Mutagenic and genotoxic effects induced by PM0.5 of different Italian towns in human cells and bacteria: The MAPEC_LIFE study.

MUTAGENIC AND GENOTOXIC EFFECTS INDUCED BY $PM_{0.5}$ OF DIFFERENT ITALIAN TOWNS IN HUMAN CELLS AND BACTERIA: THE MAPEC_LIFE STUDY

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Abstract

Particulate matter (PM) is considered an atmospheric pollutant that mostly affects human health. The finest fractions of PM ($PM_{2.5}$ or less) play a major role in causing chronic diseases.

The aim of this study was to investigate the genotoxic effects of $PM_{0.5}$ collected in five Italian towns using different bioassays. The role of chemical composition on the genotoxicity induced was also evaluated.

The present study was included in the multicentre MAPEC_LIFE project, which aimed to evaluate the associations between air pollution exposure and early biological effects in Italian children.

PM_{10} samples were collected in 2 seasons (winter and spring) using a high-volume multistage cascade impactor. The results showed that $PM_{0.5}$ represents a very high proportion of PM_{10} (range 10-63%). $PM_{0.5}$ organic extracts were chemically analysed (PAH_s, nitro-PAH_s) and tested by the comet assay (A549 and BEAS-2B cells), MN test (A549 cells) and Ames test on *Salmonella* strains (TA100, TA98, TA98NR and YG1021).

The highest concentrations of PAHs and nitro-PAHs in $PM_{0.5}$ were observed in the Torino, Brescia and Pisa samples in winter. The Ames test showed low mutagenic activity. The highest net revertants/m^3 were observed in the Torino and Brescia samples (winter), and the mutagenic effect was associated with $PM_{0.5}$ ($p<0.01$), PAH and nitro-PAH ($p<0.05$) concentrations. The YG1021 strain showed the highest sensitivity to $PM_{0.5}$ samples. No genotoxic effect of $PM_{0.5}$ extracts was observed using A549 cells except for some samples in winter (comet assay), while BEAS-2B cells showed light DNA damage in the Torino, Brescia and Pisa samples in winter, highlighting the higher sensitivity of BEAS-2B cells, which was consistent with the Ames test ($p<0.01$).

The results obtained showed that it is important to further investigate the finest fractions of PM, which represent a relevant percentage of PM_{10}, taking into account the chemical composition and the biological effects induced.

Keywords: $PM_{0.5}$, mutagenicity, genotoxicity, PAHs, nitro-PAHs

Capsule

Results highlighted the importance to further investigate the finest fractions of PM, which represent a relevant percentage of PM_{10}, taking into account its chemical composition and the biological effects induced.

1. Introduction

Atmospheric pollution poses a serious threat to human health and airborne particulate matter (PM) is one of the major contributors (Anderson et al. 2012; Cohen et al., 2017; WHO, 2016).

The causal relationship between exposure to airborne $PM_{2.5}$ and acute and/or chronic diseases is well reported in literature (EEA, 2017; Kim et al., 2015; Pope and Dockery, 2006). Moreover, the International Agency for Research on Cancer (IARC) has recently classified air pollution and fine PM as carcinogenic to humans (1 Group) (IARC, 2016).

In recent years, researcher interest in the health effects of smaller particles, the sub-micrometer particles (fine), including ultrafine particles (UFPs, $PM_{0.1}$), has considerably increased as these fractions are the most abundant particulate pollutants in urban and industrial areas (Keogh et al., 2009; Morawska et al., 2008; Schilirò et al., 2016). The greater toxicity of UFPs is related to their potential to be retained in the pulmonary alveoli, to diffuse into the blood stream and reach other organs (Nemmar et al., 2002; Peters et al., 2006) and to their greater capacity to adsorb chemicals (Wichmann et al., 2009).

The current air quality guidelines are based on the mass concentration of particles of a given aerodynamic diameter (PM_{10} or $PM_{2.5}$), but it is clear that the structure and composition of PM can also influence the biological effects (Landkocz et al., 2017. Moreover, the chemical composition of PM varies with sources of emissions, season and region of sampling and photochemical-meteorological conditions (Perrone et al., 2010; Pey et al., 2010; Pongpiachan et al., 2015; Topinka et al., 2015).

The effects of exposure to mixtures of chemicals, such as PM, are difficult to evaluate because the different chemical compounds can interact with synergistic, antagonistic or additive effects (USEPA, 2008). For a more complete evaluation of the health risk of human exposure, short-term bioassays were used to study the biological effects of chemical pollutants in urban PM (Ceretti et al., 2015; de Brito et al., 2013; Dumax-Vorzet et al., 2015; Lemos et al., 2012; Lepers et al., 2014; Palacio et al., 2016; Traversi et al., 2015). PM_1, quasi-ultrafine particles ($PM_{0.5}$; $PM_{0.4}$ and $PM_{0.3}$) and UFPs ($PM_{0.1}$) have been less extensively studied than fine ($PM_{2.5}$) and coarse ($PM_{10\text{-}2.5}$) particles. Besides the increasing epidemiological data on particles with a diameter less than 1 μm, there are still few studies on the biological effects of these fractions. Some studies have shown that UFPs are able to induce oxidative stress (Gasparotto et al., 2013), inflammation (Muller et al., 2010), apoptosis and necrosis (Sydlik et al., 2006). Moreover, cytotoxic effects (Borgie et al., 2015), release of cytokine/interleukin release (Longhin et al., 2013) and dioxin-like activity (Wichmann et al., 2009) have also been reported for quasi-ultrafine particles. However, only a few recent studies investigated the genotoxic or mutagenic effects of these finest fractions, and only some endpoints were taken into account with a

limited number of short-term assays (Landkocz et al., 2017; Topinka et al., 2015; Velali et al., 2016). Then, further studies are needed to better understand their mechanisms of action of UFPs and their involvement in the occurrence of many diseases.

The present study was included in the MAPEC_LIFE project (LIFE12 ENV/IT/000614), a multicentre Italian cohort study funded by the European Union's LIFE+ Programme that aims to evaluate the associations between air pollution (including PM) and early biological effects in 6-8-year-old Italian children. Details of the study design have been described elsewhere (Feretti et al., 2014). Briefly, oral mucosa cells of 1149 children recruited from first grade schools were collected to evaluate the frequency of MN and DNA damage. Some results on subject characteristics, diet in particular, and frequency of MN in their buccal cells have already been published (Bagordo et al., 2017; Grassi et al., 2016; Villarini et al., 2018; Zani et al., 2016). The study was conducted in different schools of five Italian towns (Figure S1) characterized by different levels of air pollution. In particular, Torino and Brescia are located in the Padana Plain in the north of Italy (one of the most polluted areas in Europe), Pisa and Perugia in central Italy (medium-low pollution area) and Lecce in southern Italy (low pollution area) (EEA, 2017; ISPRA, 2015). To evaluate children's exposure to urban air pollution, $PM_{0.5}$ was collected near each school on the same days as the biological sampling. The purpose of this work was to investigate the *in vitro* mutagenic and genotoxic effects of $PM_{0.5}$ collected in the MAPEC_LIFE study using different short-time bioassays (Ames test, comet assay, micronucleus test). The spatial and seasonal variations of the genotoxicity induced by the organic extracts of $PM_{0.5}$ were evaluated, and the role of chemical composition on the mutagenic and genotoxic effect of $PM_{0.5}$ samples was also investigated.

2. Materials and methods

2.1 Airborne particulate sampling and gravimetric analysis

PM_{10} fractions were collected in 18 sites located in the five towns involved in the MAPEC_LIFE study. The description of the sampling sites is reported in Figure S1. The sampling was performed in 3 consecutive 24-hour periods, for a total of 72 sampling hours, using a Sierra-Andersen high-volume multistage cascade impactor (AirFlow PM10-HVS sampler, AMS Analitica Srl, Pesaro, Italy) at a flow of 1160 L/min. The particle size fractions collected were as follows: 10.0-7.2, 7.2-3.0, 3.0-1.5, 1.5-0.95, 0.95-0.49, and <0.49 μm ($PM_{0.5}$). All filters were pre- and post-conditioned and weighed at controlled temperature and humidity, as previously reported (Schilirò et al., 2016).

The samplings were performed during two seasons, winter (November 2014/March 2015-winter I) and late spring (April/June 2015). Air sampling was repeated the following winter (November 2015/January 2016-winter II) only in Brescia.

2.2 Extraction of $PM_{0.5}$ components

After gravimetric analyses, the $PM_{0.5}$ filters (three for each site) were pooled to obtain a total of 40 samples. Particles were Soxhlet extracted with 200 mL of n-hexane-acetone (4:1) for 6 h to recover organic extractable compounds. Each extract was separated into different aliquots destined for chemical analysis and biological tests. The organic extracts were concentrated by rotary evaporation. For the biological tests, the samples were re-suspended in dimethyl sulfoxide (DMSO) (2 $m^3/\mu L$).

2.3 Chemical analysis of $PM_{0.5}$ organic extracts

PAH and nitro-PAH concentrations in the organic extracts of $PM_{0.5}$ were evaluated according to the EPA TO-134 1999 method. An Agilent 7690B gas chromatograph (Agilent Technologies Italia SPA) with a Rxi-17 Sil MS column (Restek) (30 m x 0.25 mm x 0.25 μm) and an Agilent 5977A mass spectrometer (single ion monitoring) were used for PAH analysis.

The following PAHs were analysed: naphthalene, acenaphthylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, perylene, dibenz(a,h)acridine, dibenz(a,j)acridine, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, dibenzo(a,l)pyrene, 7Hbenzo(c)fluorene, 5-methylchrysene, 7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene, anthanthrene, dibenz(a,e)fluoranthene, 7Hdibenzo(c,g)carbazole.

Nitro-PAH concentration was evaluated by means of GC-MS-TQ8030 (Shimadzu Europe GMBH) (multiple reaction monitoring mode) using a HP5-MS ultrainert column (Agilent) (30 m x 0.25 mm x 0.25 μm).

The nitro-PAHs analysed were 1-nitronaphthalene, 2-nitronaphthalene, 5-nitroacenaphtene, 2-nitrofluorene, 9-nitroanthracene, 1-nitropyrene, and 6-nitrochrysene.

The information about the QA/QC was reported in Supporting Information.

The comparison of the retention times and mass spectra of the different compounds with those of reference standards was used to their identification.

2.4 *Salmonella*/microsome (Ames) test on $PM_{0.5}$ organic extracts

The Ames test (Maron and Ames, 1983) was used to evaluate the mutagenicity of $PM_{0.5}$ organic extracts collected in all towns. The organic extracts were tested in duplicate at increasing doses (10, 25 and 50 m^3 of air equivalent/plate) with different *S. typhimurium* strains (TA100, TA98, TA98NR, YG1021). The TA100 and TA98 strains specifically detect base-substitution and frameshift mutations (Claxton et al., 2004). The YG1021 strain shows efficient detection of mutagenic nitroarenes and the TA98NR strain shows a reduced mutagenicity, proportional to the amount of nitroarenes present in the extract (Traversi et al., 2011).

The Ames test was performed with and without metabolic activation (±S9) to detect direct and indirect mutagens (Ceretti et al., 2015). The test was described in detail in Supporting Information. In each assay session, positive controls (10 μg/plate of 2-nitrofluorene for TA98, TA98NR and YG1021 and 10 μg/plate of sodium azide for TA100 without S9; 20 μg/plate of 2-aminofluorene for all strains with S9) and negative controls (DMSO and extracts of filter blanks) were included. The Ames test was performed by the same laboratory on all samples.

2.5 Cell culture

Two cell lines were used to evaluate the genotoxic potential of PM extracts. The human A549 cells (non-small cell lung cancer) from Interlab Cell Line Collection (Genova, IT) was used as a model for human epithelial lung cells. Human BEAS-2B cells (ATCC CRL-9609; non-cancerous cells isolated from bronchial epithelium) was used as surrogates for toxicological studies in bronchial mucosa (Courcot et al., 2012). A459 cells and BEAS-2B cell lines were cultured as previously reported (Bonetta et al. 2009; Zhang et al., 2017). The metabolic characteristics of the cells were described in detail in Supporting Information.

2.6 Comet assay on $PM_{0.5}$ organic extracts

The genotoxicity of $PM_{0.5}$ organic extracts collected in all towns in the different seasons was evaluated using the comet test on A549 cells. The samples of winter seasons (winter I and II) were also tested with BEAS-2B cell lines. The cells were cultured for 18 h in 6-well plates; then they were exposed (4 h at 37°C) to increasing doses (from 10 to 50 m^3 of air equivalent/mL) of $PM_{0.5}$ organic extracts. Cells untreated, treated with DMSO (2.5%) and treated with blank filter extracts were used as negative controls. After exposure, cell viability was assessed using the staining with trypan blue. The comet assay was performed under alkaline conditions (pH > 13) (Tice et al., 2000) as described in detail in Supporting Information. The mean percentage of DNA in the comet tail (tail intensity, TI) was used as DNA damage metric. The results obtained from control cells (DMSO) were compared with those from cells exposed to PM extracts. Statistical analyses were performed by ANOVA

combined with a *post hoc* Dunnett's test (SPSS Statistics 24.0) (IBM Corporation, Armork, NY, USA). Statistically significant differences were reported with a p value ≤0.05. The Fpg-modified comet assay was carried out as previously reported (Bonetta et al., 2009). The comet assay was performed by the same laboratory on all samples.

2.7 Cytokinesis-block MN (CBMN) test on $PM_{0.5}$ organic extracts

The CBMN test was used to evaluate the genotoxicity of $PM_{0.5}$ organic extracts collected in the five towns. The test was performed in accordance with the original method by Fenech (2000) as described in detail in Supporting Information. A549 cells were treated (24 h at 37°C with 5% CO_2) with increasing doses (10, 25 and 50 m^3 of air equivalent/mL) of the $PM_{0.5}$ organic extracts, then the viability was assessed by the trypan blue dye exclusion technique. Cells treated with DMSO (0.5%) and blank filter extracts were used as negative controls. Ethyl methanesulfonate (EMS) was used as a positive control (1.5 and 2 mM EMS). The results are expressed as the mean MN/1000 cells from two independent evaluations. Data from cell cultures exposed to control (DMSO) were compared with those from PM extracts. Statistical analyses were performed by ANOVA combined with a *post hoc* Dunnett's test (SPSS Statistics 24.0) (IBM Corporation, Armork, NY, USA). The MN test was performed by the same laboratory on all samples.

2.8 Statistical analysis

The statistical analysis was performed with the statistical package IBM SPSS Statistics 24.0 (IBM Corporation, Armork, NY, USA). Significant differences between the concentrations of PM_{10}, $PM_{0.5}$ PAHs, B(a)P and nitro-PAHs in the five towns were assessed by ANOVA and Tukey's multiple comparison tests. The differences in PM_{10}, $PM_{0.5}$ PAHs, B(a)Pyrene, nitro-PAH concentrations and genetic endpoints between winter and spring seasons were performed by Student's t-test. Significance was evaluated within 95% confidence intervals ($p \leq 0.05$). The Spearman correlation coefficient (Spearman's r) was used to assess the relationship among air pollution parameters (PAHs, B(a)pyrene and nitro-PAHs), $PM_{0.5}$ concentration and genotoxicity results.

3 Results and discussion

3.1 Size distribution of PM mass concentrations

The mass of PM samples (pooled filters) and total cubic meters of air sampled were reported in detail in Supporting Information (Table S1). The mean concentrations of PM_{10} and the other PM fractions obtained in the samples of the five towns in winter and spring seasons are reported in Figure 1.

The results of the gravimetric analysis showed that in winter samples, the mean PM_{10} concentrations were lower than the daily target of 50 $\mu g/m^3$ set by the European Air Quality Directive 2008/50/EU, except for some samples from Torino and Brescia. Often, in Italy, high PM_{10} values are observed during winter in towns located in the north of Italy, particularly in the Padana Plain, given the widespread air pollution and the general weak dispersion rate due to the territory conformation (Cadum et al., 2009; EEA, 2017).

The ANOVA underlined a significant difference in PM_{10} concentration among the samples of the five towns ($F = 6.336$, $p < 0.001$). In particular, the highest PM_{10} mass concentration values were observed in the Torino samples (winter I) ($p = 0.001$ *vs* the Perugia and Lecce samples and $p<0.01$ *vs* the Pisa samples, *post hoc* Tukey's test) and the Brescia samples (winter I and II). Conversely, as expected, the lowest value of PM_{10} was observed in the samples from Lecce. Comparing the results obtained in the Brescia samples, the PM_{10} concentration in winter I was lower than in winter II. This result could be due to the lower level of air pollution observed in winter 2014 with respect to winter 2015, which was related to the high atmospheric instability present in that season (RSA, 2017).

Although our sampling reflects only spot daily situations (3 days for each season) and does not represent long-term monitoring, the results obtained highlighted a north to south PM_{10} trend, in accordance with the Regional Agencies for Environmental Protection (ARPA) routine measurements performed in all towns during the sampling period (November 2014 – June 2015; November 2015-January 2016).

With respect to winter 2014, a significant decrease in PM_{10} concentration was observed in spring samples (spring *vs* winter $p < 0.001$, t-test). A different trend was observed only for some samples of Brescia (winter I *vs* spring). The decrease of PM_{10} in the warm season has been generally observed in urban environments (Schilirò et al., 2016).

Considering the distribution of the size fractions of PM_{10} mass in winter (Figure 1), a high particle concentration was present, especially for $PM_{0.5}$, which represented a very high proportion of PM_{10}, accounting from a minimum of 20% to a maximum of 63% of the different samples. Additionally, the fraction 0.49-0.95 represented a considerable fraction of PM_{10} although it generally showed a

lower percentage with respect to $PM_{0.5}$.

Analysing the value of the $PM_{0.5}$ concentration, the ANOVA test showed a significant difference among the samples of five towns in winter ($F = 7.277$, $p < 0.001$). As reported for PM_{10}, the highest level was found in the Torino samples ($p = 0.001$ *vs* the Perugia and Lecce samples, $p < 0.05$ *vs* the Pisa samples and $p < 0.01$ *vs* the Brescia samples, *post hoc* Tukey's test). However, the $PM_{0.5}$ level was also very high in the Brescia and Pisa samples.

The results of the statistical analyses showed a significant correlation between PM_{10} and $PM_{0.5}$ concentration in both seasons ($rS = 0.80$, $p < 0.001$ and $rS = 0.63$, $p < 0.001$ in winter and spring respectively).

Although a significant reduction in $PM_{0.5}$ concentration was observed from winter to spring in all samples ($p = 0.001$, t-test), $PM_{0.5}$ in spring also represented a considerable fraction of PM_{10}, accounting for a minimum of 10% to a maximum of 56% in the different samples.

Moreover, analysing the concentration of $PM_{0.5}$ by sampling sites (n=18), a high variability of $PM_{0.5}$ percentage was observed in the same sampling site in both seasons and from the samples of the same town.

In comparison with the few studies published on the $PM_{0.5}$ fraction, the concentrations of $PM_{0.5}$ observed in the Torino and Brescia samples in winter were similar to those observed in La Plata (Argentina) (21 $\mu g/m^3$) (Wichmann et al., 2009). Otherwise, the $PM_{0.5}$ values recorded in the samples of the other towns were similar to those found in the urban site of Prague (9.1 $\mu g/m^3$) (Topinka et al., 2013). However, the levels of $PM_{0.5}$ found in this study were generally lower than those found in other highly polluted European sites (Topinka et al., 2015) or other urban sites (Monarca et al., 1997, Velali et al., 2016).

The highest concentration of $PM_{0,5}$ during winter in comparison to spring summer was reported also in other studies for ultrafine or quasi-ultrafine fractions (Perrone et al., 2010; Perrone et al., 2013; Jalava et al., 2015; Velali et al., 2016). This trend confirmed that also this fraction was strongly influenced by seasonal meteorology in the north of Italy, where condition of atmospheric stability cause high concentrations of atmospheric pollutants (Perrone et al., 2010; Perrone et al., 2013).

As observed in our results, various studies confirmed that the finest fractions of PM are the most abundant in the atmosphere because the finest particulate pollution is homogeneously diffused (Perez et al., 2010). The high contribution of the finest fractions to the PM_{10} mass determination observed in this study was also reported in recent studies in other urban sites and has been related to traffic emissions by many authors (Topinka et al., 2015; Velali et al., 2016). Moreover, the variability of $PM_{0.5}$ percentage reported in our samples suggested, as in the study of Topinka et al. (2015), the crucial effect of the meteorological conditions. In particular, Topinka et al. (2015) highlighted the

298 day-to-day variability of PM_{10} and ultrafine particles in association with the inversion episodes.
299 Moreover, the different contributions of the most important PM sources, depending on meteorological
300 conditions, could be responsible for the relatively different amount of PM size fractions.
301
302 **3.2 Chemical analysis of PAHs and nitro-PAHs in $PM_{0.5}$**
303 The chemical analysis of the $PM_{0.5}$ organic extracts for both seasons is described in Table 1.
304 In winter I, the highest concentrations of PAHs (total and carcinogenic) and benzo(a)pyrene were
305 found in all Torino samples, in some samples from Brescia (BS2 and BS4) and in 1 sample from Pisa
306 (PI3). Considering the nitro-PAHs, out of seven nitro-PAHs analysed, only 9-nitroanthracene and 1-
307 nitropyrene were recorded in $PM_{0.5}$ samples, and the highest concentrations were found in the Pisa
308 (PI3 and PI4) and Torino samples followed by the Brescia samples (BS3 and BS4) and the Perugia
309 samples (PG2). The highest values recorded in these samples were probably related to the high
310 concentration of $PM_{0.5}$ ($\mu g/m^3$), as confirmed by the statistical analyses that indicated a linear
311 correlation between $PM_{0.5}$ levels and PAH, B(a)P and nitro-PAH concentrations in the winter season
312 ($rS = 0.86$, $p<0.001$). The results expressed as ng/µg of $PM_{0.5}$ confirmed the higher quantity of PAHs
313 (total and carcinogenic), B(a)P and nitro-PAHs in most of these samples. However, an increase in the
314 $PM_{0.5}$ level does not always correspond to a greater quantity of pollutants for µg of $PM_{0.5}$, as noted
315 by the comparison of the chemical contamination of $PM_{0.5}$ in winter I and winter II in some of the
316 Brescia samples.
317 In the spring season, as observed for $PM_{0.5}$ concentration, a significant decrease in PAH and nitro-
318 PAH concentration in $PM_{0.5}$ was reported in all samples (ten times lower than in winter for PAHs) (p
319 < 0.001, t-test). The results expressed as ng/µg of $PM_{0.5}$ confirmed the lower level of chemical
320 contaminants in spring than in the winter season, although no specific differences in this season
321 among the samples from different towns were revealed.
322 The level of PAHs observed in $PM_{0.5}$ samples of the five Italian towns was similar to that observed
323 in ultrafine particles of other European urban sites (Topinka et al., 2013; Wichmann et al., 2009). In
324 particular, PAH contamination detected in the Torino and Brescia samples was analogous to that
325 reported by Longhin et al. (2013) for $PM_{0.4}$ in another town of the Padana Plain (Milano). Considering
326 the presence of nitro-PAHs in the $PM_{0.5}$ fraction, no specific comparison with other data is possible
327 given the absence of data from other urban sites. However, the two compounds recorded in $PM_{0.5}$
328 samples (9-nitroanthracene and 1-nitropyrene) have been frequently reported in PM extracts of urban
329 environments in the literature (Carreras et al., 2013; Ladji et al., 2009; Ringuet et al., 2012).
330 The decrease in chemical contamination in spring is not surprising because of the emission decrease
331 in this season (e.g., home heating); the presence of contaminants in the PM finest fractions is also

related to the variability of atmospheric conditions between these seasons (Landlocz et al., 2017; Longhin et al., 2013). In particular, winter atmospheric conditions may promote accumulation of primary pollutants and the condensation of atmospheric pollutants in the particle phase due to the low temperature (Ebi and McGregor, 2008; Sisovic et al., 2008). The importance of atmospheric conditions on the level of chemical pollutants in the $PM_{0.5}$ fraction was also confirmed by the comparison of PAHs and nitro-PAHs for μg of $PM_{0.5}$ in Brescia in the two winter samples (winter I *vs* winter II).

3.3 Mutagenicity of $PM_{0.5}$ samples

In Table 2, the mutagenic effect of $PM_{0.5}$ extracts on bacteria is reported, expressed as net revertants/m^3 of air sampled in the TA98, TA100, TA98NR and YG1021 strains, with (+S9) and without (-S9) metabolic activation.

Overall, considering the four *S. typhimurium* strains, low mutagenic activity was observed with respect to the results obtained in other studies performed on $PM_{0.5}$ or $PM_{2.5}$ fractions in Torino and Brescia (Monarca et al., 1997; Traversi et al., 2009; Traversi et al., 2011).

In winter, the highest mutagenic activity was generally observed in the Torino and Brescia samples followed by the Pisa, Perugia and Lecce samples. The ANOVA, performed assuming mutagenicity observed with YG1021+S9 and YG1021-S9 as dependent variables and the towns as independent variables, underlined a significant difference in the mutagenic effects among the samples of the five towns (F = 18.201 and F = 13.331, $p < 0.001$, respectively). *Post hoc* Tukey's test confirmed the highest values of mutagenicity in the Torino samples (YG1021 ±S9 Torino samples *vs* Pisa/Perugia/Lecce samples $p < 0.001$ and $p < 0.01$ vs Brescia samples). This trend was probably related to the $PM_{0.5}$ concentration as confirmed by the positive correlation between mutagenic response and $PM_{0.5}$ level (YG1021 +S9 rS = 0.87, YG1021 -S9 rS = 0.76 $p < 0.001$; TA98 +S9 rS = 0.75, TA98 -S9 rS = 0.76 $p < 0.01$). The highest mutagenicity reported for the Torino and Brescia samples was also confirmed by adjusting the data for the particle mass unit (Table S2), highlighting the worse quality of the particles—in terms of mutagenic compounds (e.g., PAHs in $PM_{0.5}$ samples)—and not only the higher level of $PM_{0.5}$ concentration for each volume unit (m^3).

Comparing the results obtained with Brescia samples collected in winter I and winter II, despite the increase of $PM_{0.5}$ concentration in some samples of winter II, a similar or reduced mutagenicity was observed in winter II with respect to winter I. The lower level of chemical contamination (PAHs and nitro-PAHs) of the particles sampled in winter II was also confirmed by the lower mutagenic effect recovered after adjustment for particle mass unit.

Considering the response of the different strains, almost all $PM_{0.5}$ winter extracts (16/22) induced point mutations in the *S. typhimurium* TA98 strain (±S9). These results indicated the presence of indirect and direct mutagens. In particular, the statistical analysis used to study the associations between air pollutants and mutagenic effects confirmed a relationship between TA98 response and PAHs (TA98 +S9 rS = 0.63, $p < 0.05$) and nitro-PAHs (TA98 -S9 rS = 0.60, $p < 0.05$).

Except for two Torino samples (TO1 and TO2), the winter $PM_{0.5}$ extracts did not induce any mutagenic effects in the TA100 strain, suggesting the presence of contaminants causing frame-shift mutations, predominantly. Similar results were also found in previous studies performed in Torino and Brescia for $PM_{0.5}$ or other PM fractions (e.g., PM_{10}) (Ceretti et al., 2015; Gilli et al., 2007; Monarca et al., 1997).

As reported in other studies performed on $PM_{2.5}$ samples (Traversi et al., 2009; Traversi et al., 2015), the YG1021 strain showed the highest sensitivity to airborne pollutants. The comparison of the over producing nitroreductase strain, YG1021, with the reference TA98 strain allows quantification of the mutagenicity linked to the amplified nitroreductase activity. The $PM_{0.5}$ winter extracts determined a clear increase in the response due to amplified nitroreductase activity, which was probably related to the presence of nitroaromatic compounds, as confirmed by the significant correlation with nitro-PAH concentrations (YG1021 -S9 rS = 0.63, $p < 0.01$; YG1021 +S9 rS = 0.77, $p < 0.001$). The decrease in mutagenicity with the TA98NR strain with respect to TA98 gives further confirmation of the presence of nitroaromatic pollutants.

In the spring season, lower values of mutagenicity were recorded for all samples. Negative results were observed for TA100, TA98 and TA98NR, and the YG1021 strain showed a lower mutagenic effect than that in the winter season. A similar trend was also observed in other studies with $PM_{2.5}$ extracts (Ceretti et al., 2015; de Rainho et al., 2013; Traversi et al., 2011). The significant reduction of the mutagenic effect in the warm season (spring *vs* winter $p<0.001$ for YG1021+S9 and $p=0.001$ for YG1021-S9, t-test) was probably related to the low level of airborne contaminants in spring, as highlighted by the decrease in $PM_{0.5}$ concentration. The lower concentrations of PAHs and nitro-PAHs in spring particles were further confirmed by the lower mutagenicity of $PM_{0.5}$, adjusting the data for particle mass units.

3.4 Genotoxicity of $PM_{0.5}$ samples

3.4.1 Comet assay

No genotoxic effect of $PM_{0.5}$ was observed using the A549 cell line in almost all winter (Figure S2) and spring (Table S3) samples at all the tested doses, except for sporadic doses of a few winter samples (Figure 2). In particular, only one sample collected in Pisa in winter I (PI4) and two samples collected

399 in Brescia in winter II (BS1 and BS4) induced a significant increase in the genotoxic effect at the
400 highest tested concentration of $PM_{0.5}$ (50 m^3), but there was not dose-response relationship. Moreover,
401 the Fpg treatment did not increase the genotoxic effect, indicating there was no oxidative activity of
402 the samples analysed in both seasons (Table S3). These results highlighted that $PM_{0.5}$ samples induced
403 only light primary DNA damage in the considered cells, confirming the low level of mutagenicity
404 reported with the Ames test.
405 The comet assay on human bronchial epithelium (BEAS-2B) showed a greater genotoxic effect of
406 $PM_{0.5}$ extracts in winter samples (winter I and II) than A549 (Figure 2). In particular, two samples
407 from Torino (TO1 and TO2), three samples from Brescia (BS1, BS3 and BS4) and 2 samples from
408 Pisa (PI3 and PI4) in winter I and one sample from Brescia (BS1) in winter II showed significant
409 DNA damage, although only at the highest tested concentration (50 m^3). The highest genotoxic effect
410 was observed in Brescia samples. No dose-response relationship was observed for $PM_{0.5}$ extracts
411 except for one sample for Torino (TO1). The genotoxic effects observed for the Brescia, Torino, and
412 Pisa samples were related to the higher concentration of $PM_{0.5}$ reported in these samples and to the
413 higher level of chemical contamination (PAHs and nitro-PAHs). The linear regression used to
414 investigate the associations between DNA damage and air pollutants confirmed a significant
415 relationship between DNA damage and $PM_{0.5}$ ($rS = 0.60$, $p < 0.01$), PAHs ($rS = 0.69$, $p < 0.01$) and
416 nitro-PAHs ($rS = 0.68$, $p < 0.01$) concentrations.
417 However, the genotoxic effect reported in our study was lower than that observed in the study of
418 Velali (2016) performed on $PM_{0.5}$ collected in Thessaloniki. The difference in the genotoxic effect
419 could be related to the different pollution characteristics of the sampling sites, an urban centre located
420 in relative proximity of industrial sources, with a poor dispersion of air pollutants and a high level of
421 air contaminants. Moreover, the lower concentration of $PM_{0.5}$ per m^3 observed in our samples may
422 have contributed to the lower biological response in the presence of low levels of chemical pollutants.
423 Considering the PM_{10} fractions, some studies found that all particle size fractions induced DNA
424 damage in A549 cells, with the finer fractions (< 0.65 μm) inducing the highest damage (Healey et
425 al, 2005). In the study of Velali et al. (2016), the DNA damage (mean mass normalized) did not
426 change substantially, with the particle size being relatively higher in the 0.49-0.97 size range. This
427 behaviour could be related to the chemical pollution of the different fractions. As reported in the study
428 of Topinka et al. (2015), PAHs are mostly found to be associated with particles less than 1 μM, but
429 both the 0.5-1 μm fraction and the < 0.5 μm fraction contained high levels of PAHs, justifying the
430 genotoxic effect of fractions other than < 0.5 μm.
431 Comparing the results obtained with the comet assay using BEAS-2B and the Ames test, the
432 genotoxic effect was reported in the same samples that induced the higher mutagenic effect using the

Ames test, confirming the agreement between the two biological tests (YG1021 -S9 rS = 0.62, $p < 0.01$; YG1021 +S9 rS = 0.60, $p < 0.01$). However, with respect to the comet assay, the Ames test indicated a higher sensitivity, showing a biological effect at low levels of air pollutants with a different level of response in relation to small differences in pollutant concentration. The higher sensitivity of the Ames test than the comet assay was also reported in other studies for $PM_{2.5}$ or PM_{10} extracts (de Brito et al., 2013; ElAssouli et al., 2007). Due to the specificity of the genotoxic profile of chemical mutagens, which rarely affect different endpoints with the same efficiency, the two test used are expected to work in a complementary way, providing only partially overlapping results. Considering the two cell lines used for the comet assay, the different distribution patterns of genotoxicity among A549 and BEAS-2B after exposure to $PM_{0.5}$ extracts confirmed that the cell lines respond differently to genotoxic agents, as reported by other authors (Cavallo et al., 2013; Teoldi et al., 2017; Zhang et al., 2017). Moreover, the results obtained indicated the higher sensitivity of BEAS-2B cells with respect to A549, confirming that $PM_{0.5}$ can induce genotoxicity in normal cells, whereas cancer cells can be resistant to its adverse effects.

3.4.2 Cytokinesis-block MN test

The results of the micronucleus test using A549 cells treated with $PM_{0.5}$ organic extracts showed values similar to those of the negative control at each testing dose for both winter (Figure 3) and spring samples (Table S4) from all the towns, indicating there was no chromosomal damage detected in the considered cells. In our study, cell viability, as evaluated by the Trypan blue dye exclusion test, was always higher than 60% for all treatments. Since the cytotoxicity did not exceed the limits specified in the OECD guidelines for the in vitro micronucleus test on mammalian cell (i.e., 55 ± 5% cytotoxicity) (OECD, 2010) we considered the genotoxic response not influenced by cytotoxicity (Tables S5 and S6). Moreover, because overall cytotoxicity in cell cultures is the consequence of both cell death and cytostasis, we have also calculated the Cytokinesis-Block Proliferation Index (CBPI), as indicated in the OECD guidelines (OECD, 2010). Obtained data showed that cell proliferation was not influenced by exposure to $PM_{0.5}$ organic extracts (Tables S7 and S8).

The absence of genotoxicity with the micronucleus test confirmed the low genotoxic effect of $PM_{0.5}$ samples as also reported with the comet assay. A lower number of positive responses in the micronucleus test compared to the comet assay was also reported in other studies on PM organic extracts (Bocchi et al., 2016; Lemos et al., 2016). The authors suggested that most of the damage observed can still be repaired because the associated clastogenicity was not found in most of the samples. It is important to emphasize that genotoxicity and mutagenicity tests often give different results (Bocchi et al., 2016). Thus, the discrepancy among the tests used in this study should not be

considered as an inconsistency, but rather a consequence of the fact that the test methods address different genetic endpoints.

4. Conclusions

The results of the *in vitro* tests performed in the MAPEC_LIFE study showed that $PM_{0.5}$ samples induced low mutagenic and genotoxic effects. Although the biological effects were low, they were associated with levels of $PM_{0.5}$, PAHs and nitro-PAHs, which vary according to season and town of residence.

The lower biological effect observed in the spring season compared to winter underlines the importance of $PM_{0.5}$ chemical composition and the necessity of reducing $PM_{0.5}$ concentration to protect human health. Many epidemiological studies on other PM fractions demonstrated that a small reduction of PM_{10} or $PM_{2.5}$ can decrease premature deaths, mortality and hospital admissions for respiratory and cardiovascular disease and increase life expectancy, confirming these findings (ERS, 2010; Pope et al., 2009).

In agreement with other studies, the results obtained, emphasized the need to use a battery of assays for genotoxicity screening of air pollutants confirming that only one test could lead to a loss of information about genotoxic and mutagenic activity of airborne pollutants, as observed with the MN test. Other insights such as DNA repair study with comet assay could help to understand the different response of the biological tests (comet assay vs MN test) to PM extracts.

In contrast, the *Salmonella*/microsome assay proved to sensitively and efficiently characterize the mutagenicity of $PM_{0.5}$ samples, and the analyses of $PM_{0.5}$ using the comet assay could broaden the levels of response, complementing the findings of the *Salmonella*/microsome assay. The BEAS-2B cell line showed a greater sensitivity with respect to A549 cells (comet assay) when used with low contaminated $PM_{0.5}$ samples, and the YG1021 strain better characterized (Ames test) the mutagenicity of $PM_{0.5}$ samples compared to other strains. These findings confirmed that these models can represent the most suitable cellular models for the study of the *in vitro* effects of $PM_{0.5}$.

Historical trends confirm a decrease in the PM_{10} concentration in Italian towns, and the biological effects detected in this study were generally low. Nevertheless, it is important to further investigate the finest fractions of PM, which, also in this study, represent a relevant percentage of PM_{10}, taking into account its chemical composition and the biological effects induced. In fact, the results obtained confirmed that monitoring $PM_{0.5}$ itself could not provide sufficient information about the toxic compounds bound to the particles.

This is a relevant issue considering that different climatic conditions varying from one year to another can cause peaks of PM that could lead to different results from those observed.

The genotoxicity results evaluated in this study also require further investigations focusing on longer monitoring campaigns to better characterize the role of the $PM_{0.5}$ fraction in the determination of the biological effects in the five towns and in different climatic conditions. Moreover, further investigation of the nature of the chemical compounds and their association with the measured genotoxicity and epigenetic effects of $PM_{0.5}$ in comparison with the other PM_{10} fractions will be the aim of our future studies.

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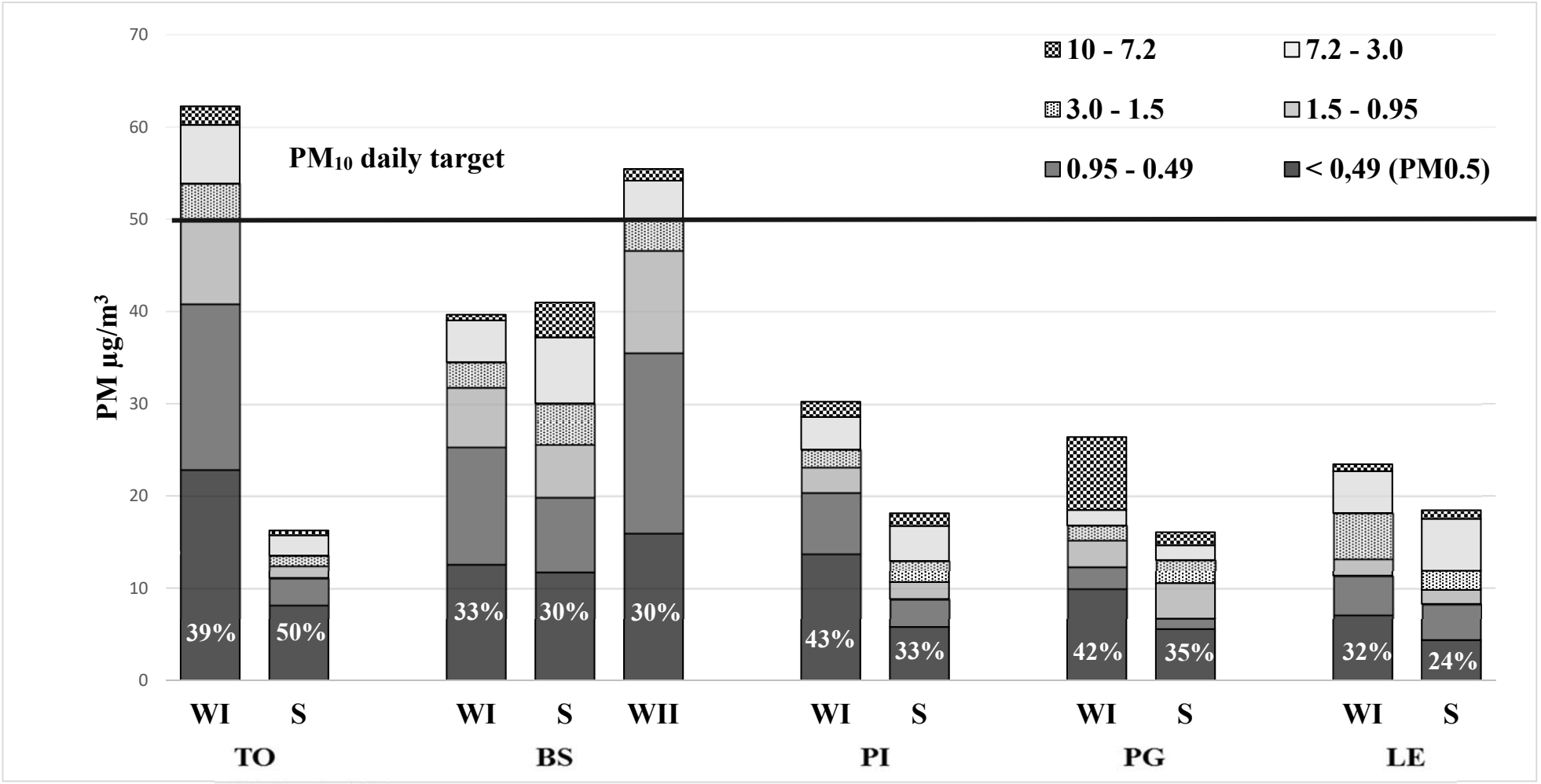
Figure 1. PM_{10} mass concentration and its fractions measured in the samples from the five towns. Data are reported as mean value of the 3-4 samples of each town in winter I (WI), spring (S) and winter II (WII). The percentages reported in the bars represent the proportion of $PM_{0.5}$ in the PM_{10} mass.



TO=Torino; BS=Brescia; PI=Pisa; PG=Perugia; LE=Lecce

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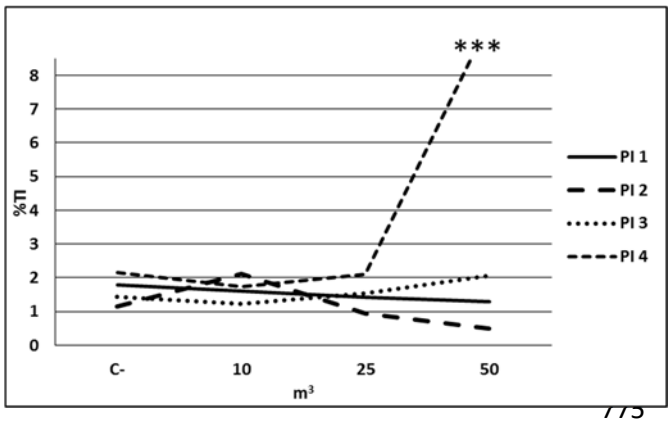
774 a)



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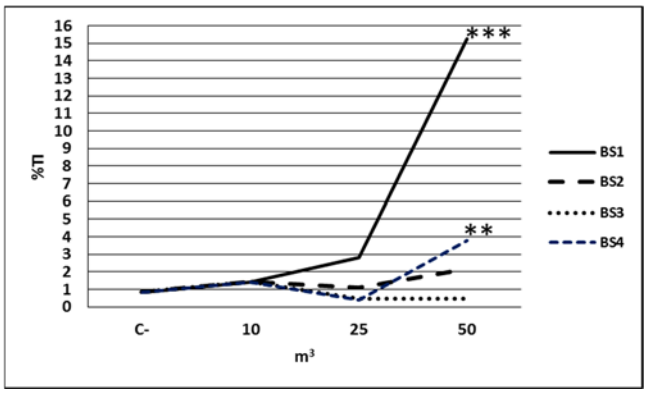

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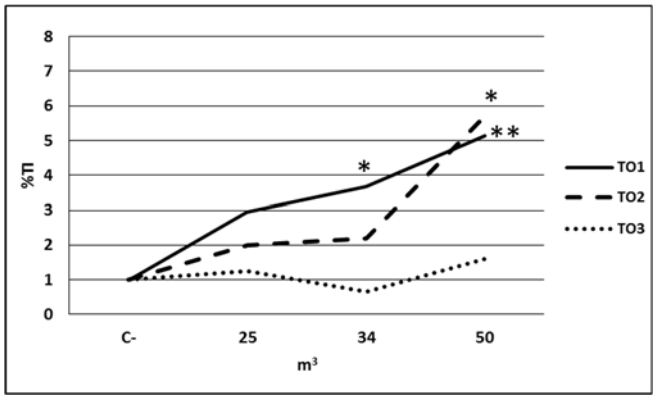

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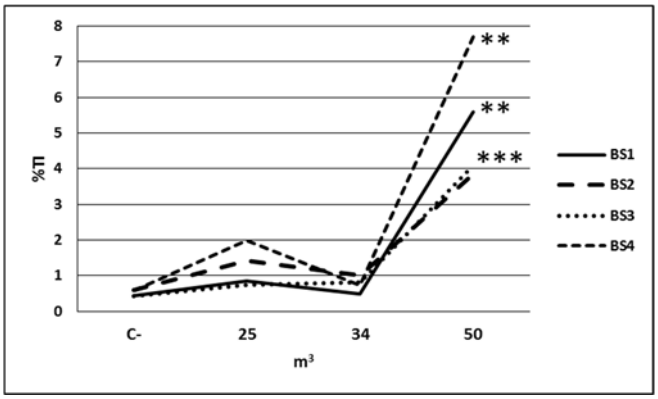

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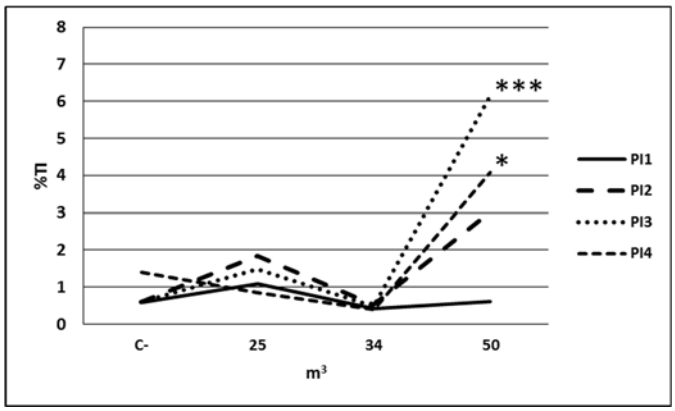
778 e)



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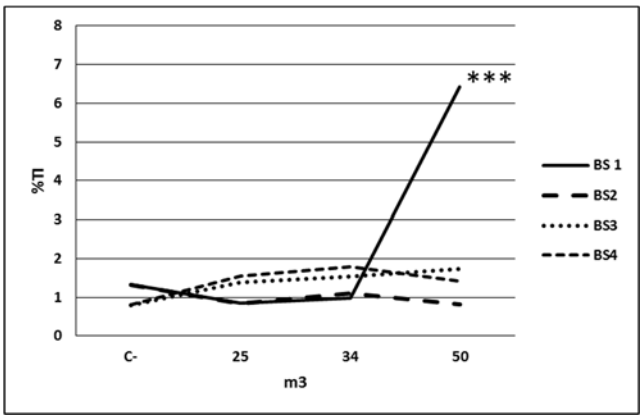

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780 **Figure 2.** Genotoxic effect (% tail DNA) in A549 cells and BEAS-2B cells exposed to $PM_{0.5}$ organic extracts
781 of winter I and II evaluated by comet assay. ***p<0.001, **p<0.01 *vs*. control cells (C-) according to ANOVA
782 combined with Dunnett's *post hoc* test. a) Pisa, winter I, A549 b) Brescia, winter II, A549 c) Torino, winter I,
783 BEAS-2B d) Brescia, winter I, BEAS-2B e) Pisa, winter I, BEAS-2B; f) Brescia, winter II, BEAS-2B.
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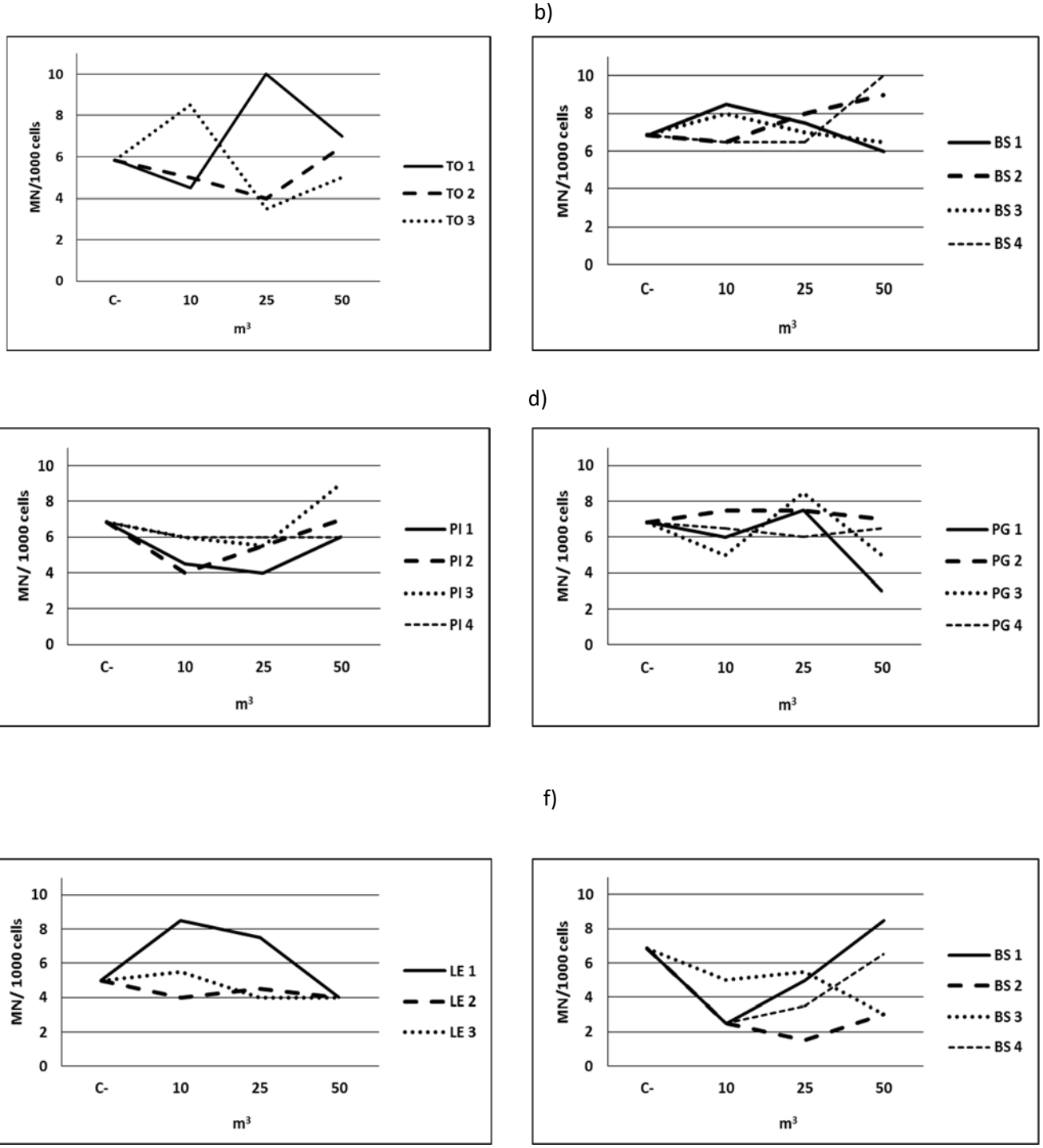

Figure 3. Genotoxic effect (MN/1000 cells) in A549 cells exposed to $PM_{0.5}$ organic extracts of winter I and II evaluated by cytokinesis-block MN test. C-: control cells; a) Torino, winter I; b) Brescia, winter I; c) Pisa, winter I; d) Perugia, winter I; e) Lecce, winter I; f) Brescia, winter II.

798 **Table 1.** Concentration of PAHs and nitro-PAHs in the $PM_{0.5}$ organic extracts sampled in winter I (WI), spring (S) and winter II (WII) in Torino, Brescia, Pisa,
799 Perugia and Lecce.

Town	Season	Site	$PM_{0.5}$ concentration ($\mu g/m^3$)	Σ PAHs[a] (ng/m^3)	B(a)P (ng/m^3)	Σ Carcinogenic PAHs[b] (ng/m^3)	Σ nitro-PAHs[a] (ng/m^3)
Torino	WI	1	22.44	12.17	1.29	6.90	0.13
Torino	WI	2	20.96	7.82	0.83	4.46	0.21
Torino	WI	3	25.12	6.13	0.60	3.46	0.16
Mean value			**22.84**	**8.71**	**0.91**	**4.94**	**0.17**
Mean value (ng/µg)			/	**0.39**	**0.04**	**0.22**	**0.75**
Brescia	WI	1	6.46	3.86	0.48	2.16	0.05
Brescia	WI	2	14.38	14.72	1.52	7.69	0.05
Brescia	WI	3	10.06	4.17	0.38	2.12	0.11
Brescia	WI	4	19.47	5.79	0.56	3.20	0.13
Mean value			**12.59**	**7.14**	**0.74**	**3.79**	**0.08**
Mean value (ng/µg)			/	**0.58**	**0.06**	**0.31**	**0.74**
Pisa	WI	1	3.69	0.55	0.03	0.23	0.02
Pisa	WI	2	12.34	3.63	0.42	2.05	0.08
Pisa	WI	3	21.09	8.47	0.90	5.24	0.45
Pisa	WI	4	17.80	2.87	0.26	1.62	0.16
Mean value			**13.73**	**3.88**	**0.40**	**2.28**	**0.18**
Mean value (ng/µg)			/	**0.25**	**0.02**	**0.14**	**1.04**
Perugia	WI	1	11.73	4.77	0.50	2.63	0.04
Perugia	WI	2	13.47	4.98	0.52	2.84	0.15
Perugia	WI	3	6.51	2.21	0.18	1.09	0.03
Perugia	WI	4	8.02	1.76	0.14	0.86	0.06
Mean value			**9.93**	**3.43**	**0.34**	**1.86**	**0.07**
Mean value (ng/µg)			/	**0.33**	**0.03**	**0.18**	**0.69**
Lecce	WI	1	6.36	1.17	0.06	0.57	0.02
Lecce	WI	2	9.39	2.76	0.17	1.50	0.06
Lecce	WI	3	5.61	0.77	0.04	0.35	0.02
Mean value			**7.12**	**1.57**	**0.09**	**0.81**	**0.03**
Mean value (ng/µg)			/	**0.21**	**0.01**	**0.10**	**0.44**
Torino	S	1	9.25	0.61	0.02	0.19	0.02
Torino	S	2	8.30	0.50	0.01	0.12	0.02
Torino	S	3	7.02	0.59	0.02	0.20	0.02

Mean value			**8.19**	**0.57**	**0.02**	**0.17**	**0.02**
Mean value (ng/µg)			/	**0.07**	**<0.01**	**0.02**	**0.28**
Brescia	S	1	6.48	0.42	0.01	0.11	0.02
Brescia	S	2	14.54	0.64	0.02	0.23	0.02
Brescia	S	3	9.02	0.37	0.01	0.08	0.02
Brescia	S	4	17.08	0.35	0.01	0.06	0.02
Mean value			**11.78**	**0.44**	**0.01**	**0.12**	**0.02**
Mean value (ng/µg)			/	**0.04**	**<0.01**	**0.01**	**0.17**
Pisa	S	1	4.40	0.34	0.01	0.09	0.02
Pisa	S	2	6.36	0.38	0.01	0.11	0.02
Pisa	S	3	9.68	0.85	0.02	0.39	0.02
Pisa	S	4	2.72	0.39	0.01	0.11	0.02
Mean value			**5.79**	**0.49**	**0.01**	**0.18**	**0.02**
Mean value (ng/µg)			/	**0.09**	**<0.01**	**0.03**	**0.38**
Perugia	S	1	7.86	0.84	0.04	0.28	0.02
Perugia	S	2	4.79	0.57	0.02	0.13	0.02
Perugia	S	3	6.50	0.52	0.01	0.09	0.02
Perugia	S	4	2.97	0.44	0.01	0.04	0.02
Mean value			**5.53**	**0.59**	**0.02**	**0.14**	**0.02**
Mean value (ng/µg)			/	**0.11**	**<0.01**	**0.02**	**0.36**
Lecce	S	1	1.83	0.56	0.02	0.18	0.02
Lecce	S	2	5.90	0.61	0.02	0.21	0.02
Lecce	S	3	5.41	0.56	0.02	0.19	0.02
Mean value			**4.38**	**0.58**	**0.02**	**0.19**	**0.02**
Mean value (ng/µg)			/	**0.17**	**0.01**	**0.06**	**0.53**
Brescia	WII	1	19.92	8.41	0.57	4.10	0.03
Brescia	WII	2	21.46	5.95	0.59	3.27	0.04
Brescia	WII	3	9.11	4.87	0.48	2.59	0.04
Brescia	WII	4	13.35	7.28	0.84	3.92	0.04
Mean value			**15.96**	**6.63**	**0.62**	**3.47**	**0.04**
Mean value (ng/µg)			/	**0.44**	**0.04**	**0.23**	**0.28**

[a]CRM percentage recovery was found to be between 48% and 147% and the uncertainty was between 24 and 26%

[b]∑ Carcinogenic PAHs: benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene.

Table 2. Mutagenic activity of $PM_{0.5}$ organic extracts in *S. typhimurium* TA100, TA98, TA98NR, and YG1021 strains with and without metabolic activation (±S9) expressed as net revertants/m^3 of air equivalent. WI=winter I; S=spring; WII=winter II.

Sites	Net revertants/m^3															
	-S9								+S9							
	TA100		TA 98		TA98NR		YG1021		TA100		TA98		TA98NR		YG1021	
	WI	S	WI	S	WI	S	WI	S	WI	S	WI	S	WI	S	WI	S
Torino																
1	4.8	-	1.3	-	1.0	-	30.8	1.7	-	-	1.5	-	0.9	-	34.3	1.6
2	3.0	-	1.5	-	1.2	-	16.5	2.3	-	-	1.9	-	0.9	-	35.8	1.5
3	-	-	0.9	-	0.6	-	17.7	0.7	-	-	1.0	-	0.7	-	36.6	0.8
Brescia																
1	-	-	0.5	-	-	-	7.7	0.8	-	-	-	-	-	-	12.9	0.7
2	-	-	0.4	-	-	-	10.7	1.8	-	-	0.9	-	-	-	16.8	2.6
3	-	-	-	-	-	-	9.7	0.9	-	-	0.6	-	-	-	14.6	1.1
4	-	-	0.6	-	-	-	7.6	0.8	-	-	1.0	-	-	-	20.0	1.0
Pisa																
1	-	-	-	-	-	-	1.9	0.9	-	-	-	-	-	-	3.0	1.0
2	-	-	-	-	-	-	2.9	0.4	-	-	0.7	-	-	-	7.0	0.6
3	-	-	-	-	-	-	7.4	2.3	-	-	0.9	-	-	-	14.3	3.5
4	-	-	0.8	-	-	-	6.8	1.0	-	-	0.8	-	-	-	19.8	0.9
Perugia																
1	-	-	0.5	-	-	-	7.2	7.1	-	-	0.9	-	-	-	16.4	1.5
2	-	-	0.3	-	-	-	7.1	0.6	-	-	0.6	-	-	-	17.8	17.8
3	-	-	-	-	-	-	3.0	0.8	-	-	-	-	-	-	7.2	7.2
4	-	-	0.4	-	-	-	3.4	0.4	-	-	-	-	-	-	10.1	0.1
Lecce																
1	-	-	0.4	-	-	-	1.7	1.7	-	-	-	-	-	-	4.8	4.7
2	-	-	0.5	-	0.4	-	4.5	4.5	-	-	0.6	-	-	-	8.2	8.2
3	-	-	-	-	-	-	1.4	1.4	-	-	-	-	-	-	2.5	2.5

	-S9				+S9			
	TA100	TA98	TA98NR	YG1021	TA100	TA98	TA98NR	YG1021
	WII	WII	WII	WII	WII	WII	WII	WII
Brescia								
1	-	0.2	-	5.8	-	0.6	-	8.9
2	-	0.5	-	11.1	-	1.0	-	9.8
3	-	0.5	-	5.4	-	0.7	-	10.8
4	-	0.3	-	6.4	-	0.7	-	14.6