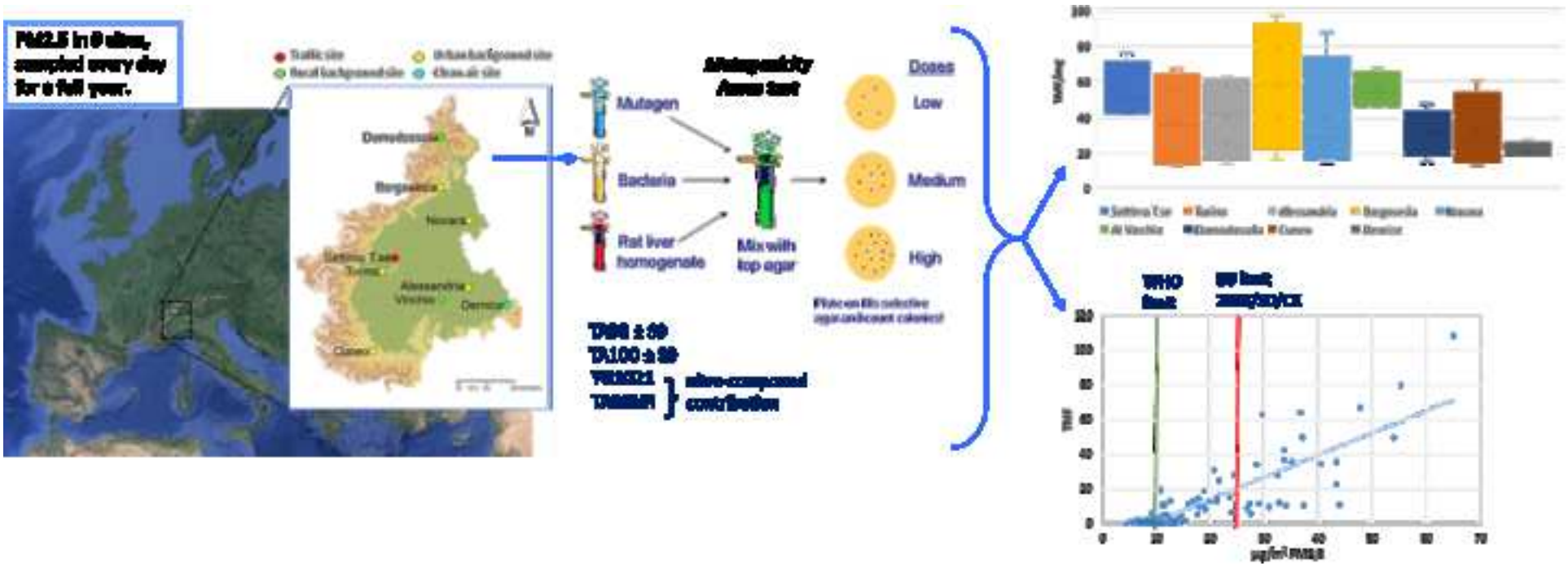

supplementary material

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

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

[a] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

[b]Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

***Corresponding author**:

Tiziana Schilirò

tiziana.schiliro@unito.it

Department of Public Health and Pediatrics,

University of Torino,

Piazza Polonia 94, 10126 Torino, Italy.

Phone: +39 011 6705822

Author statement

Daniele MARANGON: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing

Deborah TRAVERSI: Conceptualization, Supervision, Writing - original draft, Writing - review & editing

Anna Maria D'AGOSTINO: Investigation.

Marta GEA: Formal analysis, Visualization, Writing - review & editing

Marco FONTANA: Funding acquisition, Resources, Supervision

Tiziana SCHILIRÒ: Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Writing - original draft, Writing - review & editing

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

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

[a] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

[b] Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

***Corresponding author**:
Tiziana Schilirò
tiziana.schiliro@unito.it
Department of Public Health and Pediatrics,
University of Torino,
Piazza Polonia 94, 10126 Torino, Italy.
Phone: +39 011 6705822

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Competing interests statement

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