UNIVERSITÀ DEGLI STUDI DI TORINO

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Title: Inflammation response and cytotoxic effects in human THP-1 cells of size-fractionated PM10 extracts in a polluted urban site.

Article Type: Research paper

Section/Category: Environmental Toxicology and Risk Assessment

Keywords: size-fractionated PM10; THP-1 cells; LDH; IL-8; TNFalpha; CYP1A1

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Corresponding Author's Institution: University of Torino

First Author: Tiziana Schilirò

Order of Authors: Tiziana Schilirò; Luca Alessandria , PhD; Sara Bonetta, PhD; Elisabatta Carraro, Prof.; Giorgio Gilli, Prof.

Response to Reviewers: RESPONSE TO REVIEWER COMMENTS

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effects of particles. In this study, in vitro assays were performed with extracts of PM, meaning without particles present. The title and text need to be rewritten to clearly state the hypothesis driving the study of extracts instead of PM, discuss the usefulness of the extracts and different extraction methods, and then in the end discuss how the differences in toxic activity of the extracts may in part explain size-related effects observed in PM-exposure studies.

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General comments:
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The year of the study was added as required by the Reviewer.

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Ms. Ref. No.: CHEM37545

Dear Editor,

please find enclosed the revised manuscript "*Inflammation response and cytotoxic effects in human THP-1 cells of size-fractionated PM_{10} extracts in a polluted urban site.*" by Tiziana Schilirò, Luca Alessandria, Sara Bonetta, Elisabetta Carraro and Giorgio Gilli.

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

Finally, our responses to each Reviewers comments have been reported as follows, highlighted in yellow.

Best regards,

Tiziana Schilirò and Co-authors.

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T. Schilirò[*a], L. Alessandria[a^], S. Bonetta[a^], E. Carraro[a], G. Gilli[a]

[a] *Department of Public Health and Pediatrics, University of Torino, Piazza Polonia, 94 - 10126, Torino, Italy*; tiziana.schiliro@unito.it; *luca.al@hotmail.it*; *sara.bonetta@unito.it*; *elisabetta.carraro@unito.it*; *giorgio.gilli@unito.it*

^These authors contributed equally to the work.

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ABSTRACT

To contribute to a greater characterization of the airborne particulate matter's toxicity, size-fractionated PM_{10} was sampled during different seasons in a polluted urban site in Torino, a northern Italian city. Three main size fractions (PM10 - 3 µm; PM3 – 0.95 µm; PM <0.95 µm) extracts (organic and aqueous) were assayed with THP-1 cells to evaluate their effects on cell proliferation, LDH activity, TNFα, IL-8 and CYP1A1 expression.

The mean PM_{10} concentrations were statistically different in summer and in winter and the finest fraction $PM_{<0.95}$ was always higher than the others. Size-fractionated PM_{10} extracts, sampled in an urban traffic meteorological–chemical station produced size-related toxicological effects in relation to season and particles extraction. The PM summer extracts induced a significant release of LDH compared to winter and produced a size-related effect, with higher values measured with PM_{10-3}. Exposure to size-fractionated PM_{10} extracts did not induce significant expression of TNFα. IL-8 expression was influenced by exposure to size-fractionated PM_{10} extracts and statistically significant differences were found between kind of extracts for both seasons. The mean fold increases in CYP1A1 expression were statistically different in summer and in winter; winter fraction extracts produced a size-related effect, in particular for organic samples with higher values measured with $PM_{<0.95}$ extracts.

Our results confirm that the only measure of PM can be misleading for the assessment of air quality moreover we support efforts toward identifying potential effect-based tools (e.g. *in vitro* test) that could be used in the context of the different monitoring programs.

Keywords: size-fractionated PM_{10}; THP-1 cells; LDH; IL-8; TNFα; CYP1A1

1. INTRODUCTION

In the last ten years, a considerable number of new studies have been published, providing evidence on the health effects of size fractions, components and sources of particulate matter (PM). Health effects are observed with short-term and long-term exposures to airborne particles indicating an increase in cardiac and respiratory morbidity and associated mortality (WHO-Europe, 2013).

An increasing body of epidemiological and toxicological evidences have shown PM mass ($PM_{2.5}$ and PM_{10}) comprises fractions with varying types and degrees of health effects, suggesting a role for both the chemical composition (such as transition metals, polycyclic aromatic hydrocarbons - PAHs), physical properties (size, particle number and surface area) and source (Brook et al., 2010; Kroll et al., 2013; Krzyzanowski, 2008; Steenhof et al., 2011).

Airborne particles are generally heterogeneous mixtures consisting of inorganic components (e.g., transition metals), salts, carbonaceous material, volatile organic compounds (VOC), PAHs and biological materials such as endotoxins, fungal spores and pollen (Anderson et al., 2012). Although regulatory standards for PM_{10} and $PM_{2.5}$ as well as specific particle constituents exist, there is still no clear consensus among researchers regarding the mechanisms of toxicity following exposure to current levels of air pollution PM (Schwarze et al., 2007).

Many of the observed health endpoints may result, at least in part, from oxidative stress initiated by the formation of reactive oxygen species (ROS) upon the interaction of PM with epithelial cells and macrophages (Knaapen et al., 2004). Induction of cellular oxidative stress and resulting activation of pro-inflammatory mediators are considered to play a central role in the development of airway diseases (Lonkar and Dedon, 2011; Oh et al., 2011). Pulmonary inflammation is generally characterized by local recruitment of proinflammatory cells such as neutrophils and macrophages, which are involved in the up regulation of various signaling molecules, such as cytokines (TNFα, IL-6) and chemokines (IL-8) (Lauer et al., 2009; Michael et al., 2013). Based on these mechanisms, increased production and release of inflammatory mediators by cells are relevant and often used parameters suggesting PM-induced toxicity. These mediators in connection with an increasing ROS formation play a key role in the development of several inflammatory diseases like

acute lung injury, COPD, chronic bronchitis and asthma (Valavanidis et al., 2008). Besides the ability to introduce inflammation, particles may also cause oxidative DNA damage because of their physicochemical properties. Within this so-called primary genotoxicity, surface associated free radicals and transition metals are considered to play a major role (Roig et al., 2013). Moreover particle-elicited inflammation and subsequent generation of ROS can lead to oxidative DNA damage, and this pathway is defined as secondary genotoxicity (Schins and Hei, 2006). In addition, organic compounds, such as polycyclic aromatic hydrocarbons (PAH) coated onto PM may induce DNA damage, mutations and cytotoxicity at a cellular level (Bonetta et al., 2009). Upon entering the organism, PAHs are first metabolized to transdihydrodiols by the activity of the cytochromes P450 (CYP) superfamily member CYP1A1 enzyme and epoxide hydrolase and then oxidized to reactive electrophiles by two pathways (Hanzalova et al., 2010; Spink et al., 2008). Intermediates thereafter interact with DNA target sites to produce adducts, mutation, DNA strand breaks and eventually tumour initiation (Schwarze et al., 2007).

In this regards, recently, outdoor air pollution and its major component, outdoor particulate matter, were classified as carcinogenic for humans (Group1) (Loomis et al., 2013). Consequently, reducing air pollution and particle matter to the lowest amount possible is becoming a marked priority. However, in order to define the most cost-effective policies, the most health-relevant PM fractions will have to be identified based on toxicological characteristics. With this purpose, to contribute to a greater characterization of the airborne particulate matter's toxicity, the biological effects of airborne size-fractionated PM_{10} collected in a high polluted urban site during different seasons were examined. The effects of size fractionated PM_{10} extracts (organic and aqueous) from winter and summer seasons were evaluated in human cell cultured *in vitro* (THP-1 monocytes) considering as endpoints: the inhibition of cell proliferation, lactate dehydrogenase (LDH) activity and tumour necrosis factor alpha (TNFα), interleukin 8 (IL-8) and CYP1A1 expressions.

2. MATERIALS AND METHODS

2.1 Size-fractionated PM_{10} sampling and gravimetric analysis

113 The sampling site was located in Torino (northwest of the Italian Padana Plain), in a
114 meteorological–chemical station of the Environmental Protection Regional Agency (Piedmont
115 A.R.P.A.), it was representative of typical urban pollution, with high vehicle traffic impact
116 (coordinates utm, WGS84, X: 394836, Y: 4996153).
117 The samplings were made during two periods, summer (July/August 2011) and winter
118 (January/February 2012), PM_{10} samples were collected using a Sierra-Andersen high-volume
119 cascade impactor (AirFlow PM_{10}-HVS sampler which a multi-stage cascade impactor, with pre-
120 selector complying with the EN-12341 norm by Analitica Strumenti) at an electronically controlled
121 flow at 1160 L/min. In each period, sampling durations were 24 hours and were repeated on
122 Tuesday and Friday for three weeks (6 samplings and 6 filters for each fraction, for a total of 36
123 filters in each period).
124 First, PM_{10} was selected by a pre-selector, and then the multistage impactor determined the
125 division of different particle sizes of the sampled particles by the differentiation of the aerodynamic
126 diameter, which can identify the type of trajectory that particles take inside the suction flow related
127 to the three main aerodynamic factors of the particles themselves: dimension, shape and density
128 (Analitica Strumenti). Particles with sufficient inertia will impact on that particular stage collection
129 plate, whilst smaller particles will remain entrained in the air stream and pass to the next stage
130 where the process is repeated. The stages are assembled in a stack or row in order of decreasing
131 particle size. The particle size fractions are as follows: 10.0-7.2, 7.2-3.0, 3.0-1.5, 1.5-0.95, 0.95-
132 0.49, and <0.49 µm. Glass microfiber filters with splits (Type A/E; 8”x10”; Gelman Sciences, MI,
133 USA) were used to collect particles on each impactor plate; finally, glass microfiber filters (20.32 ×
134 25.40 cm; Pall Corporation, NY, USA) were present as back-up filters to collect the finest particles
135 (<0.49 µm). All of the filters were pre- and post-conditioned by placing them in a dry and dark
136 environment for 48 h, and then they were weighted in a room with a controlled temperature and
137 humidity. The PM concentrations in the air volume sampled was calculated as previously described
138 (Schilirò et al., 2010; Traversi et al., 2011), according to the European Committee for
139 Standardization (CEN, 1998).
140 ***2.2 Particles extraction***

After gravimetric analyses, one strip was cut from each fractionated PM filters. The strips were pooled into three main size fractions (PM_{10-3}: 10 - 3 µm; $PM_{3-0.95}$: 3 – 0.95 µm; and $PM_{<0.95}$: <0.95 µm) (two strips for each main size fraction) for a total of 12 strips for each main fraction both in summer and in winter. This procedure was repeated two times to perform two different extractions chosen for different abilities in the extraction of different compounds: acetone was selected as the solvent for organic-extractable compounds (e.g., PAHs) (Claxton et al., 2004) and RPMI1640, without foetal calf serum (FCS,) was chosen to extract water-soluble components (e.g., metals), theoretically comparable to the extraction at the lung cells (Hetland et al., 2004). The 12 strips for each main fraction both in summer and winter were cut into small pieces and were placed in 50 mL polypropylene sterile tubes with 15 mL of each extraction media. The tubes were placed in an ultrasonic water bath for 10 min, followed by 1 min of vortexing. This procedure was repeated 3 times (45 mL of extracts). The samples were centrifuged at 5000 x g for 10 min to remove the filter material, and supernatant was collected. Acetone extract (organic) for biological tests was evaporated with a rotary evaporator and suspended in dimethyl sulfoxide (DMSO) for the THP-1 cells tests. RPMI extracts (aqueous) were directly assayed for biological analysis (Alessandria et al., 2014; Schilirò et al., 2010). Each extraction was also performed on a QC laboratory filter (treated with the same method as the samples). Unless specified otherwise, all chemicals were purchased from Sigma, St. Louis, MO, USA.

2.3 Cell culture

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% CO_2. Serum-free RPMI1640 without phenol red constituted the experimental medium. The use of serum-free allow a better standardization of experimental conditions.THP-1 cells are more sensitive in citotoxicity tests respect to other cell lines (e.g. A549 cells) (Corsini et al., 2013).

2.4 Cell viability- MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure cell viability as previously described (Schilirò et al., 2015); briefly THP-1 cells were seeded, using experimental medium, in 24-well plates at a density of 4×10^4 cell/well and exposed to an equivalent particle concentration of 200 µg/mL. After treatment with PM extracts (72 h), the MTT (final concentration 0.5 mg/mL) was added to cells and incubated at 37°C for 4 h. Then, 1 mL 10 % SDS/0.01 M HCl was added in each well, and the cells were incubated overnight. Cell proliferation was determined by measuring absorbance at 570 nm with a micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc.). Each PM extract was tested in triplicate. QC laboratory filter extract was tested in the same manner of the PM extracts. The % of inhibition of cell proliferation was calculated comparing the absorbance of exposed cells with the absorbance of control cells (for acqueous extract non-exposed cells and for organic extracts solvent exposed cells).

2.5 Citotoxicity-LDH assay

To evaluate PM extracts cytotoxicity, LDH activities from damaged cells were measured in cell-free culture supernatants, as previously described and modified for cells in suspension (Schilirò et al., 2015). Briefly, THP-1 cells were seeded in 6-well plates at a density of 1×10^6 cells/well with experimental medium and exposed to PM extracts containing an equivalent particle concentration of 200 µg/mL (Alessandria et al., 2014). At 72 h, LDH activity was measured in the supernatant and cell lysate. LDH activity was calculated as the ratio of extracellular LDH (measured in the supernatant) and total LDH (expressed as sum of LDH measured in supernatant and cell lysate). To obtain cell lysates, cells were centrifuged to eliminate the supernatant and resuspended with 1 mL of TRAP (82.3 mM triethanolamine hydrochloride, pH 7.6) and sonicated for 10 s. Then, LDH was measured by adding 250 µL of a mix containing 0.25 mM NADH and 0.5 mM pyruvate, and consumption of NADH was measured as absorbance at 340 nm in a micro-plate reader (Benchmark Plus Microplate Reader, Biorad). Each PM extracts was tested in triplicate. QC laboratory filter extract was tested in the same manner of the PM extracts. QC laboratory filter extracts were tested in the same manner of PM_{10} extracts samples. LDH activity of exposed cells is expressed as a percentage of control cells (for acqueous extract non-exposed cells and for organic extracts solvent exposed cells).

2.6 IL-8, TNFα and CYP1A1 gene expression

For quantitative Real Time PCR (qRT-PCR), THP-1 cells were seeded in 6-well culture plates and cultured overnight. Then, the cell culture medium was replaced by a fresh experimental medium and PM extracts were tested at an equivalent particle concentration of 200 µg/mL for 48 h. Total RNA was isolated from treated and untreated THP-1 cells as previously described (Schilirò et al., 2015). Briefly total RNA (1 µg) was used for the first-strand cDNA reaction with reverse transcriptase using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative gene expression was determined by real-time PCR with a Chromo 4 Real-Time System (Bio-Rad, Hercules, CA, USA) using 1 µL of cDNA sample aliquot (10 ng total mRNA) as a template with Sso Fast EvaGreen SuperMix (Bio-Rad, Hercules, CA, USA).

Three genes were analysed, TNFα , IL-8, CYP1A1. The following primer sequences were used for relative gene expression analysis:

TNFα - f: 5′-ATGAGCACTGAAAGCATGATCCG-3′,

TNFα - r: 5′- CAGGCTTGTCACTCGGGGTTC-3′;

IL-8 - f: 5′- TGCCAAGGAGTGCTAAAG-3′,

IL-8 - r: 5′-CTCCACAACCCTCTGCAC-3′;

CYP1A1 - f: sense 5′- GGCAGATCAACCATGACCAGAAG-3′ ,

CYP1A1 - r: 5′-ACAGCAGGCATGCTTCATGGTTAG-3′.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT1) gene expression were used as the internal control as they are housekeeping genes and were analysed in each experiment for normalisation using the following primers:

GAPDH - f: 5’-CCCTTCATTGACCTCAACTACATG-3’,

GAPDH - r: 5’-TGGGATTTCCATTGATGACAAGC-3’;

HPRT1- f: 5’-TGACACTGGCAAAACAATGCA-3’,

HPRT1 - r: 5’-GGTCCTTTTCACCAGCAAGCT-3’.

Relative fold inductions were calculated using the ΔCt formula (Schefe et al., 2006). QC laboratory filter extract was tested in the same manner of PM extracts. All real-time RT-PCR assays for

relative gene expression were repeated at least three times in duplicates from independent total RNA samples for the same treatment conditions. non-exposed cells and solvent exposed cells were used as control cells for acqueous and organic extracts respectively.

2.7 Chemicals and meteo data

Chemical data (metals: As, Cd, Ni, Pb and PAHs: Benzo(a)antracene, Benzo(b+j+k)fluorantene, Benzo(a)pyrene, Indeno(1,2,3-cd)pyrene) were extracted from a specialised database provided by the Regional System for the real-time monitoring of Air Quality, AriaWeb (ARPA Piemonte, 2015). The data were obtained for the same meteorological–chemical station and for the same days as our sampling. For example the metals data represent a mean of daily data collected using the standard monitoring method EN 14902:2005 «Standard method for measurement of Pb/Cd/As/Ni in the PM_{10} fraction of suspended particulate matter» (2008/50/EC, annex VI, section B). All the adopted methods conform to the directive and were validated before being published in the AriaWeb database (ARPA Piemonte, 2015).

2.8 Statistical analysis

Statistical analyses were performed using the SPSS Package, version 21.0 for Windows. The Spearman rank order correlation coefficient was used to assess relationships between variables, a Wilcoxon test was used to compare means, and ANOVA was used for multivariate analysis in which we assumed an equal variance while using Tukey as post hoc multiple comparisons. The mean differences and correlations were considered significant at $p < 0.05$.

3. RESULTS

3.1 PM concentration and meteo-chemicals data

The mean PM_{10} concentration was 23.9 ± 8.4 µg/m^3 in summer and 83.0 ± 36.2 µg/m^3 in winter, and this difference was statistically significant (Wilcoxon test, $p < 0.01$) (Figure 1).

The winter fractions concentrations compared with the corresponding summer ones showed significant differences (Wilcoxon test, $p < 0.01$).

ANOVA analysis performed assuming the gravimetric results as a dependent variable and the different PM_{10} size fractions as the independent variable reported significant general results

(summer: F = 15.091, p < 0.001, d=5; and winter: F = 4.904, p < 0.01, d=5): the finest fraction $PM_{<0.49}$ was always significantly higher than the others in summer (Tukey test, p < 0.01) and represented about 37% of the total PM_{10} mass, while in winter was significantly higher only *vs* $PM_{10.0-7.2}$ and $PM_{3.0-1.5}$ (Tukey test, p < 0.01) and represented about 28% of the total PM_{10} mass; Figure 1 shows also the percentage size composition of PM_{10}.

Table 1 reports the descriptive analysis of the chemical and meteo-climatic parameters collected during the summer and winter samplings.

The mean temperature difference between sampling seasons was significant (Wilcoxon test, p < 0.01): the mean winter temperature was 1.2 ± 4.2°C and the mean summer temperature was 23.0 ± 1.8 °C. Also radiation was significantly different (Wilcoxon test, p < 0.01) in the two considered periods: 23 ± 3 MJ/m^2 in summer and 11 ± 3 MJ/m^2 in winter.

Neither the average humidity nor the wind speed were significantly different (Wilcoxon test, p >0.05), with 82% humidity during the winter *vs.* 63% summer and a range wind speed in both seasons of 1.0-2.0 m/s.

ANOVA analysis performed assuming the gravimetric results as a dependent variable and the three main size fractions (analyzed two by two: PM_{10-3}: 10 - 3 µm; $PM_{3-0.95}$: 3 – 0.95 µm; and $PM_{<0.95}$: 0.95 - < 0.49 µm) as the independent variable reported a significant result in which the finest fraction ($PM_{<0.95}$) was significantly higher than the others both in summer and in winter (summer: F = 11.170, p < 0.01, d=2; and winter: F = 5.790, p < 0.05, d=2). Beyond this $PM_{<0.95}$ represented about 53% of PM_{10} mass in this urban site both in summer and in winter.

Referring to the annual limits of metals set by the European Directive 2008/50/EC, these are observed even if our sampling reflects only spot seasonal situations (3 weeks in each season) and the European limits refer to yearly averages. Cd and Pb winter concentrations showed significant differences (Wilcoxon test, p < 0.05) if compared with the corresponding summer concentrations.

Moreover both total PAHs and single PAHs concentrations were higher during the winter than summer (Wilcoxon test, p < 0.05).

3.2 Cell proliferation

Figure 2a shows effects of the different extracts on cell proliferation: inhibition of THP-1 cell proliferation observed *in vitro* was significant respect to control cells for all PM extracts although the observed effect was independent of the aerodynamic diameter of the particles and of kind of PM extract. Both aqueous and organic extracts inhibited cell proliferation with similar effects (Wilcoxon test, $p > 0.05$). The mean percentage proliferation inhibition was 30.8 ± 6.0 % for summer and 28.4 ± 6.9 % for winter fraction extracts. The difference between summer and winter samples was not significant both for organic and aqueous extracts (Wilcoxon test, $p > 0.05$).

3.3 LDH release

LDH assay indicates loss in cell membrane integrity and it was used for cytotoxicity determination. In general, the summer PM extracts induced a more significant release of LDH compared to winter (54.3 ± 36.5 % and 22.6 ± 17.7 % respectively; Wilcoxon test, $p < 0.001$). Significant differences in toxicity between the three different size fraction extracts were observed: summer organic extracts produced a marked size-related effect, with higher values measured with $PM_{10-3} > PM_{3-0.95} > PM_{<0.95}$; considering the winter extracts, LDH release over the negative control was lower than summer ones, except for the finest winter fraction $PM_{< 0.95}$ of aqueous extract (Figure 2b).

3.4 IL-8, TNFα and CYP1A1 gene expression

The production of the pro-inflammatory cytokine TNFα, as well as the chemokine IL-8 were determined in the cell culture supernatant after 48h of size-fractionated PM extracts exposure; these signaling molecules constitute a major parameter in the mediation of airway inflammation (Gualtieri et al., 2010). Exposure of THP-1 cells to size-fractionated PM_{10} extracts did not induce significant expression of TNFα, except for the finest winter fraction both organic and aqueous samples (Figure 3a). On the other side, particles extracts had a stronger effect on IL-8 expression especially organic extracts in summer. Statistically significant differences were found between the two kind of extracts for both season (Wilcoxon test, $p < 0.05$) and comparing summer (4.13 ± 1.34 fold increase) and winter (2.78 ± 0.96 fold increase) extracts (Wilcoxon test, $p<0.001$) (Figure 3b). No significant differences in toxicity between the extracts of the three different size fractions were observed.

306 The mean fold increase in CYP1A1 expression for summer extracts was 3.61 ± 1.18 and for winter
307 was 3.01 ± 1.46, and this difference was significant (Figure 3c). The average effect was higher in
308 summer with the exception of the organic finest fraction extracts.
309 In general in winter the effect was higher for organic extract, indeed there were statistically
310 significant differences in the effects induced by the two type of extracts (Wilcoxon test, $p < 0.05$).
311 PM extracts produced a significant marked size-related CYP1A1 expression in winter; ANOVA
312 analysis performed assuming the CYP1A1 expression as a dependent variable and the different
313 size fractionated extracts as the independent variable showed a significant difference in the effects
314 produced by winter extracts ($F = 8.756$, $p < 0.01$, $d=2$ organic; $F = 6.291$, $p<0.01$ $d=2$ aqueous) with
315 higher values measured with $PM_{<0.95} > PM_{3\text{-}0.95} > PM_{10\text{-}3}$.
316
317 **4. DISCUSSION and CONCLUSIONS**
318 In our study size-fractionated PM_{10} was collected in a polluted urban site in Torino (northwest
319 Italian city) during different seasons. In the north of Italy, in particular in Padana Plain, there is a
320 widespread air pollution; the weak dispersion rate, observed during winter, represents a relevant
321 factor and it is principally due to the conformation of the territory (Cadum et al., 2009). Torino PM
322 concentrations are characterized by high levels of fine particles in winter: during high pollution
323 periods the $PM_{2.5}/PM_{10}$ ratio increased up to 0.87; in this area the winter PM chemical composition
324 is characterised by high levels of PAHs and metals (Romanazzi et al., 2014; Traversi et al., 2015)
325 whereas in summer PM is rich of ions such sulphates (Schilirò et al., 2015). These seasonal
326 differences could be explained as the winter contribution of domestic heating and of a lower
327 atmospheric mixing layer also the low temperatures facilitate the condensation and absorption of
328 volatile compounds on particle surfaces (Brüggemann et al., 2009); during summer, the
329 photochemical reactions, associated with elevated solar radiation, modify the PM_{10} chemical
330 constituents.
331 In the present study the observed PM_{10} winter levels are higher than both the WHO guidelines
332 (Krzyzanowski, 2008) and the EU regulations 2008/50/CE. In addition, critical particle
333 concentrations are present especially for the finest fraction PM < 0.49 µm that represented a very

high proportion of PM_{10}: 37% in summer and 28% in winter. Various studies confirmed that the finest fractions are the most abundant in the atmosphere because of the finest particulate pollution is homogeneously diffused (Perez et al., 2010).

The high temperature and radiation observed in summer respect to winter attested more frequent photochemical reactions in summer that promotes the formation of secondary particles as highlighted by the percentage of fine fraction ($PM_{<0.49}$) in summer. In general the percentage of secondary particles increases with decreasing particle size, this is in agreement with the literature data according to which the components of the inorganic fraction of the secondary particles (ammonium, nitrate and sulphate) are present mainly in the finer fraction (Pateraki et al., 2012; Perrino et al., 2014)

Among the metals monitored in this study only Cd and Pb had a seasonal trend, As and Ni had similar concentrations in winter and summer; in a previous work in this area, metals appeared to be variously distributed among all the fractions, especially in winter season: As, Ni and Pb were preferentially accumulated on the coarse fractions while Cd was equally distributed (Romanazzi et al., 2014). Contrariwise PAHs showed a marked seasonal trend and the mean winter benzo(a)pyrene concentration was higher both than the annual limit set by the European Directive 2008/50/EC (1 ng/m3) and the WHO guide line value of 0.12 ng/m3 (Krzyzanowski, 2008; WHO-Europe, 2013) even if our sampling, as for metals, reflects only a spot seasonal situation. A higher PAHs contamination was observed in the winter and this is probably due to the winter atmospheric conditions that may promote an accumulation of primary pollutants and because of low temperatures, the condensation of atmospheric pollutants in the particle phase (Ebi and McGregor, 2008); this seasonal trend was confirmed in other studies (Sisovic et al., 2008).

Size-fractionated PM_{10} extracts (organic and aqueous) exerted a significant impact on cells both in winter and in summer. The presented results indicated that different PM_{10} fraction extracts lead to clear changes in some toxicological responses.

Inhibition of proliferation showed a trend without significant differences among fraction extracts as found in other researches (Longhin et al., 2013; Wessels et al., 2010), however it was significant both in summer and in winter. The particles size influenced the cytotoxic (LDH release) and pro-

362 inflammatory potential (TNFα concentrations). Organic PM extracts induced a significant size-
363 related LDH release, with greater release by the greater fractions. LDH release was significantly
364 greater in summer than in winter with the exception of aqueous extracts of the finest fraction in
365 which transition metals are mainly accumulated (Daher et al., 2014). Both organic and aqueous
366 winter extracts of the finer fraction induced a significant TNFα expression that may be associated
367 to the presence of PAHs and transition metals respectively in the finer fractions (de Kock et al.,
368 2006).
369 Summer fraction extracts were able to trigger a pro-inflammatory response with the release of
370 significant quantity of IL-8, especially the summer greater fractions. Actually various *in vitro* data
371 showed the higher inflammatory potential of PM extracts of *coarse* fraction (Hetland et al., 2004;
372 Jalava et al., 2007). Inflammatory effect of summer PM extracts could be related to the presence
373 of biogenic compounds (Gualtieri et al., 2010; Schins et al., 2004); the role of endotoxins in leading
374 the PM inflammatory effects has been well documented (Hetland et al., 2004; Traversi et al.,
375 2010). Inflammatory effect of aqueous extracts of PM_{10} fractions could also be related to the
376 particles bound metals; the PM chemical composition describes the presence of crustal elements,
377 ions and metals, variously distributed among all the fractions (Romanazzi et al., 2014), which have
378 been showed to induce cell inflammation (Øvrevik et al., 2005; Veranth et al., 2006).
379 Apparently Torino summer PM_{10} fraction extracts in a traffic site had a higher proinflammatory
380 potential and induces stronger IL-8 responses in confront of a remote site (Schilirò et al., 2015),
381 similar results have been presented by numerous authors (Camatini et al., 2012; Duvall et al.,
382 2008; Gualtieri et al., 2010; Hetland et al., 2004).
383 PM extracts induced a significant size-related CYP1A1 expression, in particular for organic winter
384 extracts. The importance of organic compounds, with regard to CYP1A1 expression, was
385 confirmed as all the winter organic extracts induced significant size-related levels of this biological
386 marker, with greater increase by the finer fraction extracts. This can easily be associated to the link
387 between the majority of particle-bound PAHs and the fine and ultra-fine particles (Claxton et al.,
388 2004; Di Filippo et al., 2010a). In this study, CYP1A1 expression was significant also for the
389 summer extracts even if PAHs levels were lower highlighting the presence of other chemical

compounds capable to induce the CYP1A1 expression (e.g. dioxin like molecules) (Wenger et al., 2009).

Torino PM_{10} winter fractions in a traffic site showed a higher CYP1A1 expression compared to a background urban site (Schilirò et al., 2015) especially the finer fractions extracts.

No statistically significant association were found between the THP-1 endpoints and chemical (metals or PAHs) or meteo-climatic parameters collected during the summer and winter samplings. One possible explanation for the lack of correlations could be that the chemical parameters were quantified in total PM_{10} samples instead of separated fractions.

Comparing results obtained in this work considering a trafficated Torino urban site with those reported in a previous study conducted in a background Torino urban site in the same seasons (Schilirò et al., 2015), the total PM_{10} concentration was higher in the trafficated urban site in winter but showed similar values respect to background site in summer. Moreover, the biological effects were higher in the trafficated site mostly considering inhibition of cell proliferation and CYP1A1 expression in winter and LDH release and IL-8 expression in summer.

Our results show that biological responses significantly differ after exposure to equal mass concentrations of urban traffic and background Torino urban site. These observations confirm the hypothesis that particle composition, as well as source, constitute an important factor in PM induced toxicity as underlined in other studies (Michael et al., 2013).

It is now widely accepted that air pollution and its major components have a marked seasonality, and the toxic content in particulates can vary based upon the meteo-climatic conditions (Albinet et al., 2008). However, biological effects underlined the potential importance of the non-regulated pollutants to assess the air quality (Di Filippo et al., 2010a). This complexity is the reason for difficulties in solving the question of PM toxicity.

In addition, our results confirm that the only measure of PM can be misleading for the assessment of air quality (Di Filippo et al., 2010b) in fact PM_{10} or $PM_{2.5}$ monitoring itself is not informative enough on toxic compounds bound to particles (Topinka et al., 2015). Evaluation of air pollution should not be limited just to gravimetric assessment of PM but should consider also the size spectrum of more dangerous PM fractions. Because of their large and irregular specific surface,

the finest fractions can favour the adsorption of mutagenic and carcinogenic species (such as PAHs, nitro-PAHs and metals) (Claxton e tal., 2004; Topinka et al., 2015), moreover they can reach the deepest sites of the respiratory system (alveolar lung region).The use of different types of extract allows to evaluate the role of chemical composition on biological effects of different PM fraction extracts.

Moreover, the use of *in vitro* text, that integrate toxic potencies of samples including unidentified as well as identified chemicals, can give a correct estimation of the impact of a complex mixture.

Finally in this study we support efforts toward establishing more effective and source-specific regulations for mitigating PM toxicity (Daher et al., 2014) and identifying potential effect-based tools (e.g. *in vitro* test on specific cell line such as THP-1 or A549) that could be used in the context of the different monitoring programmes after being properly standardized. These kind of effect-based monitoring tools could also be valuable in toxicity identification evaluation (TIE) approaches to identify toxic fractions and provide guidance for the identification of causative agents.

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HIGHLIGHTS

The PM_{10} finest fraction PM < 0.49 μm was the highest one both in winter and in summer

The particles size influenced both LDH release and IL-8 concentrations

Size-fractionated PM_{10} extracts induced a significant size-related CYP1A1 expression

Air pollution evaluation should consider the size spectrum of dangerous PM fractions

Inflammation response and cytotoxic effects in human THP-1 cells of size-fractionated PM_{10} extracts in a polluted urban site.

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ABSTRACT

To contribute to a greater characterization of the airborne particulate matter's toxicity, size-fractionated PM_{10} was sampled during different seasons in a polluted urban site in Torino, a northern Italian city. Three main size fractions (PM10 - 3 µm; PM3 – 0.95 µm; PM <0.95 µm) extracts (organic and aqueous) were assayed with THP-1 cells to evaluate their effects on cell proliferation, LDH activity, TNFα, IL-8 and CYP1A1 expression.

The mean PM_{10} concentrations were statistically different in summer and in winter and the finest fraction $PM_{<0.95}$ was always higher than the others. Size-fractionated PM_{10} extracts, sampled in an urban traffic meteorological–chemical station produced size-related toxicological effects in relation to season and particles extraction. The PM summer extracts induced a significant release of LDH compared to winter and produced a size-related effect, with higher values measured with PM_{10-3}. Exposure to size-fractionated PM_{10} extracts did not induce significant expression of TNFα. IL-8 expression was influenced by exposure to size-fractionated PM_{10} extracts and statistically significant differences were found between kind of extracts for both seasons. The mean fold increases in CYP1A1 expression were statistically different in summer and in winter; winter fraction extracts produced a size-related effect, in particular for organic samples with higher values measured with $PM_{<0.95}$ extracts.

Our results confirm that the only measure of PM can be misleading for the assessment of air quality moreover we support efforts toward identifying potential effect-based tools (e.g. *in vitro* test) that could be used in the context of the different monitoring programs.

Keywords: size-fractionated PM_{10}; THP-1 cells; LDH; IL-8; TNFα; CYP1A1

1. INTRODUCTION

In the last ten years, a considerable number of new studies have been published, providing evidence on the health effects of size fractions, components and sources of particulate matter (PM). Health effects are observed with short-term and long-term exposures to airborne particles indicating an increase in cardiac and respiratory morbidity and associated mortality (WHO-Europe, 2013).

An increasing body of epidemiological and toxicological evidences have shown PM mass ($PM_{2.5}$ and PM_{10}) comprises fractions with varying types and degrees of health effects, suggesting a role for both the chemical composition (such as transition metals, polycyclic aromatic hydrocarbons - PAHs), physical properties (size, particle number and surface area) and source (Brook et al., 2010; Kroll et al., 2013; Krzyzanowski, 2008; Steenhof et al., 2011).

Airborne particles are generally heterogeneous mixtures consisting of inorganic components (e.g., transition metals), salts, carbonaceous material, volatile organic compounds (VOC), PAHs and biological materials such as endotoxins, fungal spores and pollen (Anderson et al., 2012). Although regulatory standards for PM_{10} and $PM_{2.5}$ as well as specific particle constituents exist, there is still no clear consensus among researchers regarding the mechanisms of toxicity following exposure to current levels of air pollution PM (Schwarze et al., 2007).

Many of the observed health endpoints may result, at least in part, from oxidative stress initiated by the formation of reactive oxygen species (ROS) upon the interaction of PM with epithelial cells and macrophages (Knaapen et al., 2004). Induction of cellular oxidative stress and resulting activation of pro-inflammatory mediators are considered to play a central role in the development of airway diseases (Lonkar and Dedon, 2011; Oh et al., 2011). Pulmonary inflammation is generally characterized by local recruitment of proinflammatory cells such as neutrophils and macrophages, which are involved in the up regulation of various signaling molecules, such as cytokines (TNFα, IL-6) and chemokines (IL-8) (Lauer et al., 2009; Michael et al., 2013). Based on these mechanisms, increased production and release of inflammatory mediators by cells are relevant and often used parameters suggesting PM-induced toxicity. These mediators in connection with an increasing ROS formation play a key role in the development of several inflammatory diseases like

acute lung injury, COPD, chronic bronchitis and asthma (Valavanidis et al., 2008). Besides the ability to introduce inflammation, particles may also cause oxidative DNA damage because of their physicochemical properties. Within this so-called primary genotoxicity, surface associated free radicals and transition metals are considered to play a major role (Roig et al., 2013). Moreover particle-elicited inflammation and subsequent generation of ROS can lead to oxidative DNA damage, and this pathway is defined as secondary genotoxicity (Schins and Hei, 2006). In addition, organic compounds, such as polycyclic aromatic hydrocarbons (PAH) coated onto PM may induce DNA damage, mutations and cytotoxicity at a cellular level (Bonetta et al., 2009). Upon entering the organism, PAHs are first metabolized to transdihydrodiols by the activity of the cytochromes P450 (CYP) superfamily member CYP1A1 enzyme and epoxide hydrolase and then oxidized to reactive electrophiles by two pathways (Hanzalova et al., 2010; Spink et al., 2008). Intermediates thereafter interact with DNA target sites to produce adducts, mutation, DNA strand breaks and eventually tumour initiation (Schwarze et al., 2007).

In this regards, recently, outdoor air pollution and its major component, outdoor particulate matter, were classified as carcinogenic for humans (Group1) (Loomis et al., 2013). Consequently, reducing air pollution and particle matter to the lowest amount possible is becoming a marked priority. However, in order to define the most cost-effective policies, the most health-relevant PM fractions will have to be identified based on toxicological characteristics. With this purpose, to contribute to a greater characterization of the airborne particulate matter's toxicity, the biological effects of airborne size-fractionated PM_{10} collected in a high polluted urban site during different seasons were examined. The effects of size fractionated PM_{10} extracts (organic and aqueous) from winter and summer seasons were evaluated in human cell cultured *in vitro* (THP-1 monocytes) considering as endpoints: the inhibition of cell proliferation, lactate dehydrogenase (LDH) activity and tumour necrosis factor alpha (TNFα), interleukin 8 (IL-8) and CYP1A1 expressions.

2. MATERIALS AND METHODS

2.1 Size-fractionated PM_{10} sampling and gravimetric analysis

113 The sampling site was located in Torino (northwest of the Italian Padana Plain), in a
114 meteorological–chemical station of the Environmental Protection Regional Agency (Piedmont
115 A.R.P.A.), it was representative of typical urban pollution, with high vehicle traffic impact
116 (coordinates utm, WGS84, X: 394836, Y: 4996153).
117 The samplings were made during two periods, summer (July/August 2011) and winter
118 (January/February 2012), PM_{10} samples were collected using a Sierra-Andersen high-volume
119 cascade impactor (AirFlow PM_{10}-HVS sampler which a multi-stage cascade impactor, with pre-
120 selector complying with the EN-12341 norm by Analitica Strumenti) at an electronically controlled
121 flow at 1160 L/min. In each period, sampling durations were 24 hours and were repeated on
122 Tuesday and Friday for three weeks (6 samplings and 6 filters for each fraction, for a total of 36
123 filters in each period).
124 First, PM_{10} was selected by a pre-selector, and then the multistage impactor determined the
125 division of different particle sizes of the sampled particles by the differentiation of the aerodynamic
126 diameter, which can identify the type of trajectory that particles take inside the suction flow related
127 to the three main aerodynamic factors of the particles themselves: dimension, shape and density
128 (Analitica Strumenti). Particles with sufficient inertia will impact on that particular stage collection
129 plate, whilst smaller particles will remain entrained in the air stream and pass to the next stage
130 where the process is repeated. The stages are assembled in a stack or row in order of decreasing
131 particle size. The particle size fractions are as follows: 10.0-7.2, 7.2-3.0, 3.0-1.5, 1.5-0.95, 0.95-
132 0.49, and <0.49 μm. Glass microfiber filters with splits (Type A/E; 8”x10”; Gelman Sciences, MI,
133 USA) were used to collect particles on each impactor plate; finally, glass microfiber filters (20.32 ×
134 25.40 cm; Pall Corporation, NY, USA) were present as back-up filters to collect the finest particles
135 (<0.49 μm). All of the filters were pre- and post-conditioned by placing them in a dry and dark
136 environment for 48 h, and then they were weighted in a room with a controlled temperature and
137 humidity. The PM concentrations in the air volume sampled was calculated as previously described
138 (Schilirò et al., 2010; Traversi et al., 2011), according to the European Committee for
139 Standardization (CEN, 1998).
140 ***2.2 Particles extraction***

After gravimetric analyses, one strip was cut from each fractionated PM filters. The strips were pooled into three main size fractions (PM_{10-3}: 10 - 3 µm; $PM_{3-0.95}$: 3 – 0.95 µm; and $PM_{<0.95}$: <0.95 µm) (two strips for each main size fraction) for a total of 12 strips for each main fraction both in summer and in winter. This procedure was repeated two times to perform two different extractions chosen for different abilities in the extraction of different compounds: acetone was selected as the solvent for organic-extractable compounds (e.g., PAHs) (Claxton et al., 2004) and RPMI1640, without foetal calf serum (FCS,) was chosen to extract water-soluble components (e.g., metals), theoretically comparable to the extraction at the lung cells (Hetland et al., 2004). The 12 strips for each main fraction both in summer and winter were cut into small pieces and were placed in 50 mL polypropylene sterile tubes with 15 mL of each extraction media. The tubes were placed in an ultrasonic water bath for 10 min, followed by 1 min of vortexing. This procedure was repeated 3 times (45 mL of extracts). The samples were centrifuged at 5000 x g for 10 min to remove the filter material, and supernatant was collected. Acetone extract (organic) for biological tests was evaporated with a rotary evaporator and suspended in dimethyl sulfoxide (DMSO) for the THP-1 cells tests. RPMI extracts (aqueous) were directly assayed for biological analysis (Alessandria et al., 2014; Schilirò et al., 2010). Each extraction was also performed on a QC laboratory filter (treated with the same method as the samples). Unless specified otherwise, all chemicals were purchased from Sigma, St. Louis, MO, USA.

2.3 Cell culture

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% CO_2. Serum-free RPMI1640 without phenol red constituted the experimental medium. The use of serum-free allow a better standardization of experimental conditions.THP-1 cells are more sensitive in citotoxicity tests respect to other cell lines (e.g. A549 cells) (Corsini et al., 2013).

2.4 Cell viability- MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure cell viability as previously described (Schilirò et al., 2015); briefly THP-1 cells were seeded, using experimental medium, in 24-well plates at a density of 4×10^4 cell/well and exposed to an equivalent particle concentration of 200 µg/mL. After treatment with PM extracts (72 h), the MTT (final concentration 0.5 mg/mL) was added to cells and incubated at 37°C for 4 h. Then, 1 mL 10 % SDS/0.01 M HCl was added in each well, and the cells were incubated overnight. Cell proliferation was determined by measuring absorbance at 570 nm with a micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc.). Each PM extract was tested in triplicate. QC laboratory filter extract was tested in the same manner of the PM extracts. The % of inhibition of cell proliferation was calculated comparing the absorbance of exposed cells with the absorbance of control cells (for acqueous extract non-exposed cells and for organic extracts solvent exposed cells).

2.5 Citotoxicity-LDH assay

To evaluate PM extracts cytotoxicity, LDH activities from damaged cells were measured in cell-free culture supernatants, as previously described and modified for cells in suspension (Schilirò et al., 2015). Briefly, THP-1 cells were seeded in 6-well plates at a density of 1×10^6 cells/well with experimental medium and exposed to PM extracts containing an equivalent particle concentration of 200 µg/mL (Alessandria et al., 2014). At 72 h, LDH activity was measured in the supernatant and cell lysate. LDH activity was calculated as the ratio of extracellular LDH (measured in the supernatant) and total LDH (expressed as sum of LDH measured in supernatant and cell lysate). To obtain cell lysates, cells were centrifuged to eliminate the supernatant and resuspended with 1 mL of TRAP (82.3 mM triethanolamine hydrochloride, pH 7.6) and sonicated for 10 s. Then, LDH was measured by adding 250 µL of a mix containing 0.25 mM NADH and 0.5 mM pyruvate, and consumption of NADH was measured as absorbance at 340 nm in a micro-plate reader (Benchmark Plus Microplate Reader, Biorad). Each PM extracts was tested in triplicate. QC laboratory filter extract was tested in the same manner of the PM extracts. QC laboratory filter extracts were tested in the same manner of PM_{10} extracts samples. LDH activity of exposed cells is expressed as a percentage of control cells (for acqueous extract non-exposed cells and for organic extracts solvent exposed cells).

2.6 IL-8, TNFα and CYP1A1 gene expression

For quantitative Real Time PCR (qRT-PCR), THP-1 cells were seeded in 6-well culture plates and cultured overnight. Then, the cell culture medium was replaced by a fresh experimental medium and PM extracts were tested at an equivalent particle concentration of 200 µg/mL for 48 h. Total RNA was isolated from treated and untreated THP-1 cells as previously described (Schilirò et al., 2015). Briefly total RNA (1 µg) was used for the first-strand cDNA reaction with reverse transcriptase using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative gene expression was determined by real-time PCR with a Chromo 4 Real-Time System (Bio-Rad, Hercules, CA, USA) using 1 µL of cDNA sample aliquot (10 ng total mRNA) as a template with Sso Fast EvaGreen SuperMix (Bio-Rad, Hercules, CA, USA).

Three genes were analysed, TNFα , IL-8, CYP1A1. The following primer sequences were used for relative gene expression analysis:

TNFα - f: 5′-ATGAGCACTGAAAGCATGATCCG-3′,

TNFα - r: 5′- CAGGCTTGTCACTCGGGGTTC-3′;

IL-8 - f: 5′- TGCCAAGGAGTGCTAAAG-3′,

IL-8 - r: 5′-CTCCACAACCCTCTGCAC-3′;

CYP1A1 - f: sense 5′- GGCAGATCAACCATGACCAGAAG-3′ ,

CYP1A1 - r: 5′-ACAGCAGGCATGCTTCATGGTTAG-3′.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT1) gene expression were used as the internal control as they are housekeeping genes and were analysed in each experiment for normalisation using the following primers:

GAPDH - f: 5’-CCCTTCATTGACCTCAACTACATG-3’,

GAPDH - r: 5’-TGGGATTTCCATTGATGACAAGC-3’;

HPRT1- f: 5’-TGACACTGGCAAAACAATGCA-3’,

HPRT1 - r: 5’-GGTCCTTTTCACCAGCAAGCT-3’.

Relative fold inductions were calculated using the ΔCt formula (Schefe et al., 2006). QC laboratory filter extract was tested in the same manner of PM extracts. All real-time RT-PCR assays for

relative gene expression were repeated at least three times in duplicates from independent total RNA samples for the same treatment conditions. non-exposed cells and solvent exposed cells were used as control cells for acqueous and organic extracts respectively.

2.7 Chemicals and meteo data

Chemical data (metals: As, Cd, Ni, Pb and PAHs: Benzo(a)antracene, Benzo(b+j+k)fluorantene, Benzo(a)pyrene, Indeno(1,2,3-cd)pyrene) were extracted from a specialised database provided by the Regional System for the real-time monitoring of Air Quality, AriaWeb (ARPA Piemonte, 2015). The data were obtained for the same meteorological–chemical station and for the same days as our sampling. For example the metals data represent a mean of daily data collected using the standard monitoring method EN 14902:2005 «Standard method for measurement of Pb/Cd/As/Ni in the PM_{10} fraction of suspended particulate matter» (2008/50/EC, annex VI, section B). All the adopted methods conform to the directive and were validated before being published in the AriaWeb database (ARPA Piemonte, 2015).

2.8 Statistical analysis

Statistical analyses were performed using the SPSS Package, version 21.0 for Windows. The Spearman rank order correlation coefficient was used to assess relationships between variables, a Wilcoxon test was used to compare means, and ANOVA was used for multivariate analysis in which we assumed an equal variance while using Tukey as post hoc multiple comparisons. The mean differences and correlations were considered significant at $p < 0.05$.

3. RESULTS

3.1 PM concentration and meteo-chemicals data

The mean PM_{10} concentration was 23.9 ± 8.4 μg/m^3 in summer and 83.0 ± 36.2 μg/m^3 in winter, and this difference was statistically significant (Wilcoxon test, $p < 0.01$) (Figure 1).

The winter fractions concentrations compared with the corresponding summer ones showed significant differences (Wilcoxon test, $p < 0.01$).

ANOVA analysis performed assuming the gravimetric results as a dependent variable and the different PM_{10} size fractions as the independent variable reported significant general results

(summer: $F = 15.091$, $p < 0.001$, $d=5$; and winter: $F = 4.904$, $p < 0.01$, $d=5$): the finest fraction $PM_{<0.49}$ was always significantly higher than the others in summer (Tukey test, $p < 0.01$) and represented about 37% of the total PM_{10} mass, while in winter was significantly higher only *vs* $PM_{10.0-7.2}$ and $PM_{3.0-1.5}$ (Tukey test, $p < 0.01$) and represented about 28% of the total PM_{10} mass; Figure 1 shows also the percentage size composition of PM_{10}.

Table 1 reports the descriptive analysis of the chemical and meteo-climatic parameters collected during the summer and winter samplings.

The mean temperature difference between sampling seasons was significant (Wilcoxon test, $p < 0.01$): the mean winter temperature was 1.2 ± 4.2°C and the mean summer temperature was 23.0 ± 1.8 °C. Also radiation was significantly different (Wilcoxon test, $p < 0.01$) in the two considered periods: 23 ± 3 MJ/m^2 in summer and 11 ± 3 MJ/m^2 in winter.

Neither the average humidity nor the wind speed were significantly different (Wilcoxon test, $p >0.05$), with 82% humidity during the winter *vs.* 63% summer and a range wind speed in both seasons of 1.0-2.0 m/s.

ANOVA analysis performed assuming the gravimetric results as a dependent variable and the three main size fractions (analyzed two by two: PM_{10-3}: 10 - 3 µm; $PM_{3-0.95}$: 3 – 0.95 µm; and $PM_{<0.95}$: 0.95 - < 0.49 µm) as the independent variable reported a significant result in which the finest fraction ($PM_{<0.95}$) was significantly higher than the others both in summer and in winter (summer: $F = 11.170$, $p < 0.01$, $d=2$; and winter: $F = 5.790$, $p < 0.05$, $d=2$). Beyond this $PM_{<0.95}$ represented about 53% of PM_{10} mass in this urban site both in summer and in winter.

Referring to the annual limits of metals set by the European Directive 2008/50/EC, these are observed even if our sampling reflects only spot seasonal situations (3 weeks in each season) and the European limits refer to yearly averages. Cd and Pb winter concentrations showed significant differences (Wilcoxon test, $p < 0.05$) if compared with the corresponding summer concentrations.

Moreover both total PAHs and single PAHs concentrations were higher during the winter than summer (Wilcoxon test, $p < 0.05$).

3.2 Cell proliferation

279 Figure 2a shows effects of the different extracts on cell proliferation: inhibition of THP-1 cell
280 proliferation observed *in vitro* was significant respect to control cells for all PM extracts although
281 the observed effect was independent of the aerodynamic diameter of the particles and of kind of
282 PM extract. Both aqueous and organic extracts inhibited cell proliferation with similar effects
283 (Wilcoxon test, $p > 0.05$). The mean percentage proliferation inhibition was 30.8 ± 6.0 % for
284 summer and 28.4 ± 6.9 % for winter fraction extracts. The difference between summer and winter
285 samples was not significant both for organic and aqueous extracts (Wilcoxon test, $p > 0.05$).

286 ***3.3 LDH release***

287 LDH assay indicates loss in cell membrane integrity and it was used for cytotoxicity determination.
288 In general, the summer PM extracts induced a more significant release of LDH compared to winter
289 (54.3 ± 36.5 % and 22.6 ± 17.7 % respectively; Wilcoxon test, $p < 0.001$). Significant differences in
290 toxicity between the three different size fraction extracts were observed: summer organic extracts
291 produced a marked size-related effect, with higher values measured with $PM_{10-3} > PM_{3-0.95} >$
292 $PM_{<0.95}$; considering the winter extracts, LDH release over the negative control was lower than
293 summer ones, except for the finest winter fraction $PM_{< 0.95}$ of aqueous extract (Figure 2b).

294 ***3.4 IL-8, TNFα and CYP1A1 gene expression***

295 The production of the pro-inflammatory cytokine TNFα, as well as the chemokine IL-8 were
296 determined in the cell culture supernatant after 48h of size-fractionated PM extracts exposure;
297 these signaling molecules constitute a major parameter in the mediation of airway inflammation
298 (Gualtieri et al., 2010). Exposure of THP-1 cells to size-fractionated PM_{10} extracts did not induce
299 significant expression of TNFα, except for the finest winter fraction both organic and aqueous
300 samples (Figure 3a). On the other side, particles extracts had a stronger effect on IL-8 expression
301 especially organic extracts in summer. Statistically significant differences were found between the
302 two kind of extracts for both season (Wilcoxon test, $p < 0.05$) and comparing summer (4.13 ± 1.34
303 fold increase) and winter (2.78 ± 0.96 fold increase) extracts (Wilcoxon test, $p<0.001$) (Figure 3b).
304 No significant differences in toxicity between the extracts of the three different size fractions were
305 observed.

306 The mean fold increase in CYP1A1 expression for summer extracts was 3.61 ± 1.18 and for winter
307 was 3.01 ± 1.46, and this difference was significant (Figure 3c). The average effect was higher in
308 summer with the exception of the organic finest fraction extracts.
309 In general in winter the effect was higher for organic extract, indeed there were statistically
310 significant differences in the effects induced by the two type of extracts (Wilcoxon test, $p < 0.05$).
311 PM extracts produced a significant marked size-related CYP1A1 expression in winter; ANOVA
312 analysis performed assuming the CYP1A1 expression as a dependent variable and the different
313 size fractionated extracts as the independent variable showed a significant difference in the effects
314 produced by winter extracts ($F = 8.756$, $p < 0.01$, $d=2$ organic; $F = 6.291$, $p<0.01$ $d=2$ aqueous) with
315 higher values measured with $PM_{<0.95} > PM_{3\text{-}0.95} > PM_{10\text{-}3}$.
316
317 **4. DISCUSSION and CONCLUSIONS**
318 In our study size-fractionated PM_{10} was collected in a polluted urban site in Torino (northwest
319 Italian city) during different seasons. In the north of Italy, in particular in Padana Plain, there is a
320 widespread air pollution; the weak dispersion rate, observed during winter, represents a relevant
321 factor and it is principally due to the conformation of the territory (Cadum et al., 2009). Torino PM
322 concentrations are characterized by high levels of fine particles in winter: during high pollution
323 periods the $PM_{2.5}/PM_{10}$ ratio increased up to 0.87; in this area the winter PM chemical composition
324 is characterised by high levels of PAHs and metals (Romanazzi et al., 2014; Traversi et al., 2015)
325 whereas in summer PM is rich of ions such sulphates (Schilirò et al., 2015). These seasonal
326 differences could be explained as the winter contribution of domestic heating and of a lower
327 atmospheric mixing layer also the low temperatures facilitate the condensation and absorption of
328 volatile compounds on particle surfaces (Brüggemann et al., 2009); during summer, the
329 photochemical reactions, associated with elevated solar radiation, modify the PM_{10} chemical
330 constituents.
331 In the present study the observed PM_{10} winter levels are higher than both the WHO guidelines
332 (Krzyzanowski, 2008) and the EU regulations 2008/50/CE. In addition, critical particle
333 concentrations are present especially for the finest fraction PM < 0.49 μm that represented a very

high proportion of PM_{10}: 37% in summer and 28% in winter. Various studies confirmed that the finest fractions are the most abundant in the atmosphere because of the finest particulate pollution is homogeneously diffused (Perez et al., 2010).

The high temperature and radiation observed in summer respect to winter attested more frequent photochemical reactions in summer that promotes the formation of secondary particles as highlighted by the percentage of fine fraction ($PM_{<0.49}$) in summer. In general the percentage of secondary particles increases with decreasing particle size, this is in agreement with the literature data according to which the components of the inorganic fraction of the secondary particles (ammonium, nitrate and sulphate) are present mainly in the finer fraction (Pateraki et al., 2012; Perrino et al., 2014)

Among the metals monitored in this study only Cd and Pb had a seasonal trend, As and Ni had similar concentrations in winter and summer; in a previous work in this area, metals appeared to be variously distributed among all the fractions, especially in winter season: As, Ni and Pb were preferentially accumulated on the coarse fractions while Cd was equally distributed (Romanazzi et al., 2014). Contrariwise PAHs showed a marked seasonal trend and the mean winter benzo(a)pyrene concentration was higher both than the annual limit set by the European Directive 2008/50/EC (1 ng/m3) and the WHO guide line value of 0.12 ng/m3 (Krzyzanowski, 2008; WHO-Europe, 2013) even if our sampling, as for metals, reflects only a spot seasonal situation. A higher PAHs contamination was observed in the winter and this is probably due to the winter atmospheric conditions that may promote an accumulation of primary pollutants and because of low temperatures, the condensation of atmospheric pollutants in the particle phase (Ebi and McGregor, 2008); this seasonal trend was confirmed in other studies (Sisovic et al., 2008).

Size-fractionated PM_{10} extracts (organic and aqueous) exerted a significant impact on cells both in winter and in summer. The presented results indicated that different PM_{10} fraction extracts lead to clear changes in some toxicological responses.

Inhibition of proliferation showed a trend without significant differences among fraction extracts as found in other researches (Longhin et al., 2013; Wessels et al., 2010), however it was significant both in summer and in winter. The particles size influenced the cytotoxic (LDH release) and pro-

362 inflammatory potential (TNFα concentrations). Organic PM extracts induced a significant size-
363 related LDH release, with greater release by the greater fractions. LDH release was significantly
364 greater in summer than in winter with the exception of aqueous extracts of the finest fraction in
365 which transition metals are mainly accumulated (Daher et al., 2014). Both organic and aqueous
366 winter extracts of the finer fraction induced a significant TNFα expression that may be associated
367 to the presence of PAHs and transition metals respectively in the finer fractions (de Kock et al.,
368 2006).
369 Summer fraction extracts were able to trigger a pro-inflammatory response with the release of
370 significant quantity of IL-8, especially the summer greater fractions. Actually various *in vitro* data
371 showed the higher inflammatory potential of PM extracts of *coarse* fraction (Hetland et al., 2004;
372 Jalava et al., 2007). Inflammatory effect of summer PM extracts could be related to the presence
373 of biogenic compounds (Gualtieri et al., 2010; Schins et al., 2004); the role of endotoxins in leading
374 the PM inflammatory effects has been well documented (Hetland et al., 2004; Traversi et al.,
375 2010). Inflammatory effect of aqueous extracts of PM_{10} fractions could also be related to the
376 particles bound metals; the PM chemical composition describes the presence of crustal elements,
377 ions and metals, variously distributed among all the fractions (Romanazzi et al., 2014), which have
378 been showed to induce cell inflammation (Øvrevik et al., 2005; Veranth et al., 2006).
379 Apparently Torino summer PM_{10} fraction extracts in a traffic site had a higher proinflammatory
380 potential and induces stronger IL-8 responses in confront of a remote site (Schilirò et al., 2015),
381 similar results have been presented by numerous authors (Camatini et al., 2012; Duvall et al.,
382 2008; Gualtieri et al., 2010; Hetland et al., 2004).
383 PM extracts induced a significant size-related CYP1A1 expression, in particular for organic winter
384 extracts. The importance of organic compounds, with regard to CYP1A1 expression, was
385 confirmed as all the winter organic extracts induced significant size-related levels of this biological
386 marker, with greater increase by the finer fraction extracts. This can easily be associated to the link
387 between the majority of particle-bound PAHs and the fine and ultra-fine particles (Claxton et al.,
388 2004; Di Filippo et al., 2010a). In this study, CYP1A1 expression was significant also for the
389 summer extracts even if PAHs levels were lower highlighting the presence of other chemical

390 compounds capable to induce the CYP1A1 expression (e.g. dioxin like molecules) (Wenger et al.,
391 2009).
392 Torino PM_{10} winter fractions in a traffic site showed a higher CYP1A1 expression compared to a
393 background urban site (Schilirò et al., 2015) especially the finer fractions extracts.
394 No statistically significant association were found between the THP-1 endpoints and chemical
395 (metals or PAHs) or meteo-climatic parameters collected during the summer and winter samplings.
396 One possible explanation for the lack of correlations could be that the chemical parameters were
397 quantified in total PM_{10} samples instead of separated fractions.
398 Comparing results obtained in this work considering a trafficated Torino urban site with those
399 reported in a previous study conducted in a background Torino urban site in the same seasons
400 (Schilirò et al., 2015), the total PM_{10} concentration was higher in the trafficated urban site in winter
401 but showed similar values respect to background site in summer. Moreover, the biological effects
402 were higher in the trafficated site mostly considering inhibition of cell proliferation and CYP1A1
403 expression in winter and LDH release and IL-8 expression in summer.
404 Our results show that biological responses significantly differ after exposure to equal mass
405 concentrations of urban traffic and background Torino urban site. These observations confirm the
406 hypothesis that particle composition, as well as source, constitute an important factor in PM
407 induced toxicity as underlined in other studies (Michael et al., 2013).
408 It is now widely accepted that air pollution and its major components have a marked seasonality,
409 and the toxic content in particulates can vary based upon the meteo-climatic conditions (Albinet et
410 al., 2008). However, biological effects underlined the potential importance of the non-regulated
411 pollutants to assess the air quality (Di Filippo et al., 2010a). This complexity is the reason for
412 difficulties in solving the question of PM toxicity.
413 In addition, our results confirm that the only measure of PM can be misleading for the assessment
414 of air quality (Di Filippo et al., 2010b) in fact PM_{10} or $PM_{2.5}$ monitoring itself is not informative
415 enough on toxic compounds bound to particles (Topinka et al., 2015). Evaluation of air pollution
416 should not be limited just to gravimetric assessment of PM but should consider also the size
417 spectrum of more dangerous PM fractions. Because of their large and irregular specific surface,

418 the finest fractions can favour the adsorption of mutagenic and carcinogenic species (such as
419 PAHs, nitro-PAHs and metals) (Claxton e tal., 2004; Topinka et al., 2015), moreover they can
420 reach the deepest sites of the respiratory system (alveolar lung region).The use of different types
421 of extract allows to evaluate the role of chemical composition on biological effects of different PM
422 fraction extracts.
423 Moreover, the use of *in vitro* text, that integrate toxic potencies of samples including unidentified as
424 well as identified chemicals, can give a correct estimation of the impact of a complex mixture.
425 Finally in this study we support efforts toward establishing more effective and source-specific
426 regulations for mitigating PM toxicity (Daher et al., 2014) and identifying potential effect-based
427 tools (e.g. *in vitro* test on specific cell line such as THP-1 or A549) that could be used in the context
428 of the different monitoring programmes after being properly standardized. These kind of effect-
429 based monitoring tools could also be valuable in toxicity identification evaluation (TIE) approaches
430 to identify toxic fractions and provide guidance for the identification of causative agents.
431

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436

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Table

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Table 1. Descriptive analysis for chemical and meteo-climatic parameters in summer and winter samplings (means with standard deviations, minimum and maximum values) in the urban meteorological–chemical station. nd: not detected

Parameters	Mean (sd)		Min		Max	
	summer	winter	summer	winter	summer	winter
Temperature (°C)	**23.0 (1.8)**	**1.2 (4.2)**	13.7	- 5.5	32.3	16.7
Wind (m/s)	**1.7 (0.3)**	**1.6 (0.4)**	1.1	1.0	2.0	2.0
Precipitations (mm)	**0.2 (sum)**	**0.0 (sum)**	0.0	0.0	0.2	0.0
Humidity (%)	**63 (20)**	**82 (21)**	16	27	96	100
Radiation (MJ/m^2)	**23 (3)**	**11 (3)**	19	6	28	12
PM 10-3 µm (µg/m^3)	**6.6 (1.0)**	**18.0 (2.3)**	4.3	10.1	10.8	23.9
PM 3 – 0.95 µm (µg/m^3)	**4.5 (0.6)**	**20.9 (5.2)**	2.9	9.2	6.6	45.0
PM < 0.95 µm (µg/m^3)	**12.7 (1.9)**	**44.1 (8.7)**	6.8	24.1	19.5	85.2
As (ng/m^3)	**0.7 (0.3)**	**0.7 (0.3)**	nd	nd	0.8	0.7
Cd (ng/m^3)	**0.2 (0.1)**	**0.5 (0.2)**	0.1	nd	0.2	0.5
Ni (ng/m^3)	**5.9 (2.5)**	**6.5 (2.6)**	nd	nd	6.3	7.6
Pb (ng/m^3)	**6.2 (2.5)**	**15.5 (1.2)**	nd	13	7.0	16
Benzo(a)antracene	**0.04 (0.01)**	**2.08 (0.93)**	nd	nd	0.04	3.16
Benzo(b+j+k)fluorantene	**0.20 (0.08)**	**4.04 (1.81)**	nd	nd	0.25	6.38
Benzo(a)pyrene	**0.08 (0.05)**	**2.20 (0.98)**	nd	nd	0.10	3.30
Indeno(1,2,3-cd)pyrene	**0.06 (0.02)**	**2.18 (0.97)**	nd	nd	0.06	3.12

FIGURE CAPTIONS

Figure 1. PM_{10} fractions concentration and percentage size composition in winter (a) and in summer (b), sampled in a polluted urban meteorological–chemical station.

Figure 2. Inhibition of proliferation (a) and LDH release (b) of THP-1 cells exposed (48h, 200µg/mL) to organic (black bars) and aqueous (grey bars) PM fraction extracts of summer and winter season. Bars represent the mean value from three replicates; error bars represent standard error of mean. *p< 0.05 *vs* control (control level is at 0%) according to Wilcoxon test.

Figure 3. TNFα (a), IL-8 (b) and CYP1A1 (c) expressions of THP-1 cells exposed (48h, 200µg/mL) to organic (black bars) and aqueous (grey bars) PM fraction extracts of summer and winter season. Bars represent the mean value from three analysis in duplicate; error bars represent standard error of mean. *p< 0.05 *vs* control cells (control level is at 1 fold increase) according to Wilcoxon test.

Figure
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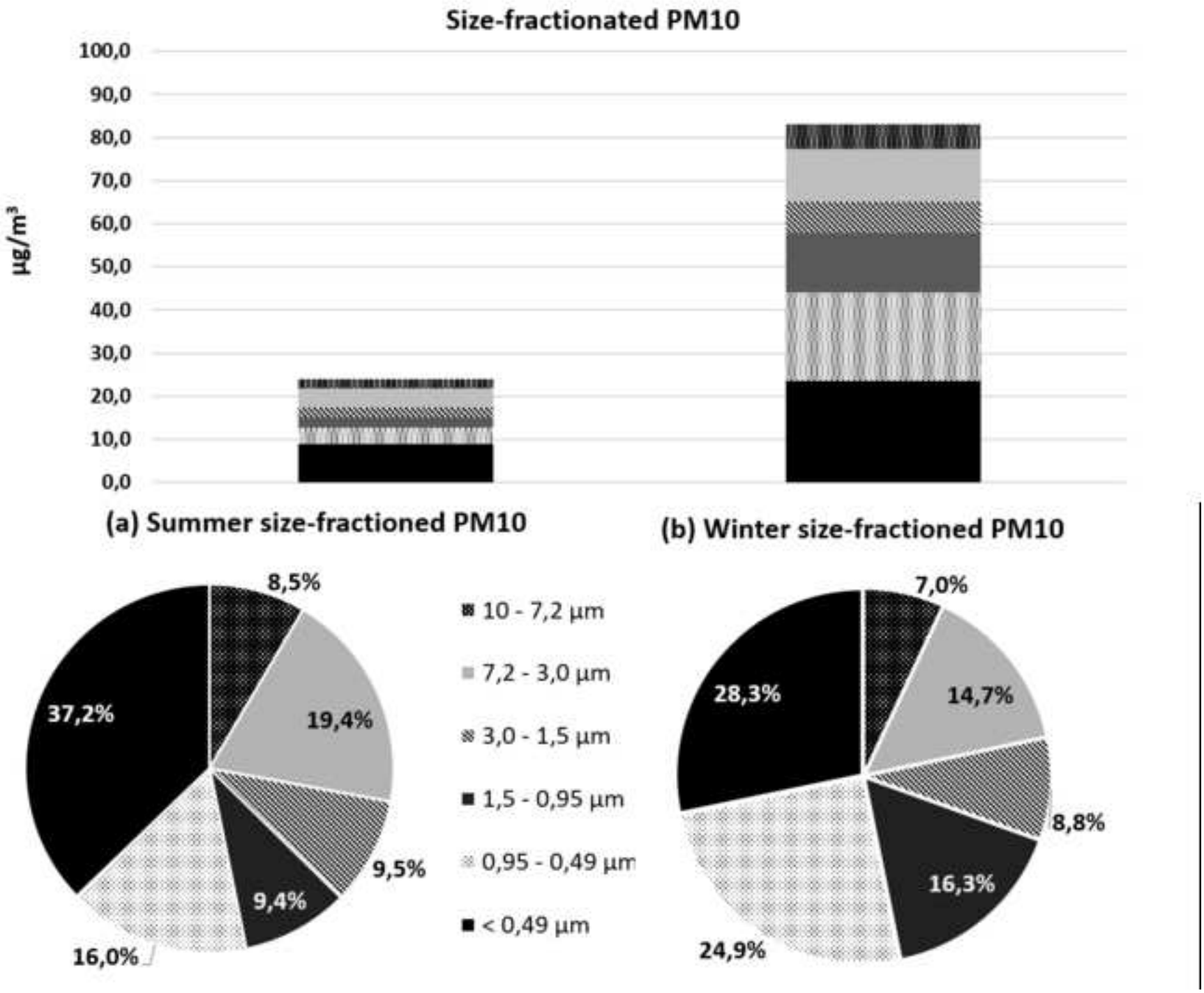

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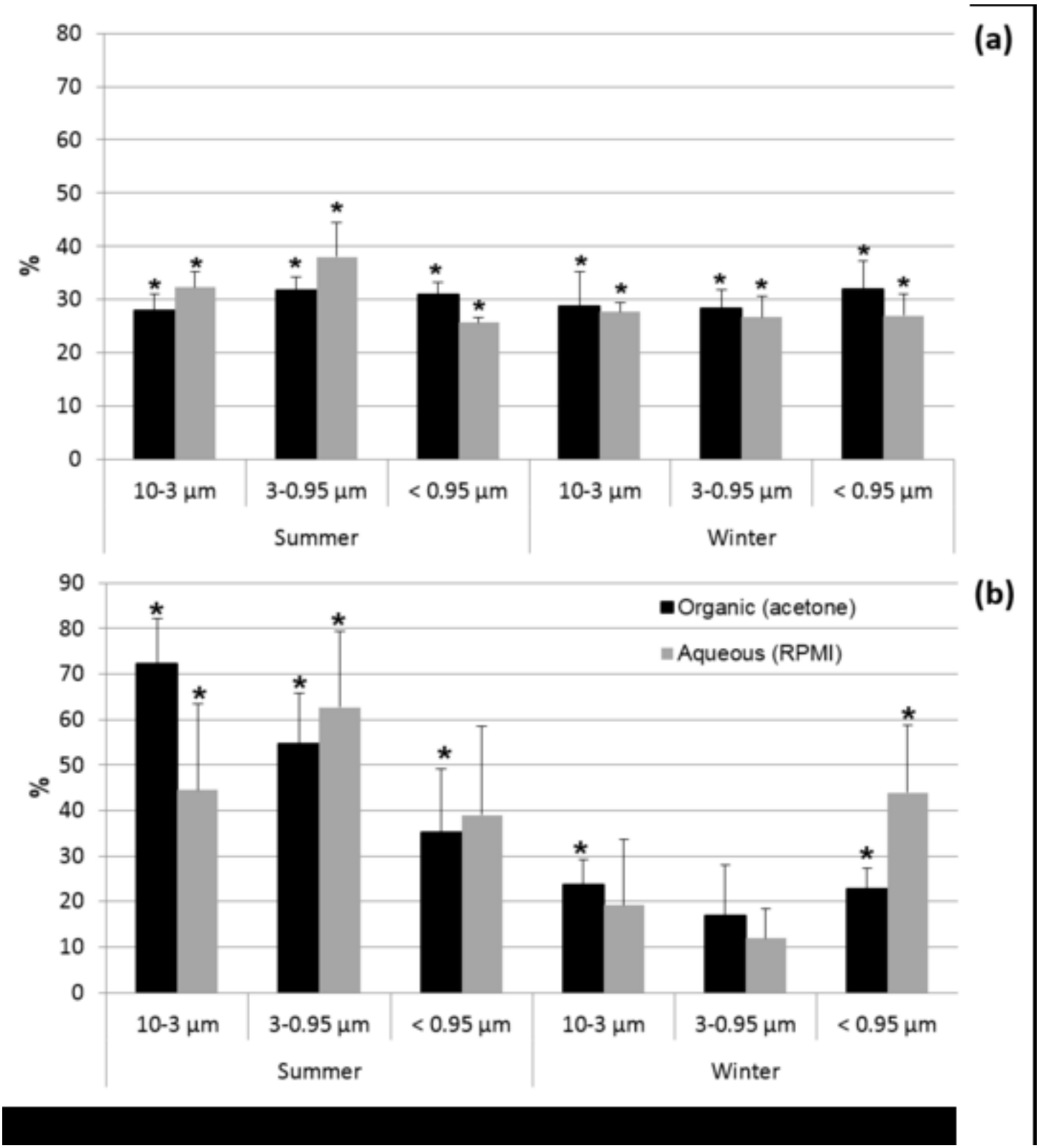

Figure

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