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

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(Article begins on next page)

1 **MUTAGENIC AND GENOTOXIC EFFECTS INDUCED BY PM<sub>0.5</sub> OF DIFFERENT**  
2 **ITALIAN TOWNS IN HUMAN CELLS AND BACTERIA: THE MAPEC\_LIFE STUDY**

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36 **Abstract**

37 Particulate matter (PM) is considered an atmospheric pollutant that mostly affects human health. The  
38 finest fractions of PM (PM<sub>2.5</sub> or less) play a major role in causing chronic diseases.

39 The aim of this study was to investigate the genotoxic effects of PM<sub>0.5</sub> collected in five Italian towns  
40 using different bioassays. The role of chemical composition on the genotoxicity induced was also  
41 evaluated.

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

44 PM<sub>10</sub> samples were collected in 2 seasons (winter and spring) using a high-volume multistage cascade  
45 impactor. The results showed that PM<sub>0.5</sub> represents a very high proportion of PM<sub>10</sub> (range 10-63%).

46 PM<sub>0.5</sub> organic extracts were chemically analysed (PAHs, nitro-PAHs) and tested by the comet assay  
47 (A549 and BEAS-2B cells), MN test (A549 cells) and Ames test on *Salmonella* strains (TA100,  
48 TA98, TA98NR and YG1021).

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

57 The results obtained showed that it is important to further investigate the finest fractions of PM, which  
58 represent a relevant percentage of PM<sub>10</sub>, taking into account the chemical composition and the  
59 biological effects induced.

60

61

62 *Keywords: PM<sub>0.5</sub>, mutagenicity, genotoxicity, PAHs, nitro-PAHs*

63

64 **Capsule**

65 Results highlighted the importance to further investigate the finest fractions of PM, which represent  
66 a relevant percentage of PM<sub>10</sub>, taking into account its chemical composition and the biological effects  
67 induced.

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69

## 70 **1. Introduction**

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

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

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

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

90 The effects of exposure to mixtures of chemicals, such as PM, are difficult to evaluate because the  
91 different chemical compounds can interact with synergistic, antagonistic or additive effects (USEPA,  
92 2008). For a more complete evaluation of the health risk of human exposure, short-term bioassays  
93 were used to study the biological effects of chemical pollutants in urban PM (Ceretti et al., 2015; de  
94 Brito et al., 2013; Dumax-Vorzet et al., 2015; Lemos et al., 2012; Lepers et al., 2014; Palacio et al.,  
95 2016; Traversi et al., 2015). PM<sub>1</sub>, quasi-ultrafine particles (PM<sub>0.5</sub>; PM<sub>0.4</sub> and PM<sub>0.3</sub>) and UFPs (PM<sub>0.1</sub>)  
96 have been less extensively studied than fine (PM<sub>2.5</sub>) and coarse (PM<sub>10-2.5</sub>) particles. Besides the  
97 increasing epidemiological data on particles with a diameter less than 1 µm, there are still few studies  
98 on the biological effects of these fractions. Some studies have shown that UFPs are able to induce  
99 oxidative stress (Gasparotto et al., 2013), inflammation (Muller et al., 2010), apoptosis and necrosis  
100 (Sydlik et al., 2006). Moreover, cytotoxic effects (Borgie et al., 2015), release of cytokine/interleukin  
101 release (Longhin et al., 2013) and dioxin-like activity (Wichmann et al., 2009) have also been reported  
102 for quasi-ultrafine particles. However, only a few recent studies investigated the genotoxic or  
103 mutagenic effects of these finest fractions, and only some endpoints were taken into account with a

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

107 The present study was included in the MAPEC\_LIFE project (LIFE12 ENV/IT/000614), a  
108 multicentre Italian cohort study funded by the European Union's LIFE+ Programme that aims to  
109 evaluate the associations between air pollution (including PM) and early biological effects in 6-8-  
110 year-old Italian children. Details of the study design have been described elsewhere (Feretti et al.,  
111 2014). Briefly, oral mucosa cells of 1149 children recruited from first grade schools were collected  
112 to evaluate the frequency of MN and DNA damage. Some results on subject characteristics, diet in  
113 particular, and frequency of MN in their buccal cells have already been published (Bagordo et al.,  
114 2017; Grassi et al., 2016; Villarini et al., 2018; Zani et al., 2016). The study was conducted in different  
115 schools of five Italian towns (Figure S1) characterized by different levels of air pollution. In  
116 particular, Torino and Brescia are located in the Padana Plain in the north of Italy (one of the most  
117 polluted areas in Europe), Pisa and Perugia in central Italy (medium-low pollution area) and Lecce in  
118 southern Italy (low pollution area) (EEA, 2017; ISPRA, 2015). To evaluate children's exposure to  
119 urban air pollution, PM<sub>0.5</sub> was collected near each school on the same days as the biological sampling.  
120 The purpose of this work was to investigate the *in vitro* mutagenic and genotoxic effects of PM<sub>0.5</sub>  
121 collected in the MAPEC\_LIFE study using different short-time bioassays (Ames test, comet assay,  
122 micronucleus test). The spatial and seasonal variations of the genotoxicity induced by the organic  
123 extracts of PM<sub>0.5</sub> were evaluated, and the role of chemical composition on the mutagenic and  
124 genotoxic effect of PM<sub>0.5</sub> samples was also investigated.

125

## 126 **2. Materials and methods**

127

### 128 **2.1 Airborne particulate sampling and gravimetric analysis**

129 PM<sub>10</sub> fractions were collected in 18 sites located in the five towns involved in the MAPEC\_LIFE  
130 study. The description of the sampling sites is reported in Figure S1. The sampling was performed in  
131 3 consecutive 24-hour periods, for a total of 72 sampling hours, using a Sierra-Andersen high-volume  
132 multistage cascade impactor (AirFlow PM10-HVS sampler, AMS Analitica Srl, Pesaro, Italy) at a  
133 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,  
134 1.5-0.95, 0.95-0.49, and <0.49 µm (PM<sub>0.5</sub>). All filters were pre- and post-conditioned and weighed at  
135 controlled temperature and humidity, as previously reported (Schilirò et al., 2016).

136 The samplings were performed during two seasons, winter (November 2014/March 2015-winter I)  
137 and late spring (April/June 2015). Air sampling was repeated the following winter (November  
138 2015/January 2016-winter II) only in Brescia.

139

## 140 **2.2 Extraction of PM<sub>0.5</sub> components**

141 After gravimetric analyses, the PM<sub>0.5</sub> filters (three for each site) were pooled to obtain a total of 40  
142 samples. Particles were Soxhlet extracted with 200 mL of n-hexane-acetone (4:1) for 6 h to recover  
143 organic extractable compounds. Each extract was separated into different aliquots destined for  
144 chemical analysis and biological tests. The organic extracts were concentrated by rotary evaporation.  
145 For the biological tests, the samples were re-suspended in dimethyl sulfoxide (DMSO) (2 m<sup>3</sup>/μL).

146

## 147 **2.3 Chemical analysis of PM<sub>0.5</sub> organic extracts**

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

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

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

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

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

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

167

## 168 **2.4 *Salmonella*/microsome (Ames) test on PM<sub>0.5</sub> organic extracts**

169 The Ames test (Maron and Ames, 1983) was used to evaluate the mutagenicity of PM<sub>0.5</sub> organic  
170 extracts collected in all towns. The organic extracts were tested in duplicate at increasing doses (10,  
171 25 and 50 m<sup>3</sup> of air equivalent/plate) with different *S. typhimurium* strains (TA100, TA98, TA98NR,  
172 YG1021). The TA100 and TA98 strains specifically detect base-substitution and frameshift mutations  
173 (Claxton et al., 2004). The YG1021 strain shows efficient detection of mutagenic nitroarenes and the  
174 TA98NR strain shows a reduced mutagenicity, proportional to the amount of nitroarenes present in  
175 the extract (Traversi et al., 2011).

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

182

## 183 2.5 Cell culture

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

191

## 192 2.6 Comet assay on PM<sub>0.5</sub> organic extracts

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

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

## 207 208 **2.7 Cytokinesis-block MN (CBMN) test on PM<sub>0.5</sub> organic extracts**

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

## 220 221 **2.8 Statistical analysis**

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

230



## 231 **3 Results and discussion**

232

### 233 **3.1 Size distribution of PM mass concentrations**

234 The mass of PM samples (pooled filters) and total cubic meters of air sampled were reported in detail in  
235 Supporting Information (Table S1). The mean concentrations of PM<sub>10</sub> and the other PM fractions  
236 obtained in the samples of the five towns in winter and spring seasons are reported in Figure 1.

237 The results of the gravimetric analysis showed that in winter samples, the mean PM<sub>10</sub> concentrations  
238 were lower than the daily target of 50 µg/m<sup>3</sup> set by the European Air Quality Directive 2008/50/EU,  
239 except for some samples from Torino and Brescia. Often, in Italy, high PM<sub>10</sub> values are observed  
240 during winter in towns located in the north of Italy, particularly in the Padana Plain, given the  
241 widespread air pollution and the general weak dispersion rate due to the territory conformation  
242 (Cadum et al., 2009; EEA, 2017).

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

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

256 With respect to winter 2014, a significant decrease in PM<sub>10</sub> concentration was observed in spring  
257 samples (spring vs winter p < 0.001, t-test). A different trend was observed only for some samples of  
258 Brescia (winter I vs spring). The decrease of PM<sub>10</sub> in the warm season has been generally observed  
259 in urban environments (Schilirò et al., 2016).

260 Considering the distribution of the size fractions of PM<sub>10</sub> mass in winter (Figure 1), a high particle  
261 concentration was present, especially for PM<sub>0.5</sub>, which represented a very high proportion of PM<sub>10</sub>,  
262 accounting from a minimum of 20% to a maximum of 63% of the different samples. Additionally,  
263 the fraction 0.49-0.95 represented a considerable fraction of PM<sub>10</sub> although it generally showed a

264 lower percentage with respect to PM<sub>0.5</sub>.

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

270 The results of the statistical analyses showed a significant correlation between PM<sub>10</sub> and PM<sub>0.5</sub>  
271 concentration in both seasons ( $rS = 0.80$ ,  $p < 0.001$  and  $rS = 0.63$ ,  $p < 0.001$  in winter and spring  
272 respectively).

273 Although a significant reduction in PM<sub>0.5</sub> concentration was observed from winter to spring in all  
274 samples ( $p = 0.001$ , t-test), PM<sub>0.5</sub> in spring also represented a considerable fraction of PM<sub>10</sub>,  
275 accounting for a minimum of 10% to a maximum of 56% in the different samples.

276 Moreover, analysing the concentration of PM<sub>0.5</sub> by sampling sites ( $n=18$ ), a high variability of PM<sub>0.5</sub>  
277 percentage was observed in the same sampling site in both seasons and from the samples of the same  
278 town.

279 In comparison with the few studies published on the PM<sub>0.5</sub> fraction, the concentrations of PM<sub>0.5</sub>  
280 observed in the Torino and Brescia samples in winter were similar to those observed in La Plata  
281 (Argentina) ( $21 \mu\text{g}/\text{m}^3$ ) (Wichmann et al., 2009). Otherwise, the PM<sub>0.5</sub> values recorded in the samples  
282 of the other towns were similar to those found in the urban site of Prague ( $9.1 \mu\text{g}/\text{m}^3$ ) (Topinka et al.,  
283 2013). However, the levels of PM<sub>0.5</sub> found in this study were generally lower than those found in  
284 other highly polluted European sites (Topinka et al., 2015) or other urban sites (Monarca et al., 1997,  
285 Velali et al., 2016).

286 The highest concentration of PM<sub>0.5</sub> during winter in comparison to spring summer was reported also  
287 in other studies for ultrafine or quasi-ultrafine fractions (Perrone et al., 2010; Perrone et al., 2013;  
288 Jalava et al., 2015; Velali et al., 2016). This trend confirmed that also this fraction was strongly  
289 influenced by seasonal meteorology in the north of Italy, where condition of atmospheric stability  
290 cause high concentrations of atmospheric pollutants (Perrone et al., 2010; Perrone et al., 2013).

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

298 day-to-day variability of PM<sub>10</sub> 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<sub>0.5</sub>**

303 The chemical analysis of the PM<sub>0.5</sub> 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<sub>0.5</sub> 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<sub>0.5</sub> (µg/m<sup>3</sup>), as confirmed by the statistical analyses that indicated a linear  
311 correlation between PM<sub>0.5</sub> 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<sub>0.5</sub> 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<sub>0.5</sub> level does not always correspond to a greater quantity of pollutants for µg of PM<sub>0.5</sub>, as noted  
315 by the comparison of the chemical contamination of PM<sub>0.5</sub> in winter I and winter II in some of the  
316 Brescia samples.

317 In the spring season, as observed for PM<sub>0.5</sub> concentration, a significant decrease in PAH and nitro-  
318 PAH concentration in PM<sub>0.5</sub> 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<sub>0.5</sub> 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<sub>0.5</sub> 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<sub>0.4</sub> in another town of the Padana Plain (Milano). Considering  
326 the presence of nitro-PAHs in the PM<sub>0.5</sub> 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<sub>0.5</sub>  
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; Ladjji 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

332 related to the variability of atmospheric conditions between these seasons (Landlocz et al., 2017;  
333 Longhin et al., 2013). In particular, winter atmospheric conditions may promote accumulation of  
334 primary pollutants and the condensation of atmospheric pollutants in the particle phase due to the low  
335 temperature (Ebi and McGregor, 2008; Sisovic et al., 2008). The importance of atmospheric  
336 conditions on the level of chemical pollutants in the PM<sub>0.5</sub> fraction was also confirmed by the  
337 comparison of PAHs and nitro-PAHs for µg of PM<sub>0.5</sub> in Brescia in the two winter samples (winter I  
338 vs winter II).

339

### 340 **3.3 Mutagenicity of PM<sub>0.5</sub> samples**

341 In Table 2, the mutagenic effect of PM<sub>0.5</sub> extracts on bacteria is reported, expressed as net  
342 revertants/m<sup>3</sup> of air sampled in the TA98, TA100, TA98NR and YG1021 strains, with (+S9) and  
343 without (-S9) metabolic activation.

344 Overall, considering the four *S. typhimurium* strains, low mutagenic activity was observed with  
345 respect to the results obtained in other studies performed on PM<sub>0.5</sub> or PM<sub>2.5</sub> fractions in Torino and  
346 Brescia (Monarca et al., 1997; Traversi et al., 2009; Traversi et al., 2011).

347 In winter, the highest mutagenic activity was generally observed in the Torino and Brescia samples  
348 followed by the Pisa, Perugia and Lecce samples. The ANOVA, performed assuming mutagenicity  
349 observed with YG1021+S9 and YG1021-S9 as dependent variables and the towns as independent  
350 variables, underlined a significant difference in the mutagenic effects among the samples of the five  
351 towns ( $F = 18.201$  and  $F = 13.331$ ,  $p < 0.001$ , respectively). *Post hoc* Tukey's test confirmed the  
352 highest values of mutagenicity in the Torino samples (YG1021 ±S9 Torino samples vs  
353 Pisa/Perugia/Lecce samples  $p < 0.001$  and  $p < 0.01$  vs Brescia samples). This trend was probably  
354 related to the PM<sub>0.5</sub> concentration as confirmed by the positive correlation between mutagenic  
355 response and PM<sub>0.5</sub> level (YG1021 +S9  $rS = 0.87$ , YG1021 -S9  $rS = 0.76$   $p < 0.001$ ; TA98 +S9  $rS =$   
356  $0.75$ , TA98 -S9  $rS = 0.76$   $p < 0.01$ ). The highest mutagenicity reported for the Torino and Brescia  
357 samples was also confirmed by adjusting the data for the particle mass unit (Table S2), highlighting  
358 the worse quality of the particles—in terms of mutagenic compounds (e.g., PAHs in PM<sub>0.5</sub> samples)  
359 —and not only the higher level of PM<sub>0.5</sub> concentration for each volume unit (m<sup>3</sup>).

360 Comparing the results obtained with Brescia samples collected in winter I and winter II, despite the  
361 increase of PM<sub>0.5</sub> concentration in some samples of winter II, a similar or reduced mutagenicity was  
362 observed in winter II with respect to winter I. The lower level of chemical contamination (PAHs and  
363 nitro-PAHs) of the particles sampled in winter II was also confirmed by the lower mutagenic effect  
364 recovered after adjustment for particle mass unit.

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

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

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

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

393

### 394 **3.4 Genotoxicity of PM<sub>0.5</sub> samples**

#### 395 **3.4.1 Comet assay**

396 No genotoxic effect of PM<sub>0.5</sub> was observed using the A549 cell line in almost all winter (Figure S2)  
397 and spring (Table S3) samples at all the tested doses, except for sporadic doses of a few winter samples  
398 (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<sub>0.5</sub> (50 m<sup>3</sup>), 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<sub>0.5</sub> 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<sub>0.5</sub> 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<sup>3</sup>). The highest genotoxic effect  
410 was observed in Brescia samples. No dose-response relationship was observed for PM<sub>0.5</sub> 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<sub>0.5</sub> 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<sub>0.5</sub> (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<sub>0.5</sub> 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<sub>0.5</sub> per m<sup>3</sup> 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<sub>10</sub> 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

433 Ames test, confirming the agreement between the two biological tests (YG1021 -S9  $rS = 0.62$ ,  $p <$   
434  $0.01$ ; YG1021 +S9  $rS = 0.60$ ,  $p < 0.01$ ). However, with respect to the comet assay, the Ames test  
435 indicated a higher sensitivity, showing a biological effect at low levels of air pollutants with a  
436 different level of response in relation to small differences in pollutant concentration. The higher  
437 sensitivity of the Ames test than the comet assay was also reported in other studies for  $PM_{2.5}$  or  $PM_{10}$   
438 extracts (de Brito et al., 2013; ElAssouli et al., 2007). Due to the specificity of the genotoxic profile  
439 of chemical mutagens, which rarely affect different endpoints with the same efficiency, the two test  
440 used are expected to work in a complementary way, providing only partially overlapping results.  
441 Considering the two cell lines used for the comet assay, the different distribution patterns of  
442 genotoxicity among A549 and BEAS-2B after exposure to  $PM_{0.5}$  extracts confirmed that the cell lines  
443 respond differently to genotoxic agents, as reported by other authors (Cavallo et al., 2013; Teoldi et  
444 al., 2017; Zhang et al., 2017). Moreover, the results obtained indicated the higher sensitivity of BEAS-  
445 2B cells with respect to A549, confirming that  $PM_{0.5}$  can induce genotoxicity in normal cells, whereas  
446 cancer cells can be resistant to its adverse effects.

447

#### 448 **3.4.2 Cytokinesis-block MN test**

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

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

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

469

#### 470 **4. Conclusions**

471 The results of the *in vitro* tests performed in the MAPEC\_LIFE study showed that PM<sub>0.5</sub> samples  
472 induced low mutagenic and genotoxic effects. Although the biological effects were low, they were  
473 associated with levels of PM<sub>0.5</sub>, PAHs and nitro-PAHs, which vary according to season and town of  
474 residence.

475 The lower biological effect observed in the spring season compared to winter underlines the  
476 importance of PM<sub>0.5</sub> chemical composition and the necessity of reducing PM<sub>0.5</sub> concentration to  
477 protect human health. Many epidemiological studies on other PM fractions demonstrated that a small  
478 reduction of PM<sub>10</sub> or PM<sub>2.5</sub> can decrease premature deaths, mortality and hospital admissions for  
479 respiratory and cardiovascular disease and increase life expectancy, confirming these findings (ERS,  
480 2010; Pope et al., 2009).

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

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

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

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



501 The genotoxicity results evaluated in this study also require further investigations focusing on longer  
502 monitoring campaigns to better characterize the role of the PM<sub>0.5</sub> fraction in the determination of the  
503 biological effects in the five towns and in different climatic conditions. Moreover, further  
504 investigation of the nature of the chemical compounds and their association with the measured  
505 genotoxicity and epigenetic effects of PM<sub>0.5</sub> in comparison with the other PM<sub>10</sub> fractions will be the  
506 aim of our future studies.

507

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514

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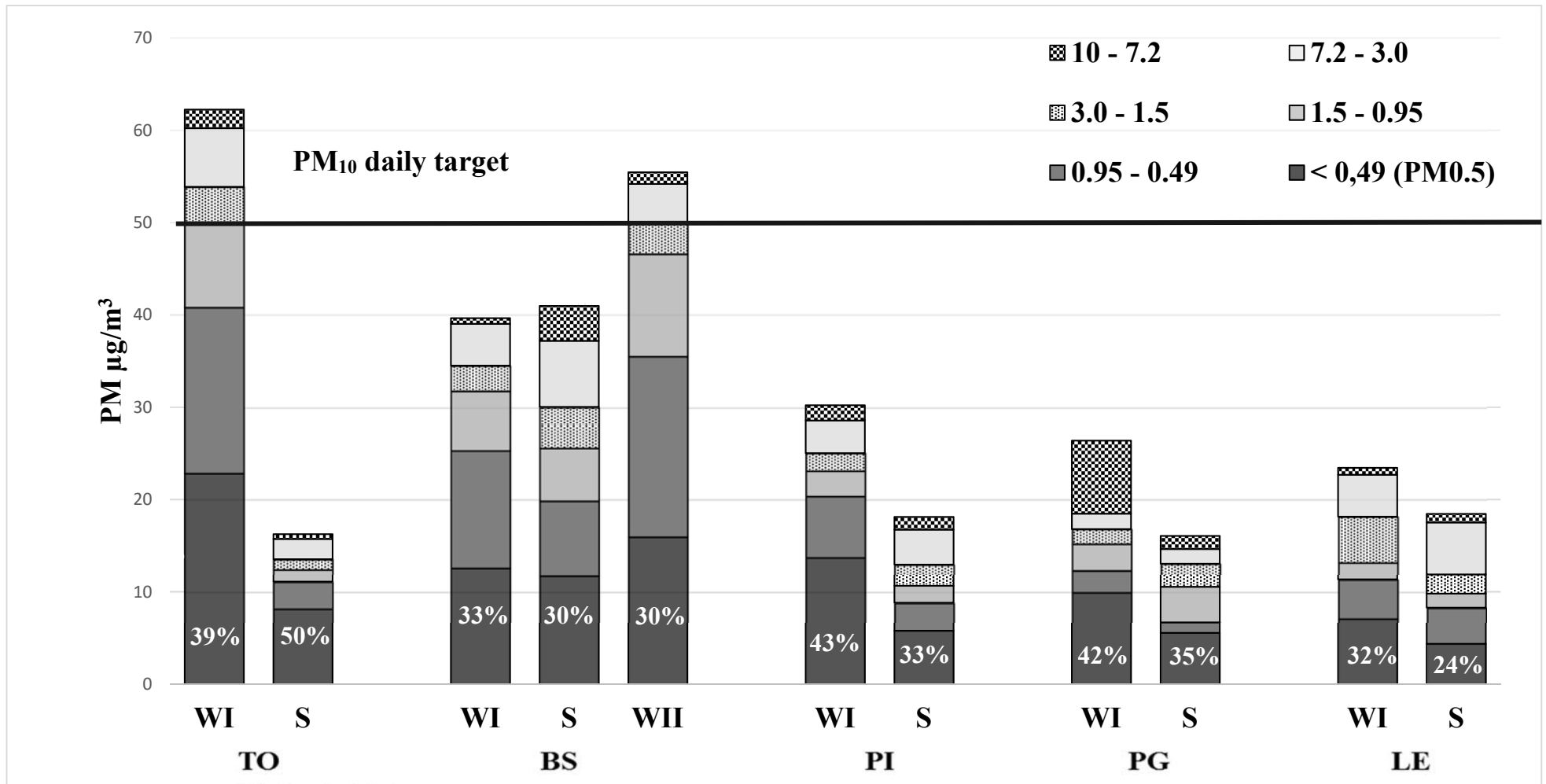
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751 **Figure 1.** PM<sub>10</sub> mass concentration and its fractions measured in the samples from the five towns. Data are reported as mean value of the 3-4 samples of each town  
 752 in winter I (WI), spring (S) and winter II (WII). The percentages reported in the bars represent the proportion of PM<sub>0.5</sub> in the PM<sub>10</sub> mass.  
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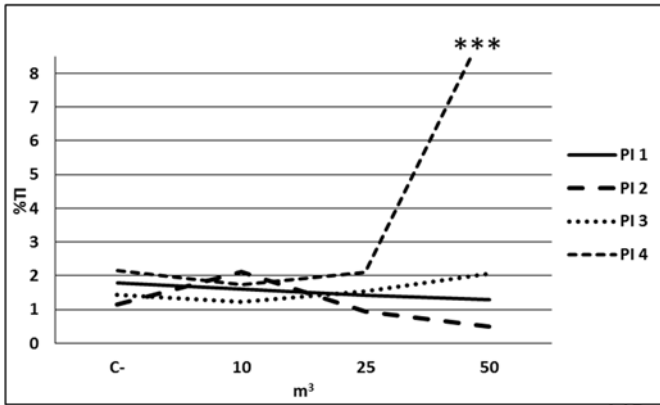


770 TO=Torino; BS=Brescia; PI=Pisa; PG=Perugia; LE=Lecce

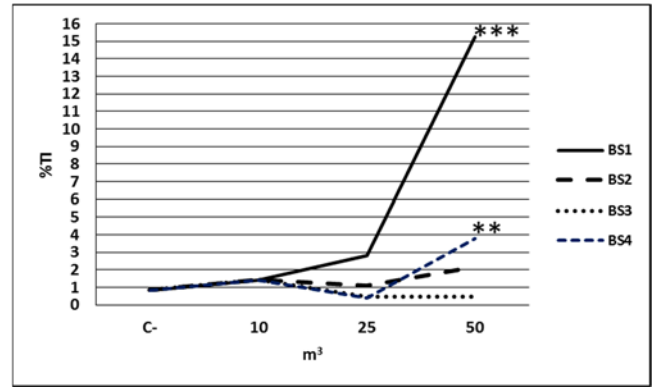
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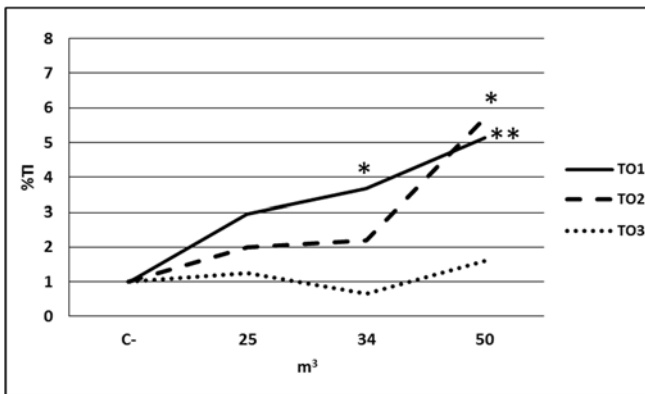
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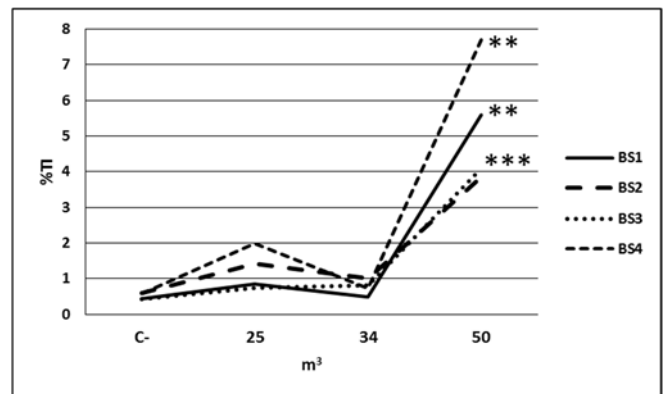
b)



776 c)

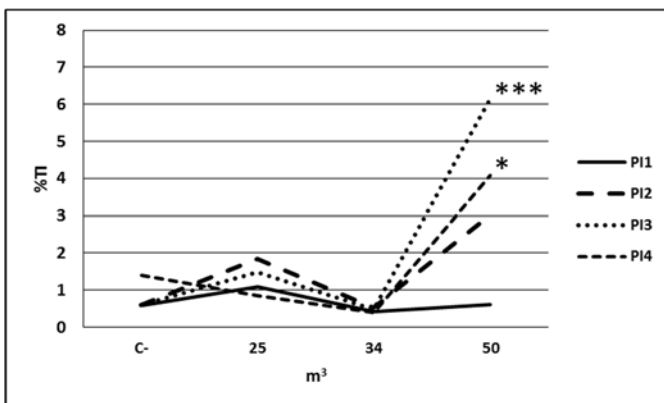


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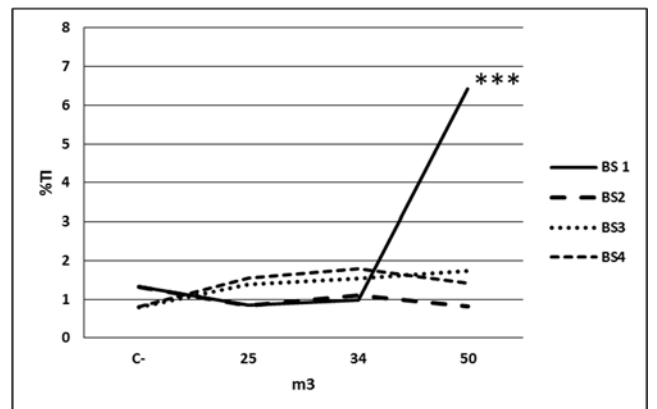


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778 e)



f)



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

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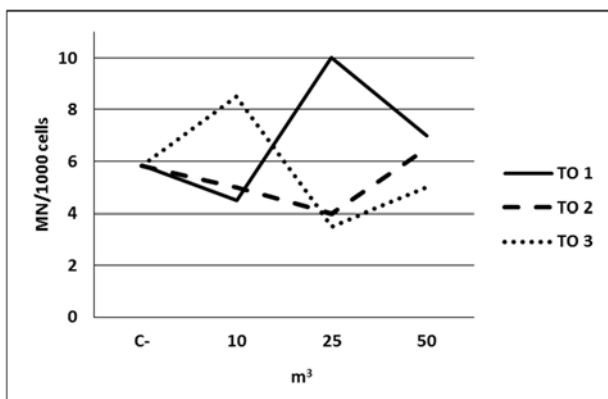
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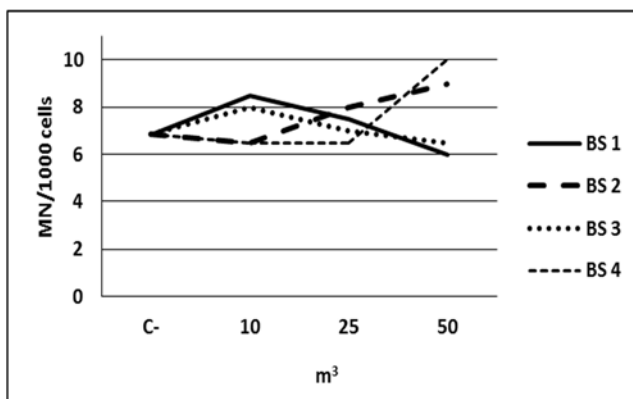
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a)



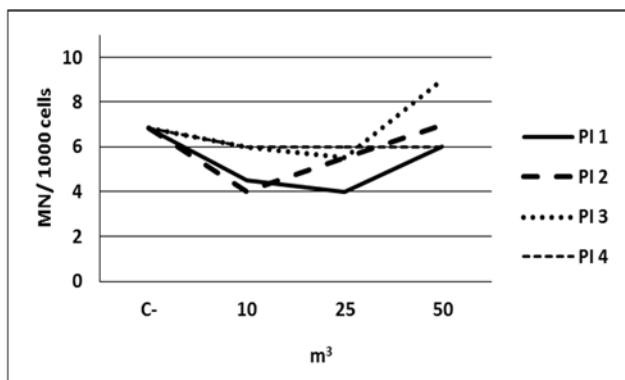
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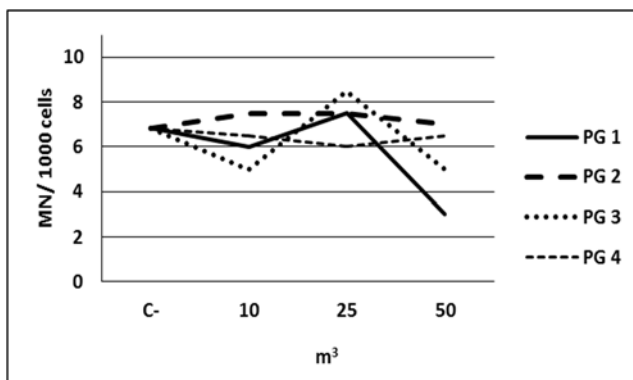
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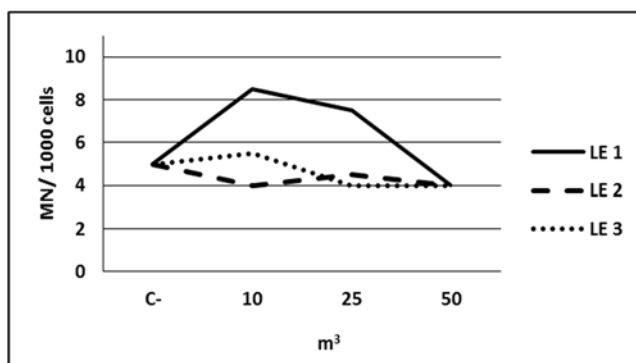


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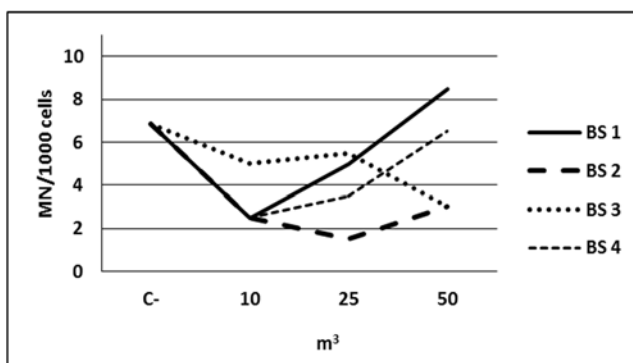
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**Figure 3.** Genotoxic effect (MN/1000 cells) in A549 cells exposed to PM<sub>0.5</sub> organic extracts of winter I and II evaluated by cytokinesis-block MN test. C-: control cells; a) Torino, winter I; b) Brescia, winter I; c) Pisa, winter I; d)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

798 **Table 1.** Concentration of PAHs and nitro-PAHs in the PM<sub>0.5</sub> 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 <sub>0.5</sub> concentration (µg/m <sup>3</sup> )	Σ PAHs <sup>a</sup> (ng/m <sup>3</sup> )	B(a)P (ng/m <sup>3</sup> )	Σ Carcinogenic PAHs <sup>b</sup> (ng/m <sup>3</sup> )	Σ nitro-PAHs <sup>a</sup> (ng/m <sup>3</sup> )
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			<b>22.84</b>	<b>8.71</b>	<b>0.91</b>	<b>4.94</b>	<b>0.17</b>
Mean value (ng/µg)			/	<b>0.39</b>	<b>0.04</b>	<b>0.22</b>	<b>0.75</b>
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			<b>12.59</b>	<b>7.14</b>	<b>0.74</b>	<b>3.79</b>	<b>0.08</b>
Mean value (ng/µg)			/	<b>0.58</b>	<b>0.06</b>	<b>0.31</b>	<b>0.74</b>
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			<b>13.73</b>	<b>3.88</b>	<b>0.40</b>	<b>2.28</b>	<b>0.18</b>
Mean value (ng/µg)			/	<b>0.25</b>	<b>0.02</b>	<b>0.14</b>	<b>1.04</b>
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			<b>9.93</b>	<b>3.43</b>	<b>0.34</b>	<b>1.86</b>	<b>0.07</b>
Mean value (ng/µg)			/	<b>0.33</b>	<b>0.03</b>	<b>0.18</b>	<b>0.69</b>
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			<b>7.12</b>	<b>1.57</b>	<b>0.09</b>	<b>0.81</b>	<b>0.03</b>
Mean value (ng/µg)			/	<b>0.21</b>	<b>0.01</b>	<b>0.10</b>	<b>0.44</b>
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			<b>8.19</b>	<b>0.57</b>	<b>0.02</b>	<b>0.17</b>	<b>0.02</b>
Mean value (ng/μg)			/	<b>0.07</b>	<b>&lt;0.01</b>	<b>0.02</b>	<b>0.28</b>
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			<b>11.78</b>	<b>0.44</b>	<b>0.01</b>	<b>0.12</b>	<b>0.02</b>
Mean value (ng/μg)			/	<b>0.04</b>	<b>&lt;0.01</b>	<b>0.01</b>	<b>0.17</b>
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			<b>5.79</b>	<b>0.49</b>	<b>0.01</b>	<b>0.18</b>	<b>0.02</b>
Mean value (ng/μg)			/	<b>0.09</b>	<b>&lt;0.01</b>	<b>0.03</b>	<b>0.38</b>
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			<b>5.53</b>	<b>0.59</b>	<b>0.02</b>	<b>0.14</b>	<b>0.02</b>
Mean value (ng/μg)			/	<b>0.11</b>	<b>&lt;0.01</b>	<b>0.02</b>	<b>0.36</b>
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			<b>4.38</b>	<b>0.58</b>	<b>0.02</b>	<b>0.19</b>	<b>0.02</b>
Mean value (ng/μg)			/	<b>0.17</b>	<b>0.01</b>	<b>0.06</b>	<b>0.53</b>
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			<b>15.96</b>	<b>6.63</b>	<b>0.62</b>	<b>3.47</b>	<b>0.04</b>
Mean value (ng/μg)			/	<b>0.44</b>	<b>0.04</b>	<b>0.23</b>	<b>0.28</b>

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<sup>a</sup>CRM percentage recovery was found to be between 48% and 147% and the uncertainty was between 24 and 26%

<sup>b</sup>∑ 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.

804 **Table 2.** Mutagenic activity of PM<sub>0.5</sub> organic extracts in *S. typhimurium* TA100, TA98, TA98NR, and YG1021 strains with and without metabolic activation ( $\pm$ S9)  
 805 expressed as net revertants/m<sup>3</sup> of air equivalent. WI=winter I; S=spring; WII=winter II.  
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Sites	Net revertants/m <sup>3</sup>															
	-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
<b>Torino</b>																
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
<b>Brescia</b>																
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
<b>Pisa</b>																
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
<b>Perugia</b>																
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
<b>Lecce</b>																
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	
<b>Brescia</b>																
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	

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