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PM10 in a background urban site: Chemical characteristics and biological effects.

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(Article begins on next page)

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Abstract: PM10 was sampled in a background urban site in Torino, a northern Italian city. PM10 extracts were tested with THP-1 and A-549 cells to evaluate their effects on cell proliferation, LDH activity, TNF α , IL8 and CYP1A1 expression, and genotoxic damage induction (Comet assay). Through Principal Component Analysis (PCA), it was observed that (1) the aqueous extracts induced the inhibition of cell proliferation in the warm season that clustered together to total ions, (2) organic extracts determined a winter cell viability reduction and (3) there was a genotoxic effect associated with PAH and metal concentrations. The analysed low PAH levels were unable to induce significant CYP1A1 expression. The results obtained confirmed that PM composition and seasonality play an important role in particle-induced toxicity. The presence of PM10-induced biological effects at a low polluted site suggested that a reduction of PM10 mass did not seem to be sufficient to reduce its toxicity.

Conflict of Interest Statement

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Dear Editor,

we are sending the manuscript "*PM₁₀ in a background urban site: biological effects and chemical characteristics*" that we submit for possible publication on *Environmental Toxicology and Pharmacology*.

PM₁₀ from a background urban site in a north Italian city, collected during different seasons have been characterised for the amount of inorganic species, metals and PAHs. The biological effects of aqueous and organic PM extracts on THP-1 monocytes and lung epithelial cell line A549 were evaluated. The role played by the season and chemical fractions on biological effects was investigated using the Principal Component Analysis (PCA). Thorough PCA was mainly observed that the aqueous extracts induced inhibition of cell proliferation in warm season that clustered together to total ions and that the organic extracts determined a winter cell viability reduction and a genotoxic effect associated with PAH and metals concentrations. The analyzed low PAHs levels were not able to induce a significant CYP1A1 expression. The different sensitivity of biological tests emphasized the need to use different *in vitro* tests for PM biological effects assessment. Results obtained showed that PCA can be considered a useful tool to analyze the association between chemical composition and biological effects of PM and confirmed the hypothesis that PM composition and seasonality play an important role in particle induced toxicity.

PM-induced biological effects also in a site with low pollution levels demonstrated that the reduction of dust does not be sufficient to reduce particles toxicity, and this is a matter of concern for all the policies aimed at the protection of human health.

In the last years others studies have been developed by the authors (1,2,3,4,5,6,7,8,9,10,11,12,13,14) in order to characterized particulate matter and to better understand its relative toxicity.

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12. Gilli G, Traversi D, Rovere R, Pignata C, Schilirò T. Chemical characteristics and mutagenic activity of PM10 in Torino, a Northern Italian City. *Sci Total Env*, (2007) 385: 97-107.
13. Gilli G, Traversi D, Rovere R, Pignata C, Schilirò T. Airborne particulate matter: ionic species role in different Italian sites. *Env Res* (2007) 103:1-8.
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Best regards
Tiziana Schilirò

Ms. Ref. No.: ETAP-D-14-00358

Dear Editor,

please find enclosed the revised manuscript “*PM10 in a background urban site: chemical characteristics and biological effects*” by Tiziana Schilirò, Sara Bonetta, Luca Alessandria, Valentina Gianotti, Elisabetta Carraro and Giorgio Gilli.

We have answered to the Reviewers’ comments, in particular relevant changes have been highlighted in yellow all over the enclosed text.

Finally, our responses to Reviewers comments have been reported as follows.

Best regards,

Tiziana Schilirò and Co-authors.

RESPONSE TO REVIEWER COMMENTS

1. Reviewer #1:

The authors investigated the PM10 extracts (aqueous and organic) on THP-1 and A-549 cells to evaluate their effects on cell proliferation, LDH activity, TNF, IL8 and CYP1A1 gene expression, and genotoxic damage induction, using Comet assay.

The Introduction and Methodology are been described the results are been presented and discussed satisfactorily. The references were make correctly.

We thank the reviewer for the comments.

2. Reviewer #3:

Dear Editor,

The manuscript ETAP-D-14-00358, entitled PM10 in a background urban site: biological effects and chemical characteristics is very interesting and worth publishing in ETAP. However, I have some major concerns regarding experiments on cell cultures and I advise major revision.

We thank the reviewer for the suggested amendments. All specific corrections have been answered and are listed in the original reviewer text below.

Major remarks:

1. THP-1 cells are not adherent cells! Both MTT and LDH procedures are described for adherent cells. However, THP-1 monocytes could be differentiated into macrophages by some chemicals (PMA), which are adherent cells. Authors should give detailed explanation about cytotoxicity tests performed on THP-1.

We thank the reviewer for the observation. THP-1 are clearly not adherent cells, they are cells in suspension! There was a mistake: the previous reported methods, although similar, were referred to adherent cells. We have corrected the sections 2.5 and 2.6, reporting both MTT and LDH method for cells in suspension.

2. Why authors performed cytotoxicity test only on THP-1 cells, but not to A549. Also, why they choose A549 cell as model for genotoxicity study (Comet and Fpg-modified comet)? This should be explained.

A549 cells were more sensitive than THP-1 cells in Comet and Fpg-modified Comet tests, while in cytotoxicity tests the two cell lines showed comparable results (Corsini et al., 2013; Danielsen et al., 2011; Danielsen et al., 2009)*. We added this consideration in the manuscript at section 2.4.

3. What was the % of DMSO in RPMI for control cells?

Negative controls were constituted by cells not exposed and cells with a maximum of 5% DMSO. We add this specific in section 2.7

4. How A549 cells were seeded for performing comet assay, in 6-well plates or 12 well plates? What was the starting concentration of the cells?

The A549 cells were cultured for 24 h in 6-well plates, we add this specific in section 2.7.

5. At page 9, paragraph 26-31 it's written that A549 cells (approximately 6×10^5 cells) were mixed with low melting point agarose (LMA)... Is this number of cells per millilitre of LMA?

It was the absolute number of cells, we add the quantity of LMA in section 2.7.

6. Why the authors choose only TNF α and IL8 among other cytokines in THP-1 cell? Also, why they choose measurement of expression of CYP1A1? This should be explained in discussion.

TNF α and IL8 are two main cytokines involved in systemic inflammation and are members of a group of cytokines that stimulate the acute phase reaction. CYP1A1 is involved in phase I xenobiotic and drug metabolism and metabolic activation of aromatic hydrocarbons. These considerations are reported in sections 3.3.3.1 and 3.3.3.2.

In the discussion we have implemented the meaning of the expression of TNF α and IL8 and CYP1A1 (lines 520-544).

7. Overall, in this research many experiments were done but discussion on these results particularly regarding biological effects is poor.

The Discussion section has been partially rewritten improving and also adding some consideration about biological effects.

Minor remarks

In my opinion it would be better to change the title into PM10 in a background urban site: chemical characteristics and biological effects, as well as order of subtitles in Materials and methods (first, chemical characterisation and then experiments on cell cultures).

Thank you for the observation, we have changed the title.

***References**

Corsini E, Budello S, Marabini L, Galbiati V, Piazzalunga A, Barbieri P, Cozzutto S, Marinovich M, Pitea D, Galli CL. Comparison of wood smoke PM_{2.5} obtained from the combustion of FIR and beech pellets on inflammation and DNA damage in A549 and THP-1 human cell lines. Arch Toxicol. 2013, 87(12):2187-99.

Danielsen PH, Møller P, Jensen KA, Sharma AK, Wallin H, Bossi R, Autrup H, Mølhave L, Ravanat JL, Briedé JJ, de Kok TM, Loft S. Oxidative stress, DNA damage, and inflammation induced by ambient air and wood smoke particulate matter in human A549 and THP-1 cell lines. Chem Res Toxicol. 2011, 24(2):168-84.

Danielsen PH, Loft S, Kocbach A, Schwarze PE, Møller P. Oxidative damage to DNA and repair induced by Norwegian wood smoke particles in human A549 and THP-1 cell lines. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2009, 674(1–2):116–122

3. Reviewer #3:

This manuscript is not particularly strong and I doubt whether it should be published in ETAP. However, if considered for publication nevertheless, it should be improved c.q. amended significantly.

All specific corrections have been answered and are listed in the original reviewer text below. We thank the reviewer for the suggested amendments.

The Results and Discussion sections are overlapping and this overlap has to be eliminated.

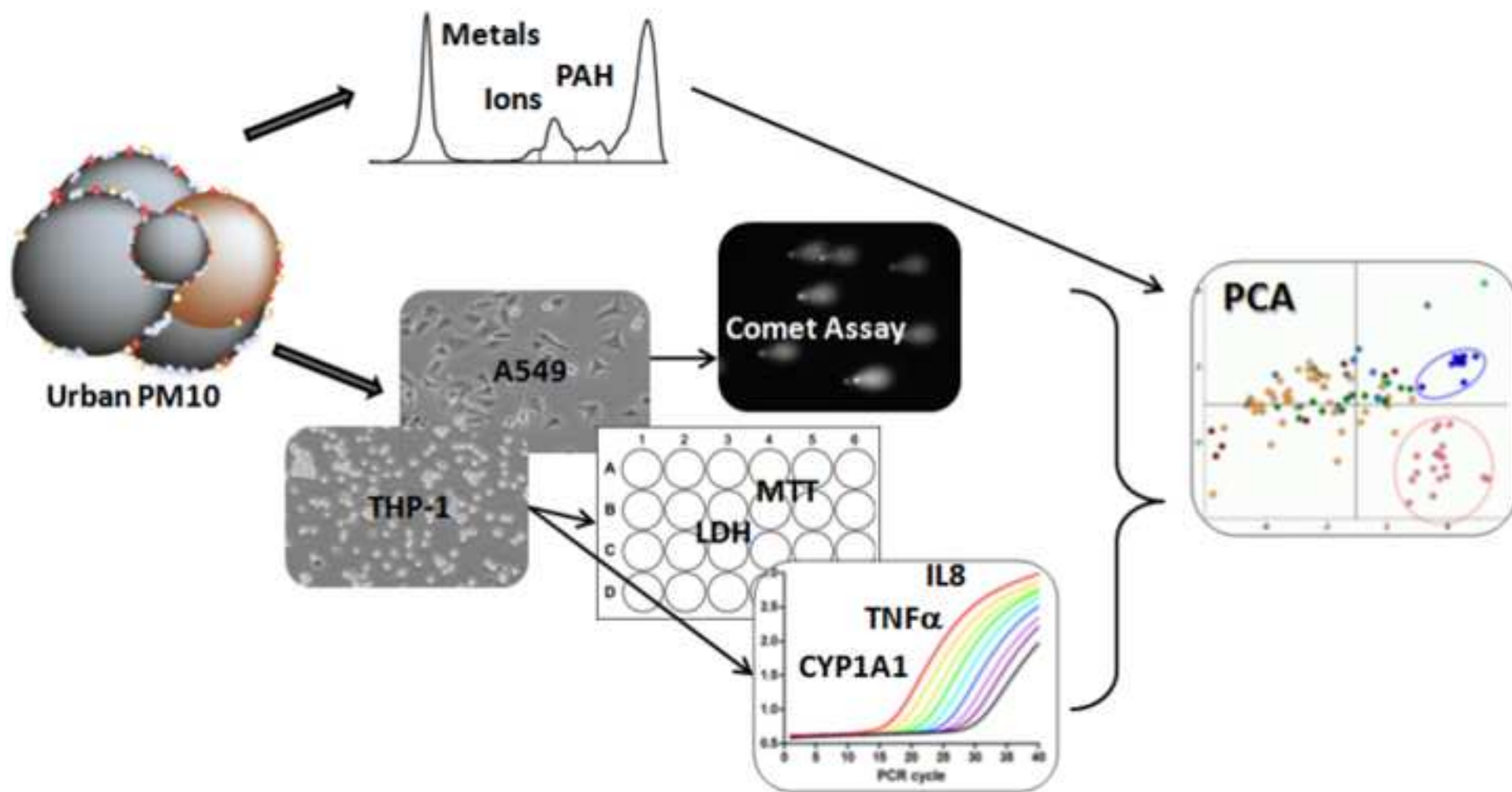
Thank you for the observation, the Results and Discussion sections have been partially modified considering the overlaps.

The number of references is too large, and can easily be reduced by 25%.

We eliminated some references (more than 16%).

The manuscript contains typographical and grammatical errors. As this is concerned, the manuscript has to be improved significantly as well.

The English language has been carefully revised for grammatical errors all over the text by an AJE English expert (we add the “editorial certificate” in supplemental material section).



PM₁₀ in a background urban site: biological effects and chemical characteristics.

HIGHLIGHTS

1. PM₁₀-induced biological effects are present in a background urban site
2. PM₁₀ aqueous extracts show cytotoxicity and high ions content in warm season
3. PM₁₀ organic extracts prove genotoxic effects and high PAHs content in cold season
4. PM₁₀ composition and seasonality play important roles in particle induced toxicity

1 **PM10 in a background urban site: chemical characteristics and biological effects**

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24 **Abstract**

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PM₁₀ was sampled in a background urban site in Torino, a northern Italian city. PM₁₀ extracts were tested with THP-1 and A-549 cells to evaluate their effects on cell proliferation, LDH activity, TNF α , IL8 and CYP1A1 expression, and genotoxic damage induction (Comet assay). Through Principal Component Analysis (PCA), it was observed that (1) the aqueous extracts induced the inhibition of cell proliferation in the warm season that clustered together to total ions, (2) organic extracts determined a winter cell viability reduction and (3) there was a genotoxic effect associated with PAH and metal concentrations. The analysed low PAH levels were unable to induce significant CYP1A1 expression. The results obtained confirmed that PM composition and seasonality play an important role in particle-induced toxicity. The presence of PM₁₀-induced biological effects at a low polluted site suggested that a reduction of PM₁₀ mass did not seem to be sufficient to reduce its toxicity.

Keywords: PM₁₀; air quality; in vitro toxicology; genotoxicity; PCA.

55 **1. Introduction**

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26 There is a large body of evidence suggesting that exposure to air pollution, even at the levels
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47 commonly achieved currently in European countries, leads to adverse health effects (Pope and
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68 Dockery, 2006). Epidemiologists have documented statistically significant associations between
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89 particulate matter (PM) mass concentrations and increased human mortality and morbidity (Brook
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160 et al., 2010). Long-term exposure to airborne PM increases the risk of lung cancer, respiratory
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1361 diseases and arteriosclerosis, while short-term exposure exacerbates several forms of respiratory
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1562 diseases, including bronchitis and asthma, as well as changes in heart rate variability (Schwarze et
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17 al., 2006).
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2064 PM is a highly complex and heterogeneous mixture of particles with natural and/or anthropogenic
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2265 origins and with different dimensions, with a chemical composition that changes with time and
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2466 space (Pope and Dockery, 2006). Major sources of PM include vehicular and industrial emissions,
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2667 power plants, crustal release, and refuse incineration. Particle composition varies between urban
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28 and rural sites. Approximately 40 % of the particle mass in an urban setting can be attributed to
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3068 fossil fuel use (Ghio et al., 2012).
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3370 It was shown that PM exposure and toxicity may be related to geographic locations and sampling
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3571 seasons and consequently to the chemical components of various PM fractions; PM chemical
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37 properties depend on the emission sources present in the examined area, the specific atmospheric
3872 chemistry and weather conditions (Perrone et al., 2013).
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4274 The respiratory system is the primary target of airborne PM, which are inhaled and tend to
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4475 accumulate in the airways. The exposure dose of the tissues depends on the PM atmospheric
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4676 concentration, aerodynamic diameter, deposition rate in the airways, clearance mechanisms and
47
48 subsequent retention of particles within the respiratory tract. Even though the problem has been
4977 intensively studied, scientists are still struggling to understand the physical and chemical properties
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5178 of PM-responsible toxicity as well as the mechanisms underlying the adverse health effects of PM
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5379 air pollution. No clear consensus exists among researchers regarding the mechanisms of toxicity
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5580 following exposure to current levels of air pollution PM (Schwarze et al., 2007); in this context,
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82 oxidative stress and inflammatory reactions seem to be of fundamental importance (Lonkar and
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23 Dedon, 2011).
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44 Some studies showed that particulate air pollution increases reactive oxygen species (ROS) levels,
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65 which may subsequently lead to cellular oxidative stress (Knaapen et al., 2004) and contribute to
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86 the genotoxic and cytotoxic effects of PM (Oh et al., 2011). Oxidative stress induces oxidative DNA
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1187 lesions, such as increased 8-hydroxy-deoxyguanosine (8-OHdG) levels and DNA strand breaks
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138 (Bonetta et al., 2009). Moreover, oxidative stress primes a cascade of events associated with
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1589 inflammation, which probably plays a key role in the air pollution PM-induced development and/or
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1790 exacerbation of acute and/or chronic lung diseases (Oh et al., 2011; Schwarze et al., 2007).
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2091 Transition metals in PM seem to be involved in this process, (Roig et al., 2013); in addition,
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2292 organic compounds, such as polycyclic aromatic hydrocarbons (PAH) and nitro-PAH, coated onto
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2493 PM may induce damage (DNA damage, mutations and cytotoxicity) at a cellular level in addition to
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2694 that caused by generating oxygen radicals through direct interaction with cellular structures. PAH
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2995 are among the most toxic extracted organic compounds because of their carcinogenic and
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3196 genotoxicity properties (Teixeira et al., 2012), which are associated with atmospheric particles. In
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3397 human lungs, PAH, which require metabolic activation to biologically reactive intermediates to elicit
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3598 their adverse health effects, are principally metabolised by the cytochromes P450 (CYP)
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3899 superfamily member CYP1A1 (Spink et al., 2008). Intermediates thereafter interact with DNA
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4000 target sites to produce adducts, mutation, DNA strand breaks and eventually tumour initiation
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4201 (Schwarze et al., 2007). Furthermore, epidemiological studies showed significant associations
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45102 between sulphates and nitrates and various health outcomes. In ambient air, sulphates and
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47103 nitrates are the major PM chemical components and are associated with secondary sources
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4904 (Schwarze et al., 2006).
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5105 To contribute to a greater understanding of the mechanism involved in PM toxicity, the biological
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53106 effects of urban air PM₁₀ collected during different seasons in Torino, a northern Italian city, were
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56107 examined. A background station was selected as a model of a low contaminated urban site. In
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5808 particular, the response and sensitivity of different endpoints in human cells cultured *in vitro* (A549
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6009 lung epithelial cells line and THP-1 monocytes) were evaluated. PM₁₀ samples were analysed for
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110 the quantity of inorganic ions, metals and PAH. Then, the effects of PM₁₀ extracts (organic and
111 aqueous) from cold and warm seasons on the inhibition of cell proliferation, tumour necrosis factor
112 alpha (TNF α), interleukin 8 (IL8) and CYP1A1 expression, lactate dehydrogenase (LDH) activity,
113 and genotoxic/oxidative damage induction (Comet assay) were evaluated. The role played by the
114 season and by chemical fractions in different biological endpoint inductions was investigated using
115 Principal Component Analysis (PCA).

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117 **2. Materials and methods**

118 *2.1. PM sampling*

119 Sampling was performed from January to December (6 filters every month) in meteorological-
120 chemical stations of the Environmental Protection Regional Agency (Piedmont A.R.P.A.) located in
121 Torino, in the northwest of the Padana Plain (Italy). The sampling site (Lingotto) was located
122 outside in a small green area within an enclosure zone classified as urban background.

123 PM₁₀ (PM passing through a size-selective inlet with a 50 % efficiency cut-off at a 10 μ m
124 aerodynamic diameter) was sampled on glass micro-fibre filters (Type A/E, 8 \times 10 in, Gelman
125 Sciences, MI, USA) with a Sierra Andersen High-Volume Sampler 1200/VFC (Andersen Sam-
126 plers, Atlanta, Georgia, USA) with a flow of 1160 L/min. The sampling duration was controlled by a
127 timer accurate to \pm 15 min over a 24 h sampling period. The exact flow rate was calculated daily
128 and corrected for variations in atmospheric pressure and actual differential pressure across the
129 filter. The filters were pre- and post-conditioned in a dry and dark environment for 48 h and
130 weighed with an analytical balance (\pm 10 μ g) before and after sampling to calculate the PM mass
131 trapped on the filter. The procedures were conducted according to the European Committee for
132 Standardization (CEN, 1998), as previously described (Gilli et al., 2007).

133 *2.2. Particles extraction*

134 After gravimetric analysis, filters were pooled and particles were extracted for chemical analysis
135 and biological assays.

136 Two different strips were cut from each PM₁₀ filter as previously reported (Alessandria et al., 2014).

137 The filter strips from each month (6 strips) were pooled to obtain two monthly samples. Briefly, the

138 two pools were treated with two different extraction media: acetone or RPMI1640 without foetal calf
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139 serum (FCS), chosen for different abilities in the extraction of different compounds. In particular,
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140 acetone was selected as the solvent for organic-extractable compounds (e.g., PAHs) (Claxton et
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141 al., 2004). RPMI without FCS was chosen to extract water-soluble components (e.g., metals),
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142 theoretically comparable to the extraction at the lung cells (Hetland et al., 2004). The monthly strips
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143 were cut into small pieces and were placed in 50 mL polypropylene sterile tubes with 15 mL of the
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144 extraction medium. The tubes were placed in an ultrasonic water bath for 10 min, followed by 1 min
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145 of vortexing. This procedure was repeated 3 times (45 mL of extracts). The samples were
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146 centrifuged at 5000 x g for 10 min to remove the filter material, and supernatant was collected.
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207 Each PM extract was separated into two aliquots destined to the chemical analysis and to
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208 biological tests. Acetone extract (organic) for biological tests was evaporated with a rotary
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149 evaporator and suspended in RPMI1640 without foetal calf serum (FCS) for the THP-1 cells tests
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150 and in dimethyl sulfoxide (DMSO) for the comet assay with A549. The other acetone extract for
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251 chemical analysis was evaporated with a rotary evaporator until 2 mL. RPMI extracts (aqueous)
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3152 were directly assayed for both biological and chemical analysis (Alessandria et al., 2014; Schilirò et
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153 al., 2010). Each extraction was also performed on a QC laboratory filter (treated with the same
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154 method as the samples). Unless specified otherwise, all chemicals were purchased from Sigma,
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155 St. Louis, MO, USA. Individual chemical species and the biological evaluation are summarised in
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156 Table 1.

2.3. ICP-MS, IC and GC-MS analysis

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158 The metal content in the aqueous extracts was measured by Inductively Coupled Plasma-Mass
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159 Spectrometry (ICP-MS) by ThermoFisher XSeries II ICP-MS (Winsford UK), software PlasmaLab
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160 V2.5.4.289. The instrumental conditions were as follows: the Main Run was Peak Jumping,
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161 Sweeps at 35 ms, Dwell Time at 10,000 ms, Channels per Mass was 1, Acquisition Duration was
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162 29412 ms and Channel Spacing is 0.02 amu. Resolution was standard. The solutions were diluted
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163 with nitric acid (Sigma Aldrich, ICP grade) to obtain solutions that contain 1% acid; next, they were
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164 filtered by polypropylene membrane filters (0.2 µm). The limit of quantification was 1 ng/mL.
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165 Analysed metals are summarised in Table 1.

166 The inorganic ions in aqueous extracts were detected using an ion chromatography (IC) analysis
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167 carried out on a Dionex LC20 ion chromatograph (Sunnyvale, CA, USA) equipped with a GP40
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168 gradient pump, an ED40 electrochemical detector and an ASRS-I anion self-regenerating
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169 suppressor, operating under chemical suppression mode, with the external chemical regenerant
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170 flowing through a Consta metric 3200 (Thermo Separation Products, San Jose, CA, USA). The
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stationary phase is a Dionex IonPac AS14A (250 mm x 4 mm) (Sunnyvale, CA, USA), equipped
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1171 with a precolumn IonPac AG14A (50 mm x 4 mm) (Sunnyvale, CA, USA). The mobile phase is a
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1172 mixture 50/50 v/v of 8 mM Na₂CO₃ and 1 mM NaHCO₃, flowing at 1 mL/min. The ionic suppression
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1173 solution is a 25 mM H₂SO₄ aqueous solution. The limit of quantification was 0.5 µg/mL. Analysed
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174 ions are summarised in Table 1.
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2276 The PAH concentration in the organic extracts was evaluated using a GC-MS Finnigan Trace GC
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Ultra-Trace DSQ (Thermo Scientific, San Jose, CA, USA) instrument with quadruple mass analyser. The stationary phase was a Thermo TR-5MS (60 m x 0.25 mm i.d.) coated with a 0.25 µm film of 5% phenyl polysilphenylene-siloxane (BPX5). The inlet temperature was 250 °C and the splitless time 1 min. Column temperature gradient was: 70 °C (4 min), 10°C/min from 70 to 120 °C and 2 °C/min from 120 to 300 °C (21 min). Helium was the carrier gas maintained at a constant flow (1 mL/min). The electron impact (EI+) mass spectra were acquired with ionization energy of 70 eV in full scan mode between 30 and 300 amu (scan rate 500 amu/s). The ion source and transfer line temperatures were set at 250 °C. Compound identification was based on the comparison of their retention times and mass spectra with those of reference standards. The limit of quantification was 0.05 µg/mL. Analysed PAH are summarised in Table 1.

2.4. Cell culture

Two cell lines were used. The human THP-1 monocytes from Interlab Cell Line Collection (Genova, IT) were used as surrogates of lung monocytes and were grown, maintained and treated in RPMI1640 supplemented with 10 % (v/v) FCS, 2 % l-glutamine 200 mM and 1 % penicillin/streptomycin 10 mg/mL, at 37 °C in an humidified atmosphere containing 5% CO₂. The human lung epithelial cell line A549 (non-small cell lung cancer) from Interlab Cell Line Collection (Genova, IT) was used as a model for human epithelial lung cells, as they exhibit characteristics

194 similar to human alveolar type II cells (Shi et al., 2006). A549 were grown as a monolayer,
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195 maintained and treated in RPMI1640 supplemented with 10 % (v/v) FCS, 2 % l-glutamine 200 mM
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196 and 1% penicillin/streptomycin 10 mg/mL, at 37 °C in an humidified atmosphere containing 5 %
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197 CO₂. A549 cells were more sensitive than THP-1 cells in genotoxicity tests, while the two cell lines
7
198 showed comparable results in cytotoxicity tests (Corsini et al., 2013; Danielsen et al., 2011).

1199 2.5. Cell viability

1200 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure cell
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1201 viability; THP-1 cells were seeded, using RPMI1640 without phenol red, in 24-well plates at a
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1202 density of 4×10⁴ cell/well and exposed to a particle concentration of 200 µg/mL, a concentration
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1203 that always induced a significant effect with samples from the same sampling site described in a
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1204 previous work (Alessandria et al., 2014). After treatment with PM₁₀ extracts (72 h), the MTT (final
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1205 concentration 0.5 mg/mL) was added to cells and incubated at 37°C for 4 h. Then, 1 ml 10 %
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1206 SDS/0.01 M HCl was added in each well, and the cells were incubated overnight. Cell proliferation
27
1207 was determined by measuring absorbance at 570 nm with a micro-plate reader (ELX 800 UV, Bio-
30
1208 Tek Instruments, Inc.). QC laboratory filter extracts were tested in the same manner of PM₁₀
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1209 extracts samples. All experiments were performed in triplicate. The % of inhibition of cell
34
1210 proliferation was calculated comparing the absorbance of exposed cultures with the absorbance of
36
1211 non-exposed cultures.

1212 2.6. LDH assay

1213 To evaluate PM₁₀ cytotoxicity, LDH activities from damaged cells were measured in cell-free
43
1214 culture supernatants, as previously described (Schilirò et al. 2010), modified for cells in
45
1215 suspension. Briefly, THP-1 cells were seeded in 6-well plates at a density of 1×10⁶ cells/well and
48
1216 exposed to PM₁₀ extracts containing 200 µg/mL particles, a concentration that always induced a
50
1217 significant effect with samples from the same sampling site described in a previous work
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1218 (Alessandria et al., 2014). QC laboratory filter extracts were tested in the same manner of PM₁₀
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1219 extracts samples. At 72 h, LDH activity was measured in the supernatant and cell lysate. LDH
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1220 activity was calculated as the ratio of extracellular LDH (measured in the supernatant) and total
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1221 LDH (expressed as sum of LDH measured in supernatant and cell lysate). To obtain cell lysates,
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222 cells were centrifuged to eliminate the supernatant and resuspended with 1 mL of TRAP (82.3 mM
1
223 triethanolamine hydrochloride, pH 7.6) and sonicated for 10 s. Then, LDH was measured by
3
224 adding 250 μ L of a mix containing 0.25 mM NADH and 0.5 mM pyruvate, and consumption of
5
225 NADH was measured as absorbance at 340 nm in a micro-plate reader (Benchmark Plus
8
226 Microplate Reader, Biorad). All experiments were performed in triplicate. LDH activity of exposed
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127 cells is expressed as a percentage of non-exposed cells.

228 2.7. Comet assay

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229 The A549 cells were cultured for 24 h in 6-well plates before exposure to PM extracts. The
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230 proportion of living cells was determined by trypan blue staining (overall > 90%). Cells were
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19
231 exposed (24 h at 37 °C) to serial dilutions of the PM₁₀ organic extracts containing a range from 200
21
232 to 500 μ g/mL of particles and PM₁₀ aqueous extracts containing 200 μ g/mL of particles. Negative
23
234 controls were constituted by cells not exposed and cells with a maximum of 5 % DMSO. Cells not
25
26
234 exposed were used as negative controls. After exposure, cell viability was checked again. The
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28
235 concentration of PM extract that decreases cell viability by more than 30 % was not used for the
30
31
236 Comet assay. The Comet assay was performed under alkaline conditions (pH > 13), according to
32
33
237 the method of Tice et al. (2000) after slight modifications (Moretti et al., 2002). A549 cells
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238 (approximately 6×10^5 cells) were mixed with 300 μ L low melting point agarose (LMA), , placed on
36
37
239 the slides coated with 1 % of normal melting agarose (NMA), with LMA added as the top layer.
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40
240 Cells were lysed at 4 °C in the dark overnight (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA
41
42
241 disodium salt, 1 % TRITON X-100 and 10% DMSO, pH 10). DNA was allowed to unwind for 20 min
43
44
242 in alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH, 10% DMSO, pH >13) and
45
46
243 subjected to electrophoresis in the same buffer for 20 min (0.78 V/cm and 300 mA). All steps for
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244 slide preparation were performed under yellow light to prevent additional DNA damage.

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245 The slides were stained with ethidium bromide (20 μ L/mL) and examined with a fluorescent
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53
246 microscope (Axiovert 100M, Zeiss). One hundred randomly selected cells per sample (2 slides)
54
55
247 were analysed using an image analysis system (CometScore). The % tail DNA was selected as the
57
248 parameter to estimate DNA damage. QC laboratory filter extracts were tested in the same manner
59
249 of PM₁₀ extracts samples. Data from unexposed cell cultures were compared with those from PM₁₀
61

250 extracts. Statistical analyses were performed by Student's t-test (SYSTAT statistical package).
1
251 Statistically significant differences were reported with P value ≤ 0.05 .

252 2.8. *Fpg-Modified Comet assay*

253 The formamidopyrimidine glycosylase (Fpg)-modified comet assay was used only for water
254 extracts. The test was carried out as described above with the exception that, after lysis, the slides
255 were washed three times for 5 min with Fpg Buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2
256 mg/mL bovine serum albumin, pH 8). Then, the slides were incubated with 1 unit of Fpg enzyme at
257 37 °C for 1 h. Control slides were incubated with buffer only. For each experimental point, the
258 mean % tail DNA from enzyme untreated cells (direct DNA damage) and mean % tail DNA for Fpg-
259 enzyme treated cells (direct and indirect DNA damage) were calculated.

260 2.9 *IL-8, TNF α and CYP1A1 gene expression*

261 For quantitative Real Time PCR (qRT-PCR), THP-1 cells were seeded in 6-well culture plates and
262 cultured overnight. Then, the cell culture medium was replaced by a fresh medium and PM₁₀
263 extracts were performed at 200 $\mu\text{g}/\text{mL}$ for 48 h. Total RNA was isolated from treated and untreated
264 THP-1 cells using GenElute Mammalian Total RNA Kit (Sigma, St. Louis, MO, USA). The RNA
265 concentration was determined spectrometrically (260/280/320 nm); total RNA (1 μg) was used for
266 the first-strand cDNA reaction with reverse transcriptase using an iScript cDNA Synthesis Kit (Bio-
267 Rad, Hercules, CA, USA). Relative gene expression was determined by real-time PCR with a
268 Chromo 4 Real-Time System (Bio-Rad, Hercules, CA, USA) using 1 μL of cDNA sample aliquot
269 (10 ng total mRNA) as a template with Sso Fast EvaGreen SuperMix (Bio-Rad, Hercules, CA,
270 USA).

271 The quality of PCR product was monitored using post-PCR melt curve analysis at the end of the
272 amplification cycles. Three genes were analysed, TNF α , IL-8, CYP1A1. The following primer
273 sequences were used for relative gene expression analysis:

274 TNF α - f: 5'-ATGAGCACTGAAAGCATGATCCG-3',

275 TNF α - r: 5'- CAGGCTTGTCACCTCGGGGTTC-3';

276 IL-8 - f: 5'- TGCCAAGGAGTGCTAAAG-3',

277 IL-8 - r: 5'-CTCCACAACCCTCTGCAC-3';

278 CYP1A1 - f: sense 5'- GGCAGATCAACCATGACCAGAAG-3' ,
1
279 CYP1A1 - r: 5'-ACAGCAGGCATGCTTCATGGTTAG-3'.
3
280 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine
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281 phosphoribosyltransferase (HPRT1) gene expression were used as the internal control as they are
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8
282 housekeeping genes and were analysed in each experiment for normalisation using the following
9
10
1283 primers:
12
1284 GAPDH - f: 5'-CCCTTCATTGACCTCAACTACATG-3',
14
1285 GAPDH - r: 5'-TGGGATTTCCATTGATGACAAGC-3';
16
1286 HPRT1- f: 5'-TGACACTGGCAAACAATGCA-3',
18
1287 HPRT1 - r: 5'-GGTCCTTTTCACCAGCAAGCT-3'.
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2288 Relative fold inductions were calculated using the ΔC_t formula (Scheffe et al., 2006). All real-time
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2289 RT-PCR assays for relative gene expression were repeated at least three times in duplicates from
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290 independent total RNA samples for the same treatment conditions.
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291 *2.10 Bivariate and PCA analysis*

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3292 The PM₁₀ samples were analysed by grouping months as follows: winter (December, January,
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3293 February), spring (March, April, May), summer (June, July, August), and autumn (September,
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3294 October, November), and also by grouping season: cold season (winter and autumn) and warm
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3295 season (spring and summer). Means were compared with the Wilcoxon-Mann-Whitney test, and
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3296 the Spearman rank correlation coefficient (r_S) was used to assess relationships between variables.
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3297 The mean difference and correlation were considered significant at $p < 0.05$. Statistical analyses
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44
3298 were performed using the SPSS Package, version 17.0 for Windows.
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3299 PCA analysis was performed on the complete dataset represented by a the X (n,p) matrix.
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3300 Samples (n) were 12 and variables (p) 18. Variables were 4 chemical components, 1 physical
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3301 parameter and 13 biological responses determined for PM samples.
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3302 Principal Component Analysis, regression models and all graphical representations were
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3303 performed by Statistica 7.1 (Statsoft Inc., USA) and Excel 2003 (Microsoft Corporation, USA).
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3304 The chemical components were total inorganic ions, total PAH, and total and transition metals. The
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3305 physical parameter was PM mass. The biological responses considered were DNA damage
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306 (organic extracts), DNA damage –Fpg (aqueous extracts), DNA damage +Fpg (aqueous extracts),
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307 inhibition of cell proliferation (organic and aqueous extracts), LDH release (organic and aqueous
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308 extracts), and TNF α , IL8 and CYP1A1 expression (organic and aqueous extracts).
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3. Results

3.1 PM concentration

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312 A total of 72 PM₁₀ filters were analysed. PM₁₀ was sampled in an urban background site placed in
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313 a green area; nevertheless, concentrations were sometimes higher than the daily target of 50
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314 $\mu\text{g}/\text{m}^3$ (Air Quality Directive, 2008/50/CE): 83 % of the samples in January and February, 33 % in
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315 December 17 % in March and November, and all of the concentrations in the spring and summer
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316 months never exceeded the daily reference limit. The mean PM₁₀ air concentrations at the
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317 sampling site are reported together with the seasonal averages in Table 2. The highest value was
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318 observed in winter (115.3 $\mu\text{g}/\text{m}^3$) while the lowest was measured in summer (11.5 $\mu\text{g}/\text{m}^3$);
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319 moreover, significant differences were found between seasonal concentrations (ANOVA: F =
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320 0.731, $p < 0.01$), with higher values in winter.
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3.2. PM₁₀ chemical characterization

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34
322 Table 2 shows concentrations of the main compounds analysed in PM₁₀ extracts.
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3.2.1. Metals

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The chemical analysis of the PM₁₀ aqueous extracts has detected the presence of 12 metals (As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Pb, Pt, V, Zn) in all samples. Among these, Fe and Cu were the most abundant, with a range of 1.95 – 62.48 ng/m³ and 11.35 – 100.53 ng/m³ and a mean value of 13.6 \pm 19.02 ng/m³ and 36.07 \pm 29.23 ng/m³, respectively. The ANOVA analysis, performed assuming metals and transition metals as dependent variables and the different seasons as independent variables, showed the general significance of the model (F = 15.711 and F = 16.346, $p < 0.05$, respectively). Post-hoc Tukey's test emphasised this seasonal trend with significant differences between winter and the other seasonal concentrations ($p < 0.05$). These compounds showed a significant positive correlation with PM₁₀ concentrations ($r_S = 0.916$ and $r_S = 0.923$, $p < 0.01$, respectively).

3.2.2. Ions

The mean concentration of ions was $16.09 \pm 7.37 \mu\text{g}/\text{m}^3$, in relation to PM_{10} . Sulphates represented 18 % of PM_{10} and 35 % of total ions, while nitrates represented 32 % of total particles and 62 % of ions. Only sulphates showed a significant seasonal trend (ANOVA, $F = 4.928$, $p < 0.05$). However, considering the concentrations of these species in relation to PM_{10} , a seasonal trend was found with higher contents of these compounds in summer. Sulphates showed a significant correlation with PM_{10} and metals concentrations ($r_S = 0.660$ and $r_S = 0.650$, $p < 0.05$, respectively).

3.2.3. PAH

The chemical analysis of the PM organic extracts showed a variable PAH contamination in the different samples (Table 2). The mean concentration was $1.02 \pm 2.43 \mu\text{g}/\text{m}^3$. The highest mean concentration both of total and carcinogenic PAH were found in January ($8.7 \text{ ng}/\text{m}^3$), in the other months, total PAH concentrations were lower by at least an order of magnitude. In the same months, no carcinogenic PAHs were detected. In May, PAHs concentrations were under the detection limit. PAH had no significant correlation with the other chemical compounds.

3.3 Biological assays

3.3.1. Cell viability

Figure 1 (a) shows the effect produced by particle extracts ($200 \mu\text{g}/\text{mL}$) on cell proliferation, as measured by MTT. Both extracts inhibited cell proliferation. Aqueous extracts inhibited cell proliferation markedly to a greater extent than organic extracts ($18.3 \pm 6.9 \%$ vs. $9.3 \pm 9.3\%$ proliferation inhibition, respectively). This difference was significant ($p < 0.05$), as confirmed by the Wilcoxon test. The QC laboratory filter extracts did not significantly inhibit cell proliferation. On a monthly basis, the maximum inhibition of cell proliferation, 32.3 %, was achieved in the organic extract in December. Proliferation inhibition of THP1 cells by aqueous extracts was significantly correlated with the Fe content of PM_{10} ($r_S = 0.685$, $p < 0.05$).

3.3.2. LDH release

Figure 1 (b) shows the effects produced by PM₁₀ extracts (200 µg/mL) on LDH release. There were no statistically significant differences in the effects produced by the two type of extracts, but both induced significant LDH release in cells exposed to particles compared to the control for all seasonal samples. The most significant increase in LDH release (67.4 %) over negative controls was obtained with spring organic sample extract; this was an unusual trend if compared to a previous study (Schilirò et al., 2010). LDH increase by THP1 due to either the organic or aqueous extract was not correlated with PM₁₀ concentrations or with any other chemical parameters. The QC laboratory filter extracts did not induce any significant LDH release.

3.3.3. Gene expression

3.3.3.1. Inflammatory response

TNF α and IL8 are two cytokines involved in systemic inflammation and are members of a group of cytokines that stimulate the acute phase reaction. PM₁₀ treatment was associated with up-regulation of these two cytokines gene. There were no differences ($p > 0.05$) in the expression induced by organic extracts or aqueous ones. TNF α (Figure 2,a) was significantly induced ($p < 0.05$) by winter and spring samples, while IL8 (Figure 2,b) expression did not show significant differences over negative control, though a greater effect was noted again with winter and spring PM₁₀ extracts. The QC laboratory filter extracts did not induce any significant TNF α and IL8 expressions.

3.3.3.2. Cytochrome expression

CYP1A1 is involved in phase I xenobiotic and drug metabolism and metabolic activation of aromatic hydrocarbons. The CYP1A1 expression (Figure 2, c) was low but significant compared to the negative control. In general, the difference between the expression induced by organic extracts or aqueous ones was significant ($p < 0.05$). The stronger expression of CYP1A1 was induced in spring samples and differences compared to the other seasons were significant (ANOVA, $F = 5.357$, $p < 0.05$). In this study, CYP1A1 expression was not correlated with PAHs levels. The QC laboratory filter extracts did not induce any significant CYP1A1 expression.

3.3.4. Genotoxic damage and oxidative stress of PM extracts

Exposure of A549 cells to PM₁₀ organic extracts showed a genotoxic effect (% tail DNA) for all the samples investigated with the exception of the sample from March, although the biological effect was in general low.

In general, results showed the highest genotoxic effects in winter and autumn samples ($p < 0.05$) (Figure 3). In particular, considering the different sample, the highest genotoxic activity was observed in October, November and December. These samples showed an increase in the % tail DNA with respect to control cells ($p < 0.05$ or $p < 0.001$) from 200 and 300 $\mu\text{g/mL}$ of PM₁₀.

For the other samples, the highest genotoxic effect was observed in PM₁₀ organic extracts of January and September (dose corresponding to 500 $\mu\text{g/mL}$). Furthermore, results of the statistical analysis showed that the genotoxic effect on A549 was correlated ($p < 0.05$) with PAH concentration.

To evaluate the direct and oxidative DNA damage of aqueous extracts, the Fpg-modified Comet assay was used. Only the samples of May and June showed the presence of a genotoxic effect in enzyme untreated cells (CL) (direct DNA damage), underlining the presence of pollutants with direct genotoxic effects (Wessels et al. 2010). Using Fpg enzyme, a significant genotoxic effect in enzyme treated cells (CLenz) (direct and indirect DNA damage) was observed in samples of April, May and June.

The subtraction of mean CL from the relative CLenz value of the exposed cells (CLenz-CL) compared with unexposed cells at each experimental point provides the intensity of the oxidative damage. A significant oxidative damage was observed only in the sample from April. The results obtained highlighted that the presence of the oxidative damage in PM₁₀ aqueous extracts seems not to be related to the metal presence in the sample ($p > 0.05$). The QC laboratory filter extracts did not induce any significant genotoxic effect.

3.4 PCA analysis

Principal Component Analysis (PCA) (Vandeginste et al., 1998) was conducted to provide a graphical representation of the data to easily evaluate similarities or differences among PM₁₀ samples. Using comparisons with score plots and loading plots, it is possible to derive the chemical

418 species and biological responses that are most responsible for a certain separation among
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419 samples.

420 PCA was first applied, after an autoscaling procedure, to the overall dataset that comprises the
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421 chemical, physical and biological parameters in which the values lower than the LOD were set to 0.

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422 The analysis shows that with principal components 1, 2 and 3 (PC1, PC2 and PC3), it is possible
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423 to explain approximately 71 % of the total variance.

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424 Both the loading plot (variables) and score plot (monthly samples) are shown in Figure 4 a and b,
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425 respectively, for PC1 and PC2.

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426 PC1 accounts for the largest amount of total variance (approximately 34%) and the corresponding
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427 loading plot (Figure 4a) shows large positive weights of the variables related to the biological
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428 response inhibition of cell proliferation (aqueous extracts). Contrarily, negative weights were
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429 observed for $TNF\alpha$, LDH and IL8 (organic and aqueous extracts).

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430 The more interesting information arises from the observation of the sample loading and variable
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431 scores along PC2 (which had a variance of approximately 26%). In fact, PC2 is characterized by
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432 the chemical variables PAH and metals and the biological responses' genotoxic effect (organic
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433 extracts), inhibition of cell proliferation (organic extracts) and $TNF\alpha$ production (aqueous extracts)
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434 (positive loadings); the negative loadings were represented by CYP1A1 expression (organic
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435 extracts), total ions, genotoxic effect (aqueous extracts) and inhibition of cell proliferation (aqueous
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436 extracts).

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437 The variables IL8, LDH and $TNF\alpha$ (organic extract) are located in the middle of the axis, thus
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438 suggesting the unimportance of this variable on PC2. Looking at the corresponding score plot
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439 (Figure 4b), a quite evident separation into two groups along PC2 can be observed: PM_{10} sampled
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440 in the cold season are all placed on PC2 positive values, whereas the PM_{10} samples from the
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441 warm season are located at PC2 negative values.

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442 Combining data from loadings and scores plots, it is possible to infer that in the cold season, PM_{10}
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443 were more contaminated from PAH and metals and exhibit a greater genotoxic effect (organic
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444 extracts), inhibition of cell proliferation (organic extracts), and $TNF\alpha$ production (aqueous extracts)
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445 with respect to the warm season sample because the variable scores and the sample location both

446 had positive values on PC2. In the same way, it is evident that these samples are characterised by
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447 low values of all the variables that are located in the negative part of the figure for PC2 values.

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4. Discussion

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450 The present study focused on the chemical characterisation and biological effects of Torino PM₁₀.
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451 The north of Italy, in particular the Padana Plain, is an area of widespread air pollution. The weak
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452 dispersion rate observed during winter due to the conformation of the territory represents a
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453 relevant factor (Cadum et al., 2009). Various air pollution indicators are above the WHO guidelines,
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454 especially for PM₁₀. Moreover, the exposure concentrations of 40 µg/m³ defined by the European
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455 Directive 2008/50/EC (*Ambient air quality and cleaner air for Europe*), which is to be met by 2015,
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456 was often exceeded. The comparison of the biological effects with the chemical properties of
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457 particles allowed for the determination of some of the features that are central in eliciting the
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458 toxicity of PM₁₀. The PM source of emissions (both natural and anthropic) is the main parameter
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459 that establishes the characteristics of particles of various size, area and season (Schwarze et al.,
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460 2006). Season-related transformations of chemicals in the atmosphere are another important
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461 parameter to be considered: during summer, the photochemical reactions, associated with
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462 elevated solar radiation, modify the PM₁₀ chemical constituents, while in winter, the low
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463 temperatures facilitate the condensation and absorption of volatile compounds on particle surfaces
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464 (Perrone et al., 2010). All of these parameters have to be taken into account in analysing the
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465 biological effects of PM₁₀, and this complexity is the reason for difficulties in solving the question of
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466 PM toxicity. In particular, the aim was to define whether the cytotoxic and genotoxic effects
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467 produced on the THP1 and A549 cells by PM₁₀ of a low contaminated urban site may be related
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468 with the chemical compounds analysed in the particle samples of different seasons.

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469 **Chemical characterisation showed metals and transition metals with a typical seasonal trend**
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470 **(Schilirò et al., 2010) with higher concentrations during cold seasons and lower ones in the warm**
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471 **seasons ($p < 0.05$).** This trend is in agreement with the results reported in other studies (Ghio et
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472 al., 2012; Kulshrestha et al., 2009). In particular, results showed atmospheric Fe concentrations
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473 with higher values during the cold season. The trend is significant according to our previous

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474 findings (Alessandria et al., 2014; Gilli et al., 2007). However, the level of metal contamination
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475 observed in this site is lower than the level reported at other sites characterised by urban pollution
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476 (Pipalatkhar et al., 2010); in particular, the Fe concentrations are surprisingly lower than those found
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477 in previous works (Alessandria et al., 2014; Schilirò et al., 2010). Such low contamination is
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478 probably related to the sampling site, a background urban station.

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479 The secondary PM₁₀ components represented approximately 50% of total PM₁₀ mass, and other
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480 studies reported similar percentages in urban sites (Lee and Hieu, 2013) and in particular in the
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481 Italian Po valley (Gilli et al., 2007). A higher content of PM₁₀ sulphates in warm seasons is normally
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482 due to the photochemical reactions that occur more frequently.

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483 A higher PAH contamination was observed in the winter and this is probably due to the winter
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484 atmospheric conditions that may promote an accumulation of primary pollutants and because of
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485 low temperatures, the condensation of atmospheric pollutants in the particle phase (Ebi and
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486 McGregor, 2008); this seasonal trend was confirmed in other studies (Sisovic et al., 2008). The low
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487 PAH concentrations may be linked to background sampling sites monitored in this study, similar to
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488 metals. Moreover, the PAH concentration was relatively low compared to other studies conducted
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489 at sites characterised by similar sources of particulates emission (Gutierrez-Castillo et al., 2006).

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490 Many studies investigated the toxicity and mechanism of PM₁₀ on airway epithelial cells
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491 (Alessandria et al., 2014; Hetland et al., 2004); in the present study, both PM₁₀ extracts (organic
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492 and aqueous extracts) exerted a significant impact on cells.

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493 Exposure to ambient air PM induced an oxidative stress that leads to a series of reactions that
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494 inhibit cell proliferation and damage the cell membrane. As reported in another study (Shang et al.,
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495 2013), MTT (Figure 1) and LDH data (Figure 2) show that PM₁₀ exposure did not produce cell
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496 death by damaging the cell membrane of THP1 cells. In some cases, the discrepancy between
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497 LDH release data and reductions in viability detected by MTT (i.e., aqueous extracts in summer
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498 and autumn or organic extracts in spring, Figure 1 and 2) suggest that impairment of viability may
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499 be due to the induction of an apoptotic cell death pathway in which the integrity of the plasma
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500 membrane is maintained. In addition, it is noteworthy that MTT assay measures cellular metabolic
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501 activity; thus, low MTT values (inhibition induced by organic extracts in spring, summer and

502 autumn, Figure 1) indicate reduced metabolic activity/mitochondrial damage or decreased cell
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503 proliferation. In this regard, the fact that different pathways probably triggered LDH release and
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504 cell proliferation inhibition was also highlighted by PCA analysis (in PC1, cell proliferation inhibition
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505 and LDH release were negatively correlated).

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506 From the PCA analysis, the different seasonal chemical composition of PM₁₀ seems to influence
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507 some biological properties. In particular, a seasonal trend was observed for aqueous extracts-
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508 induced inhibition of cell proliferation, with a greater effect in the warm season. A higher cell
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509 viability reduction for warm season samples in comparison to cold samples was also observed in
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510 other studies conducted in the Padana Plain (Alessandria et al., 2014; Perrone et al., 2010).
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511 Moreover, this biological effect clustered total ions together, and this state may be associated with
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512 the toxicity of secondary PM. Indeed, the warm season in Northern Italy is characterised by high
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513 photochemical production of secondary aerosol (Perrone et al., 2013). Moreover, PCA analysis
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514 showed a higher winter cell viability reduction associated with higher PAH concentrations,
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515 suggesting the potential role of these organic compounds in cytotoxicity induction.

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516 On the other hand, the release of LDH does not seem to vary between the cold and warm season
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517 samples (Figure 7). This trend might be associated with anomalous values observed for these
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518 parameters in the spring season or by the low sensitivity of this biological test.

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519 **In this study, the pro-inflammatory potential of PM₁₀ was assessed through the quantification of the**
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520 **relative gene expression of IL8 and TNF α .** Both PM₁₀ extracts increased gene expressions, with a
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521 low increase in summer and autumn and a high increase in spring (especially with the organic
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522 extract) and winter. Therefore, both cytokines showed similar higher effects induced by spring
45
523 PM₁₀ compared with our previous study (Schilirò et al., 2010).

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524 PCA analysis shows no seasonal differences in the expression of TNF α (organic extracts) and IL 8
50
525 (organic and aqueous extracts). This trend might be associated with values observed for these
52
526 parameters in the spring. Moreover, the PCA analysis indicates no relation between the gene
54
527 expression of IL8 or TNF α and chemical parameters, except for TNF α expression (aqueous
56
528 extract) and PM₁₀ metal concentrations. **The association between these two parameters confirms**
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529 **the role of metals and the resulting oxidative stress in the induction of inflammatory responses.**

530 Several studies confirmed the critical role of organic compounds adsorbed on PM (Oh et al., 2011),
1
531 and other investigators directly related PM biological effects to PAH and nitro- and oxygenated-
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532 PAH (Binkova et al., 2003).
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533 In this study, the involvement of PAH and nitro-PAH in inducing a PM biological effect was
7
534 investigated by CYP1A1 expression and genotoxic damage induction (Comet assay). From the
8
10 PCA analysis, seasonality for different biological effects was observed, with a greater genotoxic
1535 effect of organic extracts for cold season compared to hot season samples. Moreover, in the
12
1536 organic extracts of the cold season samples, a lower CYP1A1 expression was detected. The lack
14
1537 of correlation between CYP1A1 expression and PAH, indicated by the opposite position in the
16
1538 loading plot (Figure 4a), might be due to low PAH levels observed at this site that were unable to
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1539 induce a change of CYP1A1 expression. Indeed, the induction of CYP1A1 expression was
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240 relatively low compared to that recorded at other urban sites (Gualtieri et al., 2012). The similarity
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241 between gene expression induced by the two extracts may be related to the low concentration of
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242 contaminants that activated this enzyme.
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3144 The genotoxic effect of organic extracts and some chemical components of PM samples (e.g. PAH
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3345 and metals) clustered all together in the upper part of the loading plot. The seasonal trend
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3546 observed for PAH and genotoxic effects and significant associations ($p < 0.05$) between these
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3747 parameters suggested that PAH might contribute to explaining the genotoxic effect of these
38
4048 extracts. Several studies reported a positive correlation between the PAH content of PM and the
41
4249 particle ability to induce a significant increase in genotoxic damage (Bonetta et al., 2009; Teixeira
43
4450 et al., 2012). The biological genotoxic effect observed might also be influenced by the presence of
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4651 other airborne organic pollutants (Claxton et al., 2004) that were not monitored in this study
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48
4952 because a greater genotoxic effect was observed in some months (in particular October and
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5153 November) when low PAH concentrations were present. Moreover, the different genotoxic effects
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5354 noted in the different months might be ascribed to the low levels of total and carcinogenic PAHs,
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5555 which are likely related to the characteristics of the sampling site (background station). The low
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5756 contamination at the site might also explain the opposite position in the plot of metals and
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59
6057 genotoxic effects of aqueous extracts. Although transition metals may contribute to oxidative stress
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558 induced by PM (Bonetta et al., 2009), in this study the presence of the oxidative damage in PM
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559 water extracts seems not to be related to metals present in the sample.

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561 **5. Conclusions**

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562 The results obtained showed that PCA may be considered a useful tool to analyse the association
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563 between the chemical composition and the biological effects of PM. The data confirmed the
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564 hypothesis that PM composition and seasonality play an important role in particle-induced toxicity.
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565 This finding, recently reported by numerous researchers on this topic in different urban
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566 environments, was also confirmed in this low PM₁₀ contaminated urban site.

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567 Although the toxicity of several chemical compounds is well established, it is essential to consider
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568 the synergic biological effects of hundreds of compounds adsorbed on particles. In this study, the
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569 biological tests showed a different sensitivity: cell viability, cytotoxicity and comet assay seemed to
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570 describe the PM mixture well, while gene expressions and modified comet assay were less
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571 sensitive. These results emphasise the need to use more than one *in vitro* test for biological effects
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572 assessments of PM samples.

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573 Finally, the PM-induced biological effects at a site with low pollution levels showed that reductions
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574 in PM₁₀ mass did not seem to be sufficient to decrease its toxicity, and this is a matter of concern
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575 for all policies aimed at the protection of human health.

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576

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43
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579

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723 **FIGURE CAPTIONS**

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725 **Figure 1.** Seasonal differences in (a) the inhibition (%) of THP1 cell proliferation as measured by
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726 MTT and (b) the LDH release (%) of THP1 cells after exposure (48 h, 200 µg/mL) to PM₁₀ organic
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727 and aqueous extracts (black and grey bars, respectively). Control level is at 0%. * indicates
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1728 statistically significant differences from the control, $p < 0.05$ (ANOVA).

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730 **Figure 2.** Seasonal differences in (a) TNF α expression (fold increase), (b) IL8 expression (fold
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731 increase) and (c) CYP1A1 expression (fold increase) of THP1 cells after exposure (48 h, 200
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732 µg/mL) to PM₁₀ organic and aqueous extracts (black and grey bars, respectively). Control level is
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2733 at 1-fold increase. * indicates statistically significant differences from the control, $p < 0.05$
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734 (ANOVA).

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736 **Figure 3.** Seasonal differences in the mean % DNA tail value evaluated by the alkaline version of
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3737 the Comet assay after exposure to PM₁₀ organic and aqueous extracts (24 h, 500 µg/mL or 200
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3738 µg/mL, respectively). Control levels are at 0.6, 0.3 and 0.5 mean % DNA tail, respectively, for the
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739 Comet assay (organic and aqueous extracts) and Fpg modified Comet assay (aqueous extracts). *
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3740 indicates statistically significant differences from the control, $p < 0.05$ (t-test).

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4242 **Figure 4.** (a) Loading plot from PCA (PC1 vs PC2; Acet: acetone, organic extracts; RPMI: aqueous
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743 extracts); (b) score plot from PCA (PC1 vs PC2; 1: January, 2: February, 3: March, 4: April, 5: May,
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744 6: June, 7: July, 8: August, 9: September, 10: October, 11: November, 12: December; C: cold
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745 season, W: warm season).

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Figure 1. Seasonal differences in **(a)** the inhibition (%) of THP1 cells proliferation and **(b)** the LDH release (%) of THP1 cells after exposure (48h, 200 $\mu\text{g}/\text{mL}$) to PM_{10} organic and aqueous extracts (black and grey bars respectively). Control level is at 0%. * indicate statistically significant differences from the control, $p < 0.05$ (ANOVA).

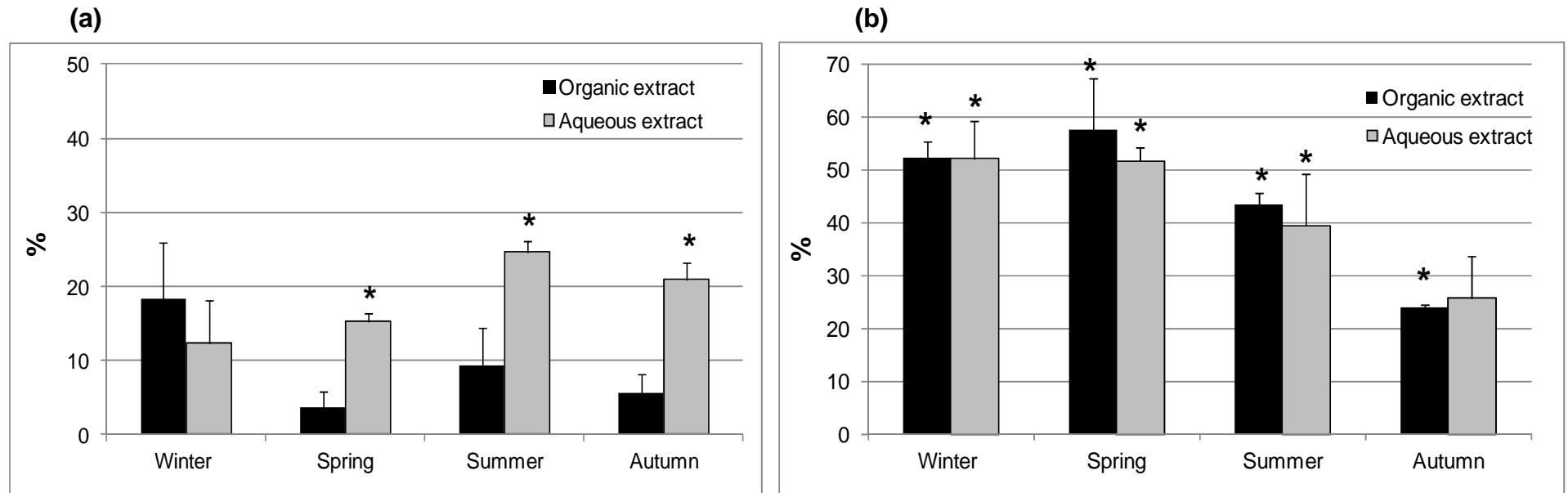


Figure 2. Seasonal differences in **(a)** TNF α expression (fold increase), **(b)** IL8 expression (fold increase) and **(c)** CYP1A1 expression (fold increase) of THP1 cells after exposure (48h, 200 $\mu\text{g}/\text{mL}$) to PM $_{10}$ organic and aqueous extracts (black and grey bars respectively). Control level is at 1 fold increase. * indicate statistically significant differences from the control, $p < 0.05$ (ANOVA).

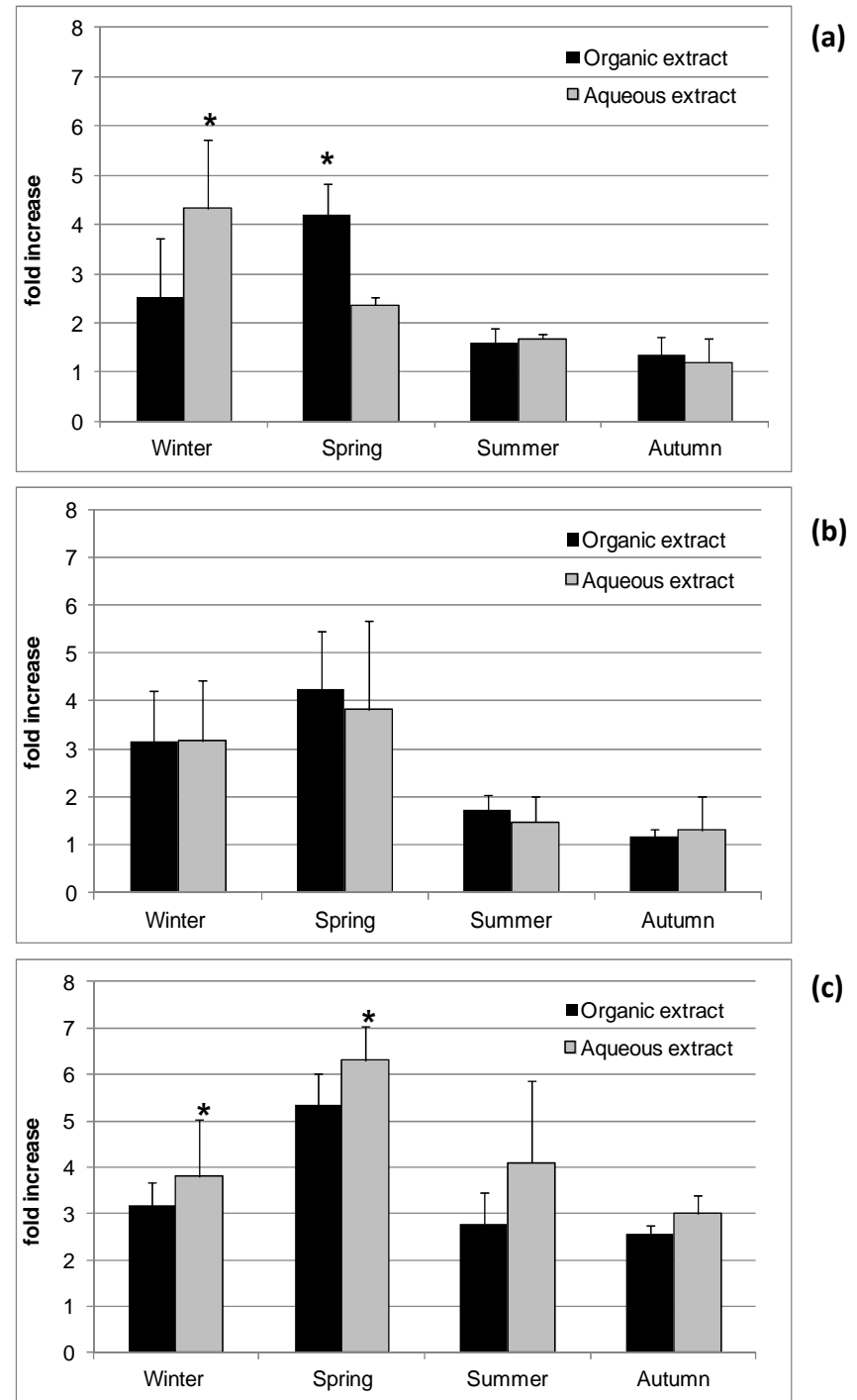


Figure 3. Seasonal differences in the mean % DNA tail value evaluated by alkaline version of the Comet assay after exposure to PM₁₀ organic and aqueous extracts (24h, 500 µg/mL or 200 µg/mL respectively). Control level is at 0.6, 0.3 and 0.5 mean % DNA tail respectively for Comet assay (organic and aqueous extracts) and Fpg modified Comet assay (aqueous extracts). * indicate statistically significant differences from the control, $p < 0.05$ (t-test).

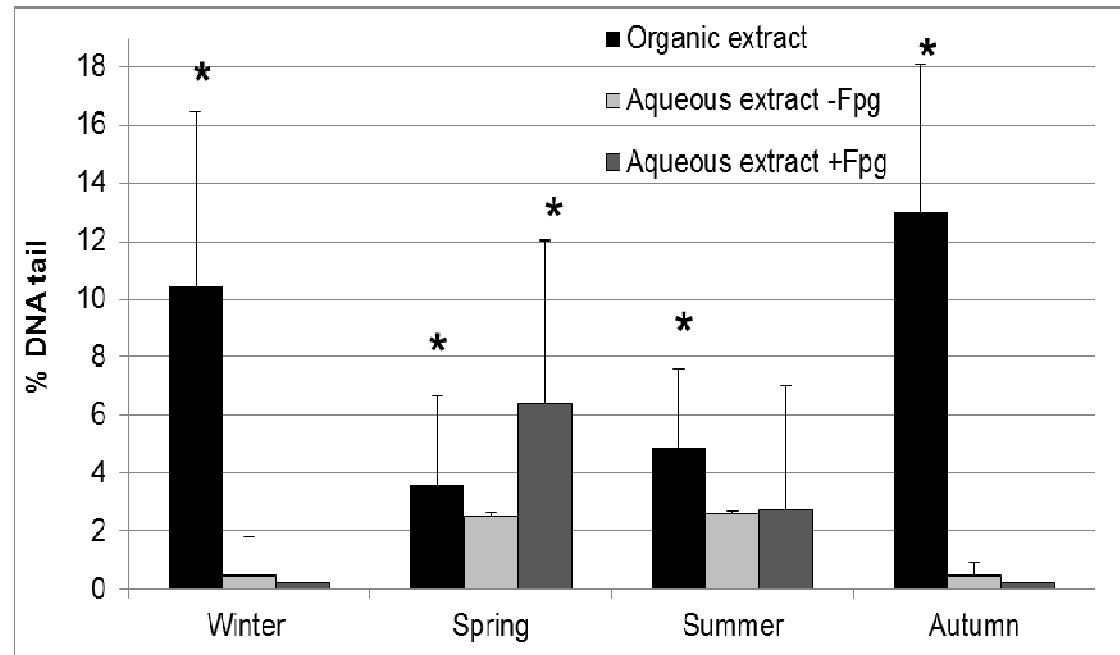
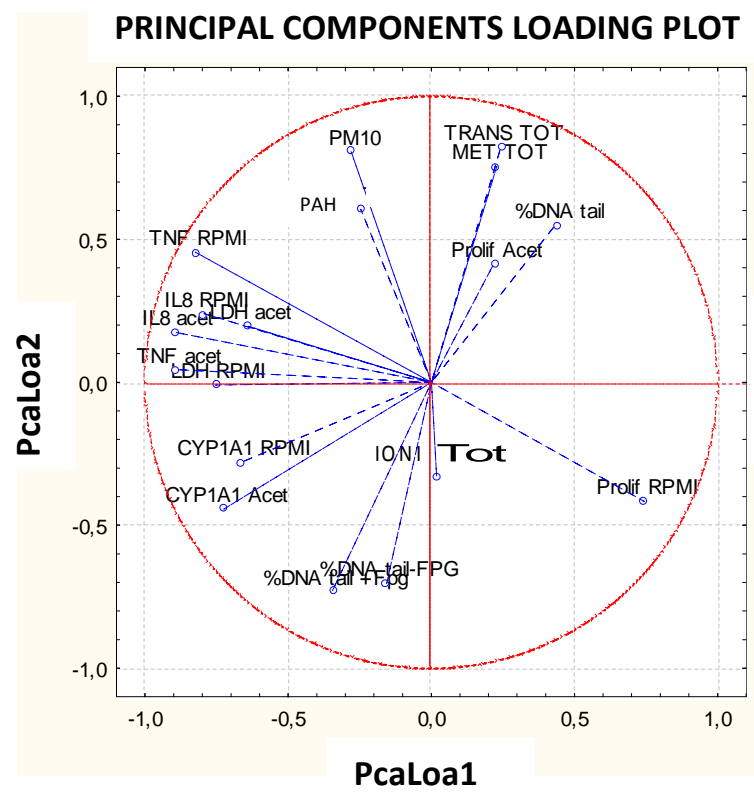


Figure 4. (a) Loading plot from PCA (PC1 vs PC2; Acet: acetone, organic extracts; RPMI: aqueous extracts); (b) score plot from PCA (PC1 vs PC2; 1: January, 2: February, 3: March, 4: April, 5: May, 6: June, 7: July, 8: August, 9: September, 10: October, 11: November, 12: December; C: cold season, W: warm season).

a)



b)

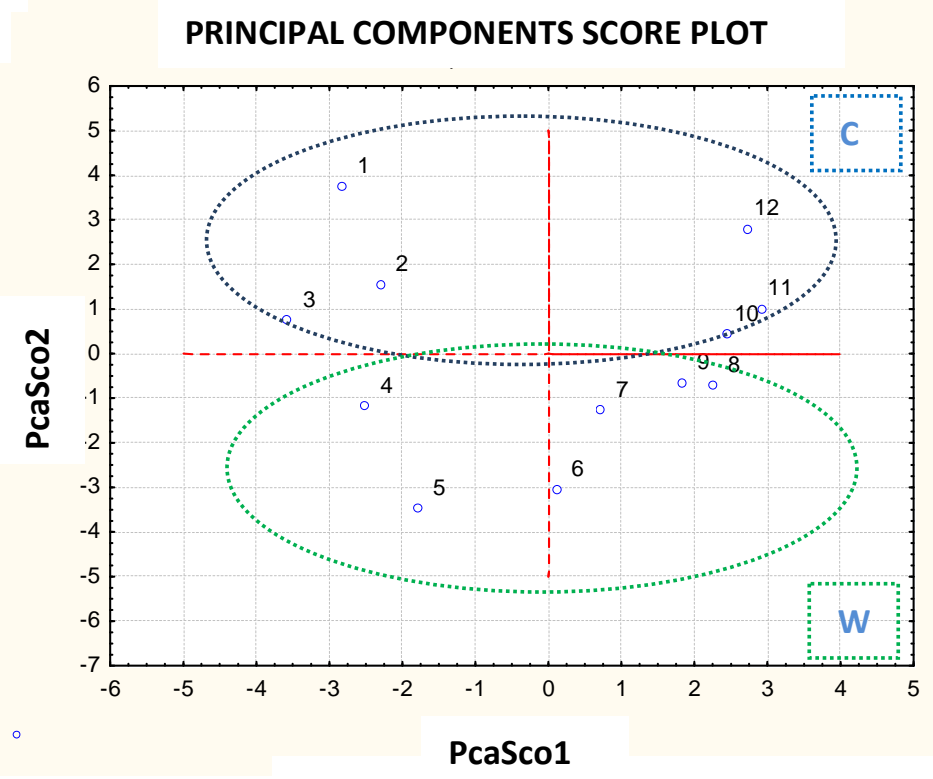


Table 1. Summary description of PM₁₀ samples, chemical measurements and biological responses.

PM samples	Chemical measurements
PM ₁₀	<u>Polycyclic aromatic hydrocarbons PAH_s</u>
Urban background station	Naphthalene
12 month (January-December 2009)	Acenaphthylene
Chemical measurements	Acenaphthene
<u>Metals</u>	Fluorene
Aluminium Al	Phenanthrene
Arsenic As	Anthracene
Barium Ba	Fluoranthene
Cadmium Cd	Pyrene
Chromium Cr	Chrysene
Cobalt Co	Benzo(a)anthracene ^a
Copper Cu	Benzo(b+j)fluoranthene ^a
Gold Au	Benzo(k)fluoranthene ^a
Indium In	Benzo(a)pyrene ^a
Iron Fe	Benzo(e)pyrene
Lead Pb	Perylene
Manganese Mn	Indeno(1.2.3cd)pyrene ^a
Nickel Ni	Dibenz(ah)anthracene ^a
Palladium Pd	Benzo(ghi)perylene
Platinum Pt	Biological responses
Scandium Sc	<u>Cytotoxicity</u>
Selenium Se	Cell proliferation - MTT (THP-1)
Silver Ag	Lactate dehydrogenase - LDH (THP-1)
Strontium Sr	
Vanadium V	<u>Inflammatory response</u>
Zinc Zn	TNF expression (THP-1)
	IL8 expression (THP-1)
<u>Ions</u>	<u>Metabolic activation</u>
Fluoride F ⁻	Cytochrome CYP1A1 expression (THP-1)
Chloride Cl ⁻	
Bromide Br ⁻	<u>DNA damage</u>
Nitrate NO ₃ ⁻	Genotoxicity - Comet assay (A549)
Phosphate PO ₄ ⁻	Oxidative stress - Comet assay w/o Fpg (A549)
Sulphate SO ₄ ⁻	

^a Carcinogenic PAH_s (US-EPA)

Table 2 Means (\pm standard deviations) of PM₁₀, metals, total ions, sulphates, nitrates and PAH concentrations of the whole sampling period and divided by seasons expressed as ng/m³ and ng/100 μ g of particles.

Sampling period	PM10 $\mu\text{g}/\text{m}^3$	Transition Metals ng/m^3 $\text{ng}/100\mu\text{g}$	Metals ng/m^3 $\text{ng}/100\mu\text{g}$	Ions $\mu\text{g}/\text{m}^3$ $\mu\text{g}/100\mu\text{g}$	Sulphates $\mu\text{g}/\text{m}^3$ $\mu\text{g}/100\mu\text{g}$	Nitrates $\mu\text{g}/\text{m}^3$ $\mu\text{g}/100\mu\text{g}$	PAH ng/m^3 $\text{ng}/100\mu\text{g}$
Winter	60.7 \pm 18.9 ^a	121.54 \pm 23.67 ^a	141.84 \pm 23.27 ^a	23.26 \pm 5.59	6.62 \pm 1.47 ^a	15.26 \pm 3.57	3.40 \pm 4.59
	/	213.84 \pm 70.20	249.23 \pm 75.28	39.43 \pm 3.52	11.39 \pm 2.33	25.88 \pm 2.53	4.7 \pm 5.47
Summer	23.1 \pm 3.5	28.24 \pm 13.60	44.62 \pm 19.24	11.75 \pm 6.57	3.94 \pm 0.36	7.51 \pm 6.98	0.13 \pm 0.15
	/	91.05 \pm 15.11	148.89 \pm 14.27	66.30 \pm 46.78	23.39 \pm 4.09	41.42 \pm 43.09	0.77 \pm 0.38
Spring	21.5 \pm 2.6	19.61 \pm 3.93	32.04 \pm 4.59	13.60 \pm 8.03	4.98 \pm 0.54	8.30 \pm 7.79	0.17 \pm 0.11
	/	118.15 \pm 38.12	187.51 \pm 50.99	48.74 \pm 19.14	17.41 \pm 4.09	29.96 \pm 23.26	0.70 \pm 0.82
Autumn	29.9 \pm 10.9	50.21 \pm 28.30	65.43 \pm 30.27	15.73 \pm 6.72	5.41 \pm 0.62	9.95 \pm 5.99	0.40 \pm 0.17
	/	157.97 \pm 56.32	212.68 \pm 45.12	51.52 \pm 6.67	19.61 \pm 6.12	30.68 \pm 11.78	1.80 \pm 1.47
Whole year	33.7 \pm 18.8	54.90 \pm 45.10	70.98 \pm 48.13	16.09 \pm 7.37	5.24 \pm 1.24	10.25 \pm 6.22	1.02 \pm 2.43
	/	145.25 \pm 64.09	199.58 \pm 58.05	51.50 \pm 24.01	17.95 \pm 5.87	31.98 \pm 22.32	1.99 \pm 2.98

^a statistically significant differences (autumn/winter vs spring/summer) $p < 0.05$ (Wilcoxon test).