PM10 in a background urban site: Chemicalcharacteristics and biological effects.

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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, 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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***Cover Letter**

Dear Editor,

we are sending the manuscript "*PM_{10} in a background urban site: biological effects and chemical characteristics"* that we submit for possible publication on *Environmental Toxicology and Pharmacology.*

PM_{10} 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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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 de 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 citotoxicity 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 $6x10^5$ 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 TNFα and IL8 among other cytokines in THP-1 cell? Also, why they choose measurement of expression of CYP1A1? This should be explained in discussion.

TNFalpha 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 consideration 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 TNFalpha 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**

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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.

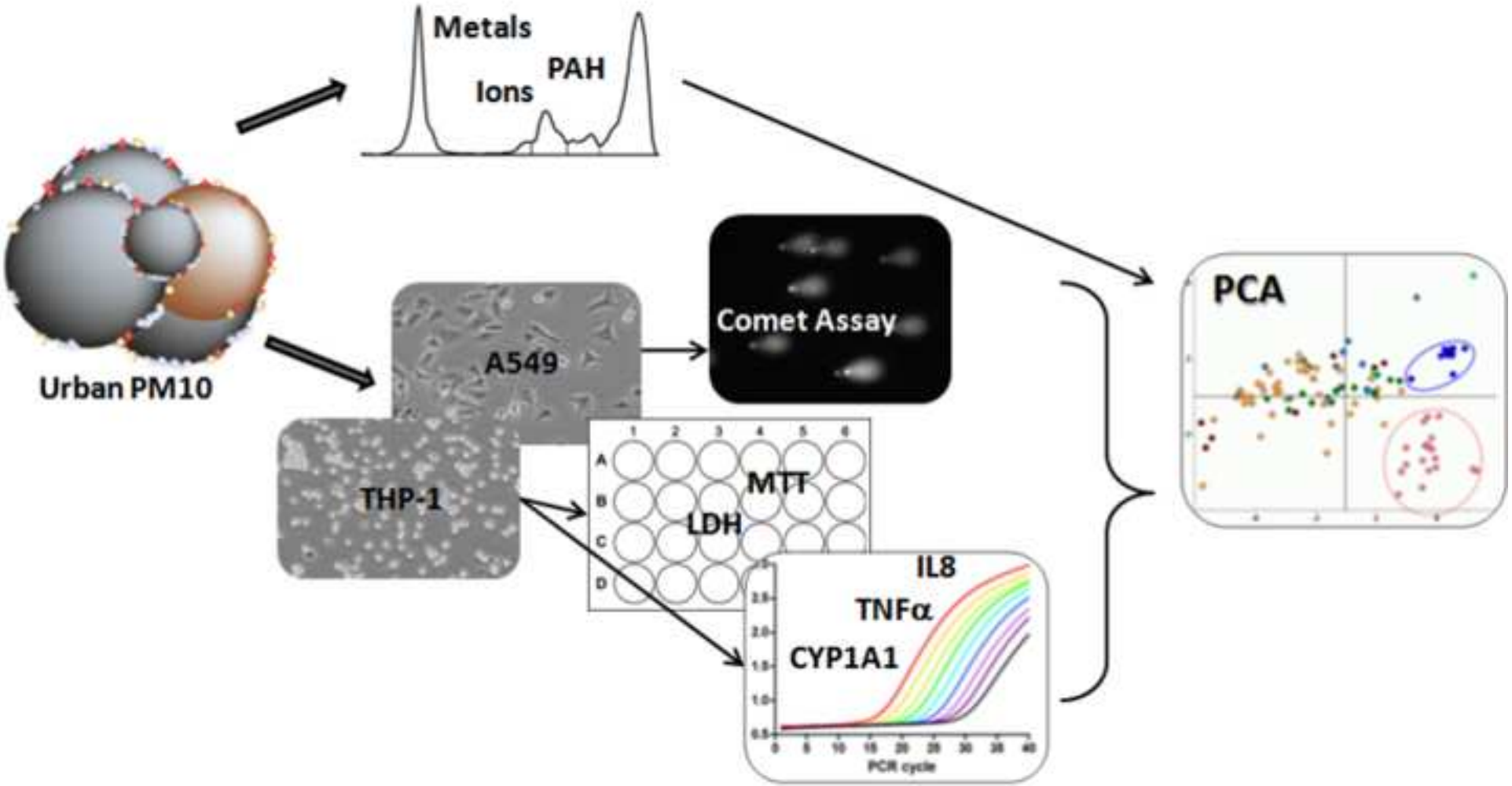
The number of references is too large, and can easily be reduced by 25%.

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).

Graphical Abstract (for review)



PM_{10} in a background urban site: biological effects and chemical characteristics.

HIGHLIGHTS

1. PM_{10}-induced biological effects are present in a background urban site
2. PM_{10} aqueous extracts show cytotoxicity and high ions content in warm season
3. PM_{10} organic extracts prove genotoxic effects and high PAHs content in cold season
4. PM_{10} composition and seasonality play important roles in particle induced toxicity

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

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Abstract

PM_{10} was sampled in a background urban site in Torino, a northern Italian city. PM_{10} extracts were tested with THP-1 and A-549 cells to evaluate their effects on cell proliferation, LDH activity, TNFα, 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 PM_{10}-induced biological effects at a low polluted site suggested that a reduction of PM_{10} mass did not seem to be sufficient to reduce its toxicity.

Keywords: PM_{10}; air quality; in vitro toxicology; genotoxicity; PCA.

1. Introduction

There is a large body of evidence suggesting that exposure to air pollution, even at the levels commonly achieved currently in European countries, leads to adverse health effects (Pope and Dockery, 2006). Epidemiologists have documented statistically significant associations between particulate matter (PM) mass concentrations and increased human mortality and morbidity (Brook et al., 2010). Long-term exposure to airborne PM increases the risk of lung cancer, respiratory diseases and arteriosclerosis, while short-term exposure exacerbates several forms of respiratory diseases, including bronchitis and asthma, as well as changes in heart rate variability (Schwarze et al., 2006).

PM is a highly complex and heterogeneous mixture of particles with natural and/or anthropogenic origins and with different dimensions, with a chemical composition that changes with time and space (Pope and Dockery, 2006). Major sources of PM include vehicular and industrial emissions, power plants, crustal release, and refuse incineration. Particle composition varies between urban and rural sites. Approximately 40 % of the particle mass in an urban setting can be attributed to fossil fuel use (Ghio et al., 2012).

It was shown that PM exposure and toxicity may be related to geographic locations and sampling seasons and consequently to the chemical components of various PM fractions; PM chemical properties depend on the emission sources present in the examined area, the specific atmospheric chemistry and weather conditions (Perrone et al., 2013).

The respiratory system is the primary target of airborne PM, which are inhaled and tend to accumulate in the airways. The exposure dose of the tissues depends on the PM atmospheric concentration, aerodynamic diameter, deposition rate in the airways, clearance mechanisms and subsequent retention of particles within the respiratory tract. Even though the problem has been intensively studied, scientists are still struggling to understand the physical and chemical properties of PM-responsible toxicity as well as the mechanisms underlying the adverse health effects of PM air pollution. No clear consensus exists among researchers regarding the mechanisms of toxicity following exposure to current levels of air pollution PM (Schwarze et al., 2007); in this context,

oxidative stress and inflammatory reactions seem to be of fundamental importance (Lonkar and Dedon, 2011).

Some studies showed that particulate air pollution increases reactive oxygen species (ROS) levels, which may subsequently lead to cellular oxidative stress (Knaapen et al., 2004) and contribute to the genotoxic and cytotoxic effects of PM (Oh et al., 2011). Oxidative stress induces oxidative DNA lesions, such as increased 8-hydroxy-deoxyguanosine (8-OHdG) levels and DNA strand breaks (Bonetta et al., 2009). Moreover, oxidative stress primes a cascade of events associated with inflammation, which probably plays a key role in the air pollution PM-induced development and/or exacerbation of acute and/or chronic lung diseases (Oh et al., 2011; Schwarze et al., 2007). Transition metals in PM seem to be involved in this process, (Roig et al., 2013); in addition, organic compounds, such as polycyclic aromatic hydrocarbons (PAH) and nitro-PAH, coated onto PM may induce damage (DNA damage, mutations and cytotoxicity) at a cellular level in addition to that caused by generating oxygen radicals though direct interaction with cellular structures. PAH are among the most toxic extracted organic compounds because of their carcinogenic and genotoxicity properties (Teixeira et al., 2012), which are associated with atmospheric particles. In human lungs, PAH, which require metabolic activation to biologically reactive intermediates to elicit their adverse health effects, are principally metabolised by the cytochromes P450 (CYP) superfamily member CYP1A1 (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). Furthermore, epidemiological studies showed significant associations between sulphates and nitrates and various health outcomes. In ambient air, sulphates and nitrates are the major PM chemical components and are associated with secondary sources (Schwarze et al., 2006).

To contribute to a greater understanding of the mechanism involved in PM toxicity, the biological effects of urban air PM_{10} collected during different seasons in Torino, a northern Italian city, were examined. A background station was selected as a model of a low contaminated urban site. In particular, the response and sensitivity of different endpoints in human cells cultured *in vitro* (A549 lung epithelial cells line and THP-1 monocytes) were evaluated. PM_{10} samples were analysed for

the quantity of inorganic ions, metals and PAH. Then, the effects of PM_{10} extracts (organic and aqueous) from cold and warm seasons on the inhibition of cell proliferation, tumour necrosis factor alpha (TNFα), interleukin 8 (IL8) and CYP1A1 expression, lactate dehydrogenase (LDH) activity, and genotoxic/oxidative damage induction (Comet assay) were evaluated. The role played by the season and by chemical fractions in different biological endpoint inductions was investigated using Principal Component Analysis (PCA).

2. Materials and methods

2.1. PM sampling

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

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

2.2. Particles extraction

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

Two different strips were cut from each PM_{10} filter as previously reported (Alessandria et al., 2014). The filter strips from each month (6 strips) were pooled to obtain two monthly samples. Briefly, the

two pools were treated with two different extraction media: acetone or RPMI1640 without foetal calf serum (FCS), chosen for different abilities in the extraction of different compounds. In particular, acetone was selected as the solvent for organic-extractable compounds (e.g., PAHs) (Claxton et al., 2004). RPMI without 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 monthly strips were cut into small pieces and were placed in 50 mL polypropylene sterile tubes with 15 mL of the extraction medium. 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. Each PM extract was separated into two aliquots destined to the chemical analysis and to biological tests. Acetone extract (organic) for biological tests was evaporated with a rotary evaporator and suspended in RPMI1640 without foetal calf serum (FCS) for the THP-1 cells tests and in dimethyl sulfoxide (DMSO) for the comet assay with A549. The other acetone extract for chemical analysis was evaporated with a rotary evaporator until 2 mL. RPMI extracts (aqueous) were directly assayed for both biological and chemical 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. Individual chemical species and the biological evaluation are summarised in Table 1.

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

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

The inorganic ions in aqueous extracts were detected using an ion chromatography (IC) analysis carried out on a Dionex LC20 ion chromatograph (Sunnyvale, CA, USA) equipped with a GP40 gradient pump, an ED40 electrochemical detector and an ASRS-I anion self-regenerating suppressor, operating under chemical suppression mode, with the external chemical regenerant flowing through a Consta metric 3200 (Thermo Separation Products, San Jose, CA, USA). The stationary phase is a Dionex IonPac AS14A (250 mm x 4 mm) (Sunnyvale, CA, USA), equipped with a precolumn IonPac AG14A (50 mm x 4 mm) (Sunnyvale, CA, USA). The mobile phase is a mixture 50/50 v/v of 8 mM Na_2CO_3 and 1 mM $NaHCO_3$, flowing at 1 mL/min. The ionic suppression solution is a 25 mM H_2SO_4 aqueous solution. The limit of quantification was 0.5 µg/mL. Analysed ions are summarised in Table 1.

The PAH concentration in the organic extracts was evaluated using a GC-MS Finnigan Trace GC 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% CO_2. 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

similar to human alveolar type II cells (Shi et al., 2006). A549 were grown as a monolayer, 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. A549 cells were more sensitive than THP-1 cells in genotoxicity tests, while the two cell lines showed comparable results in citotoxicity tests (Corsini et al., 2013; Danielsen et al., 2011).

2.5. Cell viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure cell viability; THP-1 cells were seeded, using RPMI1640 without phenol red, in 24-well plates at a density of 4×10^4 cell/well and exposed to a particle concentration of 200 µg/mL, a concentration that always induced a significant effect with samples from the same sampling site described in a previous work (Alessandria et al., 2014). After treatment with PM_{10} 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.). QC laboratory filter extracts were tested in the same manner of PM_{10} extracts samples. All experiments were performed in triplicate. The % of inhibition of cell proliferation was calculated comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures.

2.6. LDH assay

To evaluate PM_{10} cytotoxicity, LDH activities from damaged cells were measured in cell-free culture supernatants, as previously described (Schilirò et al. 2010), modified for cells in suspension. Briefly, THP-1 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and exposed to PM_{10} extracts containing 200 µg/mL particles, a concentration that always induced a significant effect with samples from the same sampling site described in a previous work (Alessandria et al., 2014). QC laboratory filter extracts were tested in the same manner of PM_{10} extracts samples. 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). All experiments were performed in triplicate. LDH activity of exposed cells is expressed as a percentage of non-exposed cells.

2.7. Comet assay

The A549 cells were cultured for 24 h in 6-well plates before exposure to PM extracts. The proportion of living cells was determined by trypan blue staining (overall > 90%). Cells were exposed (24 h at 37 °C) to serial dilutions of the PM_{10} organic extracts containing a range from 200 to 500 µg/mL of particles and PM_{10} aqueous extracts containing 200 µg/mL of particles. Negative controls were constituted by cells not exposed and cells with a maximum of 5 % DMSO.Cells not exposed were used as negative controls. After exposure, cell viability was checked again. The concentration of PM extract that decreases cell viability by more than 30 % was not used for the Comet assay. The Comet assay was performed under alkaline conditions (pH > 13), according to the method of Tice et al. (2000) after slight modifications (Moretti et al., 2002). A549 cells (approximately 6×10^5 cells) were mixed with 300 µL low melting point agarose (LMA), , placed on the slides coated with 1 % of normal melting agarose (NMA), with LMA added as the top layer. Cells were lysed at 4 °C in the dark overnight (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA disodium salt, 1 % TRITON X-100 and 10% DMSO, pH 10). DNA was allowed to unwind for 20 min in alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH, 10% DMSO, pH >13) and subjected to electrophoresis in the same buffer for 20 min (0.78 V/cm and 300 mA). All steps for slide preparation were performed under yellow light to prevent additional DNA damage.

The slides were stained with ethidium bromide (20 µL/mL) and examined with a fluorescent microscope (Axiovert 100M, Zeiss). One hundred randomly selected cells per sample (2 slides) were analysed using an image analysis system (CometScore). The % tail DNA was selected as the parameter to estimate DNA damage. QC laboratory filter extracts were tested in the same manner of PM_{10} extracts samples. Data from unexposed cell cultures were compared with those from PM_{10}

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

2.8. Fpg-Modified Comet assay

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

2.9 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 medium and PM_{10} extracts were performed at 200 µg/mL for 48 h. Total RNA was isolated from treated and untreated THP-1 cells using GenElute Mammalian Total RNA Kit (Sigma, St. Louis, MO, USA). The RNA concentration was determined spectrometrically (260/280/320 nm); 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).

The quality of PCR product was monitored using post-PCR melt curve analysis at the end of the amplification cycles. 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). 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.

2.10 Bivariate and PCA analysis

The PM_{10} samples were analysed by grouping months as follows: winter (December, January, February), spring (March, April, May), summer (June, July, August), and autumn (September, October, November), and also by grouping season: cold season (winter and autumn) and warm season (spring and summer). Means were compared with the Wilcoxon-Mann-Whitney test, and the Spearman rank correlation coefficient (rS) was used to assess relationships between variables. The mean difference and correlation were considered significant at $p < 0.05$. Statistical analyses were performed using the SPSS Package, version 17.0 for Windows.

PCA analysis was performed on the complete dataset represented by a the X (n,p) matrix. Samples (n) were 12 and variables (p) 18. Variables were 4 chemical components, 1 physical parameter and 13 biological responses determined for PM samples.

Principal Component Analysis, regression models and all graphical representations were performed by Statistica 7.1 (Statsoft Inc., USA) and Excel 2003 (Microsoft Corporation, USA).

The chemical components were total inorganic ions, total PAH, and total and transition metals. The physical parameter was PM mass. The biological responses considered were DNA damage

(organic extracts), DNA damage –Fpg (aqueous extracts), DNA damage +Fpg (aqueous extracts), inhibition of cell proliferation (organic and aqueous extracts), LDH release (organic and aqueous extracts), and TNFα, IL8 and CYP1A1 expression (organic and aqueous extracts).

3. Results

3.1 PM concentration

A total of 72 PM_{10} filters were analysed. PM_{10} was sampled in an urban background site placed in a green area; nevertheless, concentrations were sometimes higher than the daily target of 50 µg/m^3 (Air Quality Directive, 2008/50/CE): 83 % of the samples in January and February, 33 % in December 17 % in March and November, and all of the concentrations in the spring and summer months never exceeded the daily reference limit. The mean PM_{10} air concentrations at the sampling site are reported together with the seasonal averages in Table 2. The highest value was observed in winter (115.3 µg/m^3) while the lowest was measured in summer (11.5 µg/m^3); moreover, significant differences were found between seasonal concentrations (ANOVA: F = 0.731, $p < 0.01$), with higher values in winter.

3.2. PM_{10} chemical characterization

Table 2 shows concentrations of the main compounds analysed in PM_{10} extracts.

3.2.1. Metals

The chemical analysis of the PM_{10} 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/m^3 and 11.35 – 100.53 ng/m^3 and a mean value of 13.6 ± 19.02 ng/m^3 and 36.07 ± 29.23 ng/m^3, 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 PM_{10} concentrations (rS = 0.916 and rS = 0.923, $p < 0.01$, respectively).

3.2.2. Ions

The mean concentration of ions was 16.09 ± 7.37 μg/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 (rS = 0.660 and rS = 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 ± 2.43 μg/m^3. The highest mean concentration both of total and carcinogenic PAH were found in January (8.7 ng/m^3), in the other mounths, 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 μg/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 ± 6.9 % vs. 9.3 ± 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} (rS = 0.685, $p < 0.05$).

3.3.2. LDH release

Figure 1 (b) shows the effects produced by PM_{10} 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 PM_{10} 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

TNFα and IL8 are two cytokines involved in systemic inflammation and are members of a group of cytokines that stimulate the acute phase reaction. PM_{10} 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. TNFα (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 PM_{10} extracts. The QC laboratory filter extracts did not induce any significant TNFα 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 PM_{10} 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 µg/mL of PM_{10}.

For the other samples, the highest genotoxic effect was observed in PM_{10} organic extracts of January and September (dose corresponding to 500 µg/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 PM_{10} 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 PM_{10} samples. Using comparisons with score plots and loading plots, it is possible to derive the chemical

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

PCA was first applied, after an autoscaling procedure, to the overall dataset that comprises the chemical, physical and biological parameters in which the values lower than the LOD were set to 0. The analysis shows that with principal components 1, 2 and 3 (PC1, PC2 and PC3), it is possible to explain approximately 71 % of the total variance.

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

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

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

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

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

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

4. Discussion

The present study focused on the chemical characterisation and biological effects of Torino PM_{10}. The north of Italy, in particular the Padana Plain, is an area of widespread air pollution. The weak dispersion rate observed during winter due to the conformation of the territory represents a relevant factor (Cadum et al., 2009). Various air pollution indicators are above the WHO guidelines, especially for PM_{10}. Moreover, the exposure concentrations of 40 µg/m^3 defined by the European Directive 2008/50/EC (*Ambient air quality and cleaner air for Europe*), which is to be met by 2015, was often exceeded. The comparison of the biological effects with the chemical properties of particles allowed for the determination of some of the features that are central in eliciting the toxicity of PM_{10}. The PM source of emissions (both natural and anthropic) is the main parameter that establishes the characteristics of particles of various size, area and season (Schwarze et al., 2006). Season-related transformations of chemicals in the atmosphere are another important parameter to be considered: during summer, the photochemical reactions, associated with elevated solar radiation, modify the PM_{10} chemical constituents, while in winter, the low temperatures facilitate the condensation and absorption of volatile compounds on particle surfaces (Perrone et al., 2010). All of these parameters have to be taken into account in analysing the biological effects of PM_{10}, and this complexity is the reason for difficulties in solving the question of PM toxicity. In particular, the aim was to define whether the cytotoxic and genotoxic effects produced on the THP1 and A549 cells by PM_{10} of a low contaminated urban site may be related with the chemical compounds analysed in the particle samples of different seasons.

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

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

The secondary PM_{10} components represented approximately 50% of total PM_{10} mass, and other studies reported similar percentages in urban sites (Lee and Hieu, 2013) and in particular in the Italian Po valley (Gilli et al., 2007). A higher content of PM_{10} sulphates in warm seasons is normally due to the photochemical reactions that occur more frequently.

A higher PAH 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). The low PAH concentrations may be linked to background sampling sites monitored in this study, similar to metals. Moreover, the PAH concentration was relatively low compared to other studies conducted at sites characterised by similar sources of particulates emission (Gutierrez-Castillo et al., 2006).

Many studies investigated the toxicity and mechanism of PM_{10} on airway epithelial cells (Alessandria et al., 2014; Hetland et al., 2004); in the present study, both PM_{10} extracts (organic and aqueous extracts) exerted a significant impact on cells.

Exposure to ambient air PM induced an oxidative stress that leads to a series of reactions that inhibit cell proliferation and damage the cell membrane. As reported in another study (Shang et al., 2013), MTT (Figure 1) and LDH data (Figure 2) show that PM_{10} exposure did not produce cell death by damaging the cell membrane of THP1 cells. In some cases, the discrepancy between LDH release data and reductions in viability detected by MTT (i.e., aqueous extracts in summer and autumn or organic extracts in spring, Figure 1 and 2) suggest that impairment of viability may be due to the induction of an apoptotic cell death pathway in which the integrity of the plasma membrane is maintained. In addition, it is noteworthy that MTT assay measures cellular metabolic activity; thus, low MTT values (inhibition induced by organic extracts in spring, summer and

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

From the PCA analysis, the different seasonal chemical composition of PM_{10} seems to influence some biological properties. In particular, a seasonal trend was observed for aqueous extracts-induced inhibition of cell proliferation, with a greater effect in the warm season. A higher cell viability reduction for warm season samples in comparison to cold samples was also observed in other studies conducted in the Padana Plain (Alessandria et al., 2014; Perrone et al., 2010). Moreover, this biological effect clustered total ions together, and this state may be associated with the toxicity of secondary PM. Indeed, the warm season in Northern Italy is characterised by high photochemical production of secondary aerosol (Perrone et al., 2013). Moreover, PCA analysis showed a higher winter cell viability reduction associated with higher PAH concentrations, suggesting the potential role of these organic compounds in cytotoxicity induction.

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

In this study, the pro-inflammatory potential of PM_{10} was assessed through the quantification of the relative gene expression of IL8 and TNFα. Both PM_{10} extracts increased gene expressions, with a low increase in summer and autumn and a high increase in spring (especially with the organic extract) and winter. Therefore, both cytokines showed similar higher effects induced by spring PM_{10} compared with our previous study (Schilirò et al., 2010).

PCA analysis shows no seasonal differences in the expression of TNFα (organic extracts) and IL 8 (organic and aqueous extracts). This trend might be associated with values observed for these parameters in the spring. Moreover, the PCA analysis indicates no relation between the gene expression of IL8 or TNFα and chemical parameters, except for TNFα expression (aqueous extract) and PM_{10} metal concentrations. The association between these two parameters confirms the role of metals and the resulting oxidative stress in the induction of inflammatory responses.

Several studies confirmed the critical role of organic compounds adsorbed on PM (Oh et al., 2011), and other investigators directly related PM biological effects to PAH and nitro- and oxygenated-PAH (Binkova et al., 2003).

In this study, the involvement of PAH and nitro-PAH in inducing a PM biological effect was investigated by CYP1A1 expression and genotoxic damage induction (Comet assay). From the PCA analysis, seasonality for different biological effects was observed, with a greater genotoxic effect of organic extracts for cold season compared to hot season samples. Moreover, in the organic extracts of the cold season samples, a lower CYP1A1 expression was detected. The lack of correlation between CYP1A1 expression and PAH, indicated by the opposite position in the loading plot (Figure 4a), might be due to low PAH levels observed at this site that were unable to induce a change of CYP1A1 expression. Indeed, the induction of CYP1A1 expression was relatively low compared to that recorded at other urban sites (Gualtieri et al., 2012). The similarity between gene expression induced by the two extracts may be related to the low concentration of contaminants that activated this enzyme.

The genotoxic effect of organic extracts and some chemical components of PM samples (e.g. PAH and metals) clustered all together in the upper part of the loading plot. The seasonal trend observed for PAH and genotoxic effects and significant associations ($p < 0.05$) between these parameters suggested that PAH might contribute to explaining the genotoxic effect of these extracts. Several studies reported a positive correlation between the PAH content of PM and the particle ability to induce a significant increase in genotoxic damage (Bonetta et al., 2009; Teixeira et al., 2012). The biological genotoxic effect observed might also be influenced by the presence of other airborne organic pollutants (Claxton et al., 2004) that were not monitored in this study because a greater genotoxic effect was observed in some months (in particular October and November) when low PAH concentrations were present. Moreover, the different genotoxic effects noted in the different months might be ascribed to the low levels of total and carcinogenic PAHs, which are likely related to the characteristics of the sampling site (background station). The low contamination at the site might also explain the opposite position in the plot of metals and genotoxic effects of aqueous extracts. Although transition metals may contribute to oxidative stress

induced by PM (Bonetta et al., 2009), in this study the presence of the oxidative damage in PM water extracts seems not to be related to metals present in the sample.

5. Conclusions

The results obtained showed that PCA may be considered a useful tool to analyse the association between the chemical composition and the biological effects of PM. The data confirmed the hypothesis that PM composition and seasonality play an important role in particle-induced toxicity. This finding, recently reported by numerous researchers on this topic in different urban environments, was also confirmed in this low PM_{10} contaminated urban site.

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

Finally, the PM-induced biological effects at a site with low pollution levels showed that reductions in PM_{10} mass did not seem to be sufficient to decrease its toxicity, and this is a matter of concern for all policies aimed at the protection of human health.

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FIGURE CAPTIONS

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

Figure 2. Seasonal differences in (a) TNFa expression (fold increase), (b) IL8 expression (fold increase) and (c) CYP1A1 expression (fold increase) of THP1 cells after exposure (48 h, 200 µg/mL) to PM_{10} organic and aqueous extracts (black and grey bars, respectively). Control level is at 1-fold increase. * indicates statistically significant differences from the control, $p < 0.05$ (ANOVA).

Figure 3. Seasonal differences in the mean % DNA tail value evaluated by the alkaline version of the Comet assay after exposure to PM_{10} organic and aqueous extracts (24 h, 500 µg/mL or 200 µg/mL, respectively). Control levels are at 0.6, 0.3 and 0.5 mean % DNA tail, respectively, for the Comet assay (organic and aqueous extracts) and Fpg modified Comet assay (aqueous extracts). * indicates 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).

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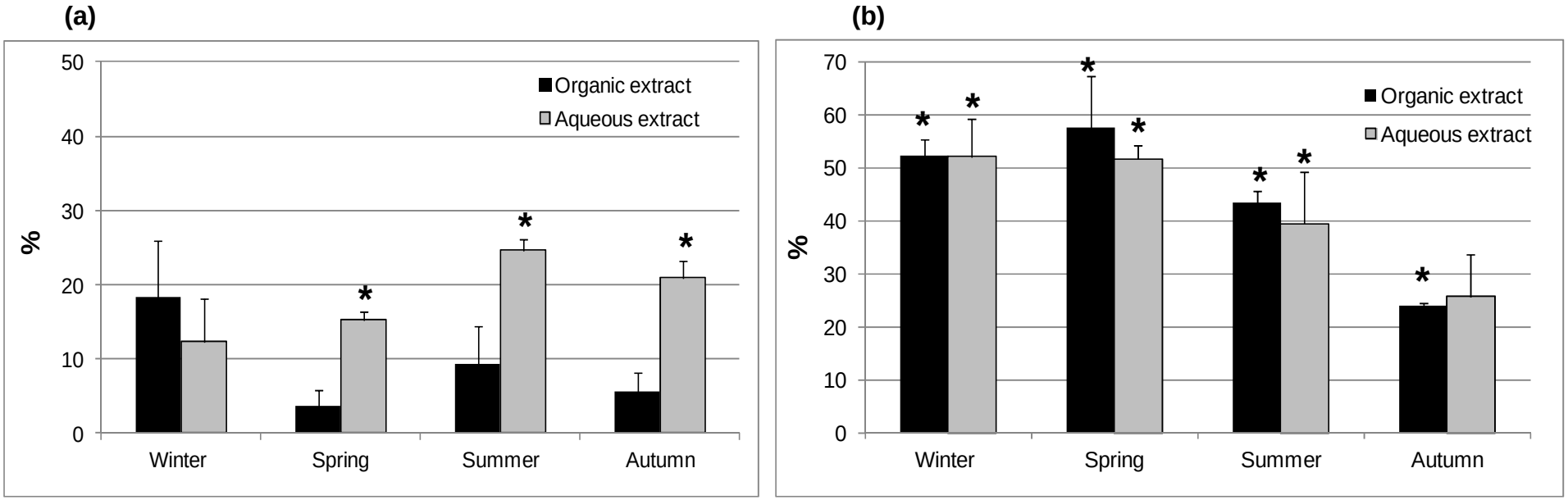
Figure

Figure 1. Seasonal differences in **(a)** the inhibition (%) of THP1 cells proliferation and **(b)** the LDH release (%) of THP1 cells after exposure (48h, 200 µg/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).



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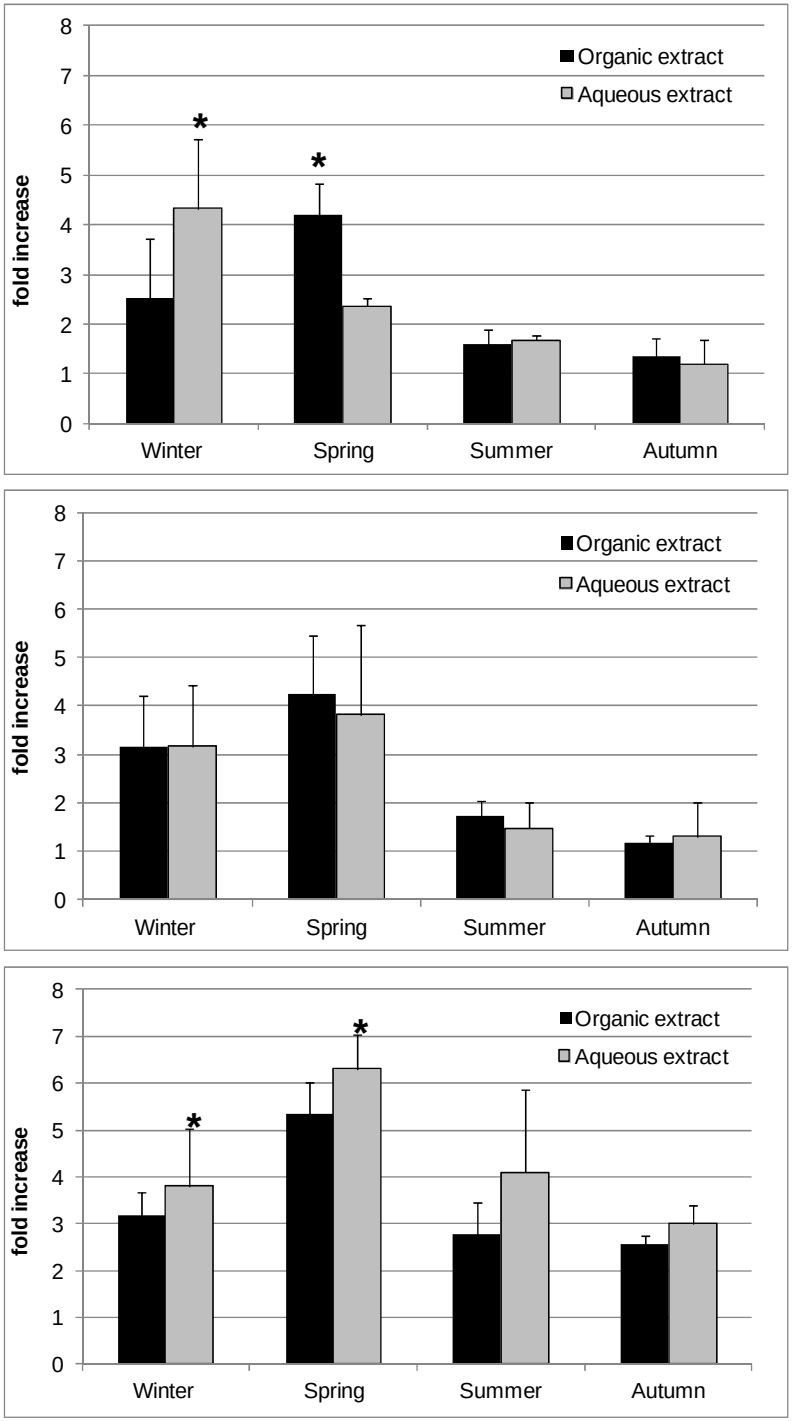

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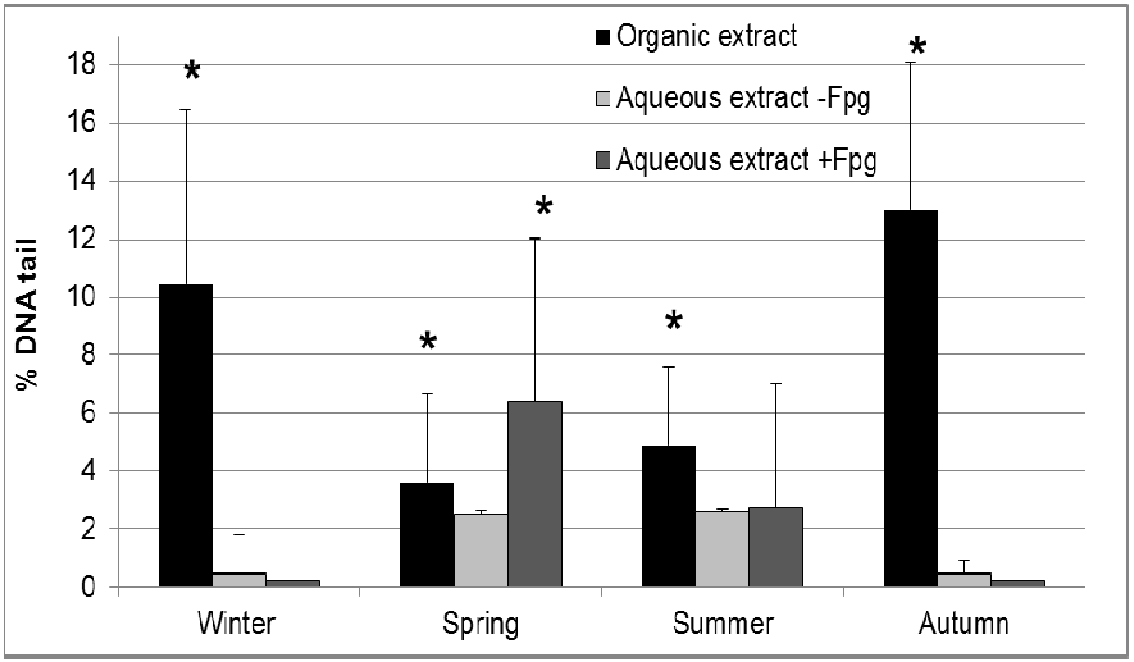

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a) **b)**

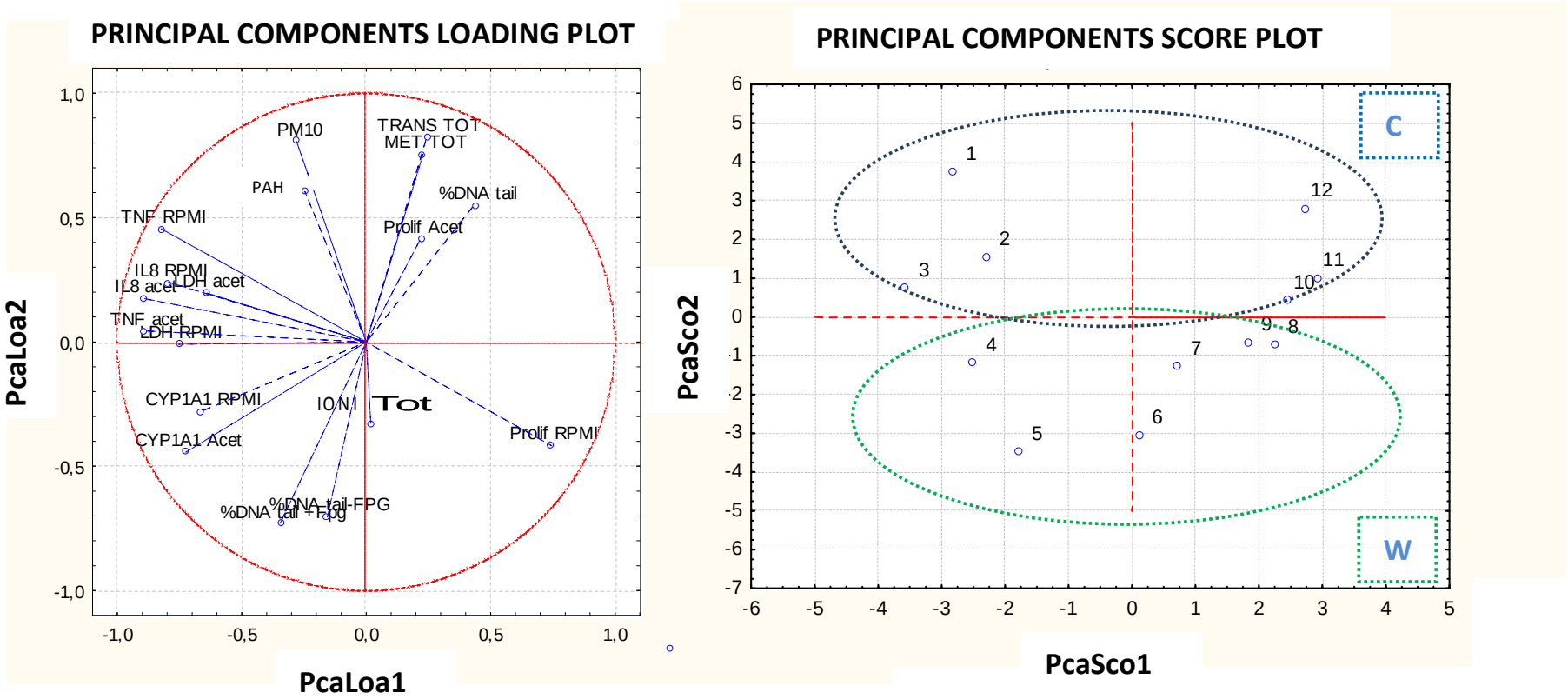

Table 1. Summary description of PM_{10} samples, chemical measurements and biological responses.

PM samples	**Chemical measurements**
PM_{10}	*Polyciclic aromatic hydrocarbons PAH_s*
Urban background station	Naphthalene
12 month (January-December 2009)	Acenaphthylene
Chemical measurements	Acenaphthene
Metals	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
Platimun Pt	***Biological responses***
Scandium Sc	*Cytotoxicity*
Selenium Se	Cell proliferation - MTT (THP-1)
Silver Ag	Lactate dehydrogenase - LDH (THP-1)
Strontium Sr	
Vanadium V	*Inflammatory response*
Zinc Zn	TNF expression (THP-1)
	IL8 expression (THP-1)
Ions	
Fluoride F^-	*Metabolic activation*
Chloride Cl^-	Cytochrome CYP1A1 expression (THP-1)
Bromide Br^-	
Nitrate NO_3^{--}	*DNA damage*
Phosphate PO_4^{--}	Genotoxicity - Comet assay (A549)
Sulphate SO_4^{--}	Oxidative stress - Comet assay w/o Fpg (A549)

[a] Carcinogenic PAH_s (US-EPA)

Table 2 Means (± standard deviations) of PM_{10}, metals, total ions, sulphates, nitrates and PAH concentrations of the whole sampling period and divided by seasons expressed as ng/m^3 and ng/100 µg of particles.

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

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