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Cytotoxic response in human lung epithelial cells and ion characteristics of urban-air particles from Torino, a northern Italian city

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Abstract

Recently, much attention has been devoted to urban air pollution because epidemiological studies have reported health impacts related to particulate matter (PM). PM10 and PM2.5 were collected during different seasons in Torino, a northern Italian city, and were characterised by inorganic chemical species (secondary particulates and bio-available iron). The biological effects of aqueous and organic solvent PM extracts on human epithelial lung A549 were evaluated, and the effects on cell proliferation and lactate dehydrogenase (LDH) release were assayed. The average PM10 concentration during the sampling period was 47.9 ± 18.0 μg/m^3; the secondary particles accounted for 49 % ± 9 % of the PM10 total mass, and the bio-available iron concentration was 0.067 ± 0.045 μg/m^3. The PM2.5/PM10 ratio in Torino ranged from 0.47 to 0.90 and was higher in cold months than in warm months. The PM10 and PM2.5 extracts inhibited cell proliferation and induced LDH release in a dose-dependent manner with a seasonal trend. The PM10 extract had a stronger effect on LDH release, whereas the PM2.5 extract more strongly inhibited cell proliferation. No significant differences were observed in the effects induced by the two extracts, and no significant correlations were found between the biological effects and the PM components evaluated in this study, thus emphasising the importance of the entire mixture in inducing a cytotoxic response.

Keywords

PM
A549
LDH
Metals
Secondary particulate
Cytotoxicity

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Introduction

Particulate matter (PM) is an air-suspended mixture of solid and liquid particles that vary in number, size, shape, surface area, chemical composition, solubility and origin (Pope and Dockery **2006**); PM can be classified as PM10 (particles with an aerodynamic diameter Ø less than 10 μm), PM2.5 (Ø < 2.5 μm), and PM1 (Ø < 1 μm). Currently, major sources of ambient PM include vehicular and industrial emissions, power plants, refuse incineration and geological material. The fine fractions (PM2.5 and PM1) are dominated by combustion-derived particles, whereas the coarse fraction (PM10) contains a major component of mineral compounds and certain adsorbed endotoxins. Anthropogenic contributions, i.e. organic carbon, are greater in urban environments, and specific metals may also be more prevalent in these areas. In an urban setting, approximately 40 % of the particle mass can be attributed to fossil fuel use (Ghio et al. 2012).

The respiratory system is the primary target of airborne particles, which are inhaled and tend to accumulate in the airways. The exposure dose in the tissues depends on the PM atmospheric concentration, the deposition rate in the airways, the clearance mechanisms and the subsequent retention of particles within the respiratory tract. The epidemiological associations are well established between PM mass concentrations and increased human mortality and morbidity (Brook et al. **2010**, Pope and Dockery **2006**). Currently, the hypothesis that long-term exposure to air pollution can produce human morbidity and mortality is unanimously accepted, and epidemiological evidence suggests an increased risk of lung cancer for people living in urban areas (Nerriere et al. **2005**, Sun et al. **2008**). Long-term exposure to air pollution PM increases the risk of lung cancer, respiratory diseases and arteriosclerosis, whereas short-term exposure can exacerbate several forms of respiratory diseases, i.e. bronchitis and asthma, and changes in heart rate variability (Dominici et al. **2007**; Peacock et al. **2011**; Pope et al. **2009**; Raaschou-Nielsen et al. **2011**; Rusconi et al. **2011**). However, fundamental uncertainties still exist with respect to the underlying mechanisms of toxicity that are responsible for the mortality or morbidity increases following exposure to current levels of air pollution PM (Schwarze et al. **2007**).

The PM-induced human health effects are still not completely understood, but oxidative stress and inflammatory reaction seem to be of fundamental importance (Baulig et al. **2009**. Garcon et al. **2006**. Lodovici and Bigagli **2011**. Lonkar and Dedon **2011**). Experimental and epidemiological data indicate that lung disorders are often associated with a pro-oxidant/antioxidant imbalance and inflammatory reaction, and there is increasing evidence that air pollution via PM induces acute responses and exacerbates existing

inflammatory diseases in the lungs (Maestrelli et al. **2011**. Peacock et al. **2011**. Raaschou-Nielsen et al. **2011**. Rusconi et al. **2011**. Terzano et al. **2010**). Under physiological conditions, the normal production of reactive oxygen species (ROS) is counteracted by antioxidant scavengers and enzymes, which include both enzymes and non-enzymatic scavengers (de Kok et al. **2006**; Garcon et al. **2006**; Sun et al. **2008**). However, under abnormal conditions, excessive levels of ROS exceed the detoxification capacity of the antioxidant defences, thereby causing a change in the redox status of the cell (Jomova and Valko **2011**; Lonkar and Dedon **2011**; Ziech et al. **2011**). Oxidative stress subsequently triggers a cascade of events closely associated with inflammation, which is believed to play a key role in the air pollution/PM-induced development and/or exacerbation of acute and/or chronic lung diseases (Dagher et al. **2005**, **2006**, **2007**‘ Garcon et al. **2006**‘ Lodovici and Bigagli **2011**; Oh et al. **2011**; Schwarze et al. **2007**). Transition metals (i.e. Fe) (Carter et al. **1997**; Hutchison et al. **2005**; Roig et al. **2013**'; Schins et al. **2004**), organics (i.e. polycyclic aromatic hydrocarbons or PAHs) (Billet et al. **2008**) and endotoxins (Becker et al. **2003**; Longhin et al. **2013**; Oberdorster et al. **2000**; Traversi et al. **2010**) in the PM seem to be involved in this process (Donaldson et al. **2003**; Schwarze et al. **2006**; Sorensen et al. **2003**). Moreover, although secondary inorganic aerosols show less toxic activity when tested under controlled laboratory conditions, epidemiological studies demonstrate significant associations between sulphates and nitrates and various health outcomes. In ambient air, this fraction may act as a carrier for other components or as a surrogate for PM emitted from the combustion of sulphur-containing fuels (Schwarze et al. **2006**).

The PM size is another important parameter that influences biological effects. The PM10 fraction has been associated with pro-inflammatory and cytotoxic effects (Gualtieri et al. **2010**; Hetland et al. **2005**), and the PM2.5 fraction has been associated primarily with a higher genotoxic potential (Billet et al **2008**) also related to higher PAH and metal contents. Moreover, positive relationships were found between the formation of ROS and the induction of DNA damage. Depending on the amount formed, ROS may result in necrosis as well as apoptosis (de Kok et al. **2006**; Gualtieri et al. **2011**).

The aim of the present study was to contribute to a better knowledge of the cytotoxic effects induced by PM extracts. The PM10 and PM2.5 materials were sampled in traffic conditions and at an urban background station. The inhalable fraction of ambient particulate matter (PM10) is widely used as an air quality indicator (European Commission (EC) Directive **2008**; WHO 2006a); moreover, PM10 and PM2.5 pollution is specifically related to urban environments (Pelucchi et al. **2009**). The PM samples were analysed in terms of the amounts of inorganic species (bio-available iron and secondary particulates of sulphates and nitrates). Additionally, A549 cells were exposed to increasing concentrations of PM10 and PM2.5 (aqueous and organic solvent) extracts, and the experiments were performed at concentrations according to previous data (Schilir~~o~~ò et al. **2010**). Next, the effects on cell proliferation and LDH release induced by extracts from cold and warm season samples were evaluated. Another aim of this work was to define whether the cytotoxic effects produced in the A549 cells by extracts of PM10 and PM2.5 might be related to the chemical compounds evaluated on the particles.

The chemical and in vitro toxicological characterisations of urban-air PM could provide a basis for environmental and sanitary actions, particularly in one of the most industrialised areas of Italy (Hazenkamp-von Arx et al. **2003**; Marcazzan et al. **2003**), with high levels of particulate matter among European nations (WHO **2006b**).

Material and methods

PM sampling

Sampling was performed from January 2007 to December 2008 at two meteorological–chemical stations of the Environmental Protection Regional Agency (Piedmont A.R.P.A.) located in Torino, a city in the northwest of the Padana Plain (Italy). The first sampling site (station 1) was located outdoors in a small green area within an enclosure zone classified as urban background. The second sampling site was located in the centre of the city (station 2) in a traffic-regulated street and was classified as a traffic station (Putaud et al. **2010**). The PM10 (PM passing through a size-selective inlet with a 50 % efficiency cut-off at 10 μm aerodynamic diameter) material was sampled at station 2 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) at a flow rate of 1.16 L/min. The sampling duration was controlled using a timer accurate to ±15 min over a 24-h sampling period. The PM2.5 was sampled at station 1 with glass micro-fibre filters (Type A/E 47 mm, Gelman Sciences, MI, USA) using a PM2.5 MicroVol 1100 Low-Volume Air Sampler with a flow rate of approximately 32 L/min. This sampler is certified in compliance with norm EN-14907. The exact flow rate was calculated daily and corrected for variations in atmospheric pressure and the actual differential pressure across the filter. The filters were conditioned for 48 h and were 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. **2007c**).

Particles extractions

The PM10 filter strips were pooled to obtain one weekly sample, and particles were extracted as previously described (Schilirò et al. **2010** In brief, each pool was treated with two different extraction media, acetone or RPMI1640 without foetal calf serum (FCS), which were chosen for different capabilities for the extraction of different compounds. Acetone was chosen as the solvent for organic-extractable compounds, and RPMI1640 without FCS was chosen to extract a water-soluble component theoretically comparable to the extraction in the lung cells (Schilirò et al. **2010**).

Each portion of the filter was cut into thin strips and placed in a 50-mL polypropylene sterile tube 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 three times. The samples were centrifuged at 5,000×*g* for 10 min to remove the filter material, and the supernatant was collected. Acetone extracts were evaporated with a rotary evaporator and re-suspended in culture medium. Aqueous extracts were directly assayed.

The PM2.5 filters were pooled to obtain one monthly sample, and the same extraction procedure was followed. Unless otherwise specified, all chemicals were purchased from Sigma, St. Louis, MO, USA.

Iron and bio-available iron

Iron determination was performed according to the procedure of Gilli et al. (**2007b**). In brief, the metals were extracted from the filter strips (3.18 cm by 20.32 cm) using a nitric acid solution. After cooling, the sample was mixed and centrifuged, and the trace element concentrations in each sample were determined by atomic absorption spectrometry (Varian GTA-96).

The bio-available iron from urban particulates was determined as previously described (Lund and Aust **1990**) with certain modifications (Gilli et al. **2007b**; Smith and Aust **1997**). In brief, filter strips (3.18 cm by 20.32 cm) were suspended in 50 nM NaCl, mixed, and adjusted to pH 7.5. Citrate was added to the samples to a final concentration of 1 mM, and all samples were placed on a wrist-action shaker in the dark for 24 h. Samples of 1 mL were withdrawn and centrifuged to remove the particulates. The amount of iron mobilised as the citrate/Fe complex in the supernatant was determined using a spectrophotometric total non-heme iron assay (Brumby and Massey **1967**).

Sulphates and nitrates

Sulphates and nitrates were measured according to the procedure of Gilli et al. (**2007a**), **2007b**). In brief, each filter strip (3.18 cm by 20.32 cm) was extracted in 15 mL of distilled and deionised water via 30-min sonication, 30-min agitation, and overnight refrigeration at 4 °C. Prior to the analysis, the samples were centrifuged to remove particles. Ion chromatography was used to determine the soluble ion content of sulphate (SO_4^{2-}) and nitrate (NO_3^-) using a Dionex DX-100 ion chromatograph with 0.3 mM NaHCO3 and 2.7 mM Na2CO3 2.7 for the eluent and IonPac analytical column S12A for the anions. The applied standards, i.e. sodium sulphate and sodium nitrate (71959 and 71759 FLUKA, respectively), ranged from 0.1 to 100 g/ml. The ions were identified by their elution/retention times (~8.75 min and 12.5 min for nitrates and sulphates, respectively) and were quantified by the conductivity peak area or peak height (300A method, US EPA 1996).

Cell culture

The human lung epithelial cell line A549 (non-small cell lung cancer) from the Interlab Cell Line Collection (Genova, IT) was used as a model for human epithelial lung cells. Cells were grown as a monolayer and were 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 a humidified atmosphere containing 5 % CO_2.

Cell viability

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to

measure cell viability (Gualtieri et al. **2010**). Cells were seeded in 24-well plates at a density of 4×10^4 cells/well and exposed to PM extracts equivalent to particle concentrations of 50, 100 and 200 µg/ml (Schilirοò et al. **2010**). After treatment with PM10 and PM2.5, the medium was discharged; cells were rinsed with PBS, and MTT (final concentration of 0.5 mg/ml) reagent was added for 4 h. The medium was removed, and the MTT reduction product (formazan crystals) was dissolved in 300 µl of DMSO. Cell proliferation was determined after 72 h of exposure by measuring the absorbance at 570 nm using a micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc.). Blank filters were used as negative controls, and all experiments were performed in triplicate. The percentage of cell proliferation was calculated by comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures.

LDH assay

To evaluate PM cytotoxicity, the released LDH activities from damaged cells were measured in cell-free culture supernatants as previously described (Schilirοò et al. **2010**). In brief, A549 cells were seeded in six-well plates at a density of 1×10^6 cells/well and exposed to PM10 and PM2.5 extracts containing an equivalent of 50, 100 and 200 µg/ml of particles. Blank filters were treated in the same manner. At 72 h, LDH activity was measured in the supernatant and cell lysate. The LDH activity, the ratio of extracellular LDH (measured in the supernatant) and total LDH (expressed as the sum of the LDH measured in the supernatant and cell lysate) were measured as consumption of NADH in terms of the absorbance at 340 nm, read using a micro-plate reader (Benchmark Plus Microplate Reader, Biorad). All experiments were performed in triplicate. The LDH activity of the exposed cells is expressed as a percentage of that of non-exposed cells.

Statistical analysis

Statistical analyses were performed using the SPSS Package (version 17.0) for Windows. The mean values were compared with the Wilcoxon–Mann–Whitney *U* test, and the Spearman rank correlation coefficient (rS) was used to assess the relationships between variables. The mean difference and correlation were considered significant at $p < 0.05$.

Results

PM concentration

A total of 117 PM10 filters and 362 PM2.5 filters were analysed during 2007 and 2008. The annual means for PM10 and PM2.5 were 47.9 ± 18.0 and 35.2 ± 22.7 µg/m^3, respectively.

The PM2.5/PM10 ratio ranged from 0.47 to 0.90 and was higher in winter/autumn than in spring/summer. This ratio is affected by the different sampling sites in terms of traffic pressure but is comparable to those values reported in the literature (Querol et al. **2004**).

The particulate matters often showed concentrations higher than the daily and annual targets proposed in the Air Quality Directive 2008/50/CE (EUEC, 2008). The highest values both for PM10 and PM2.5 were observed in winter, whereas the lowest were

measured in summer(Figure 1). Moreover, significant differences ($p < 0.05$) were observed between the seasonal (autumn/winter vs. spring/summer) PM10 and PM2.5 concentrations (Table 1).

Table 1

PM10, PM2.5, sulphates, nitrates and Fe concentrations (micrograms per cibuc metre), means ± standar

	PM10	PM2.5	$SO_4^=$	NO_3^-	$SO_4^=$	NO_3^-	Fe total	
	($\mu g/m^3$)	($\mu g/m^3$)	($\mu g/m^3$)	($\mu g/m^3$)	(% PM10)	(% PM10)	($\mu g/m^3$)	(
Whole period	47.9 ± 18.0	35.2 ± 22.7	12.5 ± 3.5	9.5 ± 5.5	29.7 ± 10.4	19.3 ± 5.4	1.953 ± 0.680	(
Winter/autumn	58.4 ± 18.6*	52.7 ± 19.8*	11.5 ± 3.3	12.1 ± 5.9	22.7 ± 9.5*	20.5 ± 5.9	2.085 ± 0.954	(
Summer/spring	37.3 ± 10.5*	17.7 ± 2.8*	13.5 ± 3.8	6.7 ± 3.8	36.7 ± 5.7*	18.2 ± 5.1	1.821 ± 0.254	(

*$p < 0.05$ level of statistically significant differences (autumn/winter vs. spring/summer)(Mann–Wh

Iron and bio-available iron

The total iron mean concentration was 1.953 ± 0.680 $\mu g/m^3$. The highest value was observed in winter (4.263 $\mu g/m^3$), whereas the lowest was observed in summer (0.600 $\mu g/m^3$). Table 1 also shows the mean concentrations of bio-available iron, and 3.9 ± 3.2 % of the total iron was bio-available. Statistically significant differences were observed for the mean seasonal concentrations ($p < 0.01$). The bio-available iron showed significant positive correlations with the PM10 and PM2.5 concentrations (respectively, rS = 0.711, $p < 0.05$ and rS = 0.846, $p < 0.01$).

Sulphates and nitrates

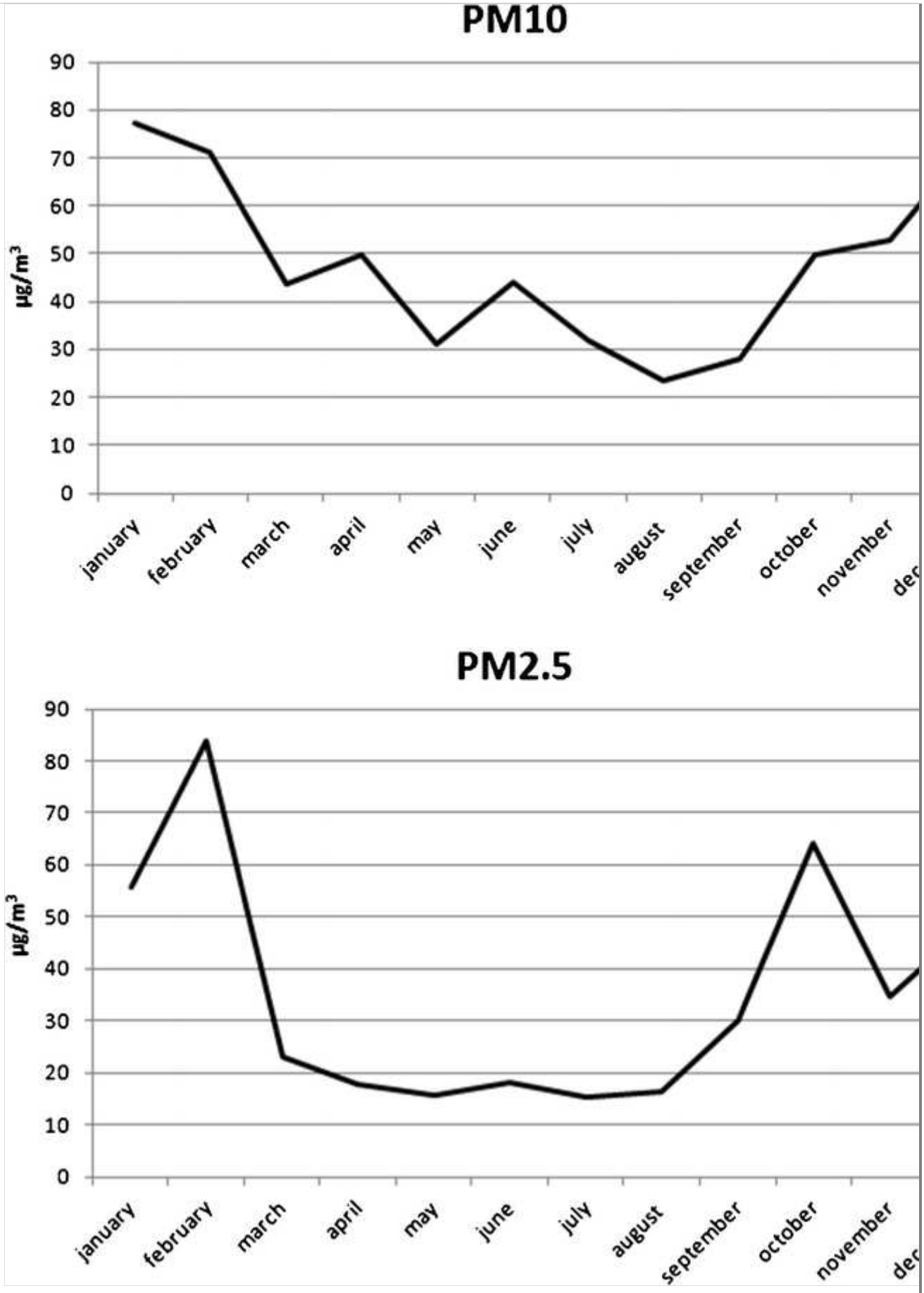
The mean concentration of sulphates was 12.5 ± 3.5 $\mu g/m^3$ relative to PM10, such that sulphates represented 29.7 ± 10.4 % of the total PM10 mass. The mean concentration of nitrates was 9.5 ± 5.5 $\mu g/m^3$, such that nitrates represented 19.3 ± 5.4 % of the total PM10 mass. Considering the sum of sulphates and nitrates as the principal components of secondary particulate matter, these materials accounted for 49 ± 9 % of the PM10 total mass (Table 1). No statistically significant differences were found for the seasonal concentrations for both sulphates and nitrates expressed as micrograms per cubic metre. Considering the concentrations of the two species relative to PM10 (percentage), it was observed that the sulphate content (36.7 ± 5.7 %) in the hot season was significantly elevated compared with that in the cold season (22.7 ± 9.5 %, $p < 0.05$) whereas, for the

nitrates, the amounts were similar in the two seasons.

As expected, the sulphates and nitrates showed significant correlations with the particulate matter concentrations. More specifically, the sulphates displayed a negative correlation with both PM10 and PM2.5, whereas the nitrates had a positive correlation ~~(Fig. 1)~~.

Fig. 1

Monthly trends of the PM10 (*top*) and PM2.5 (*bottom*) concentrations



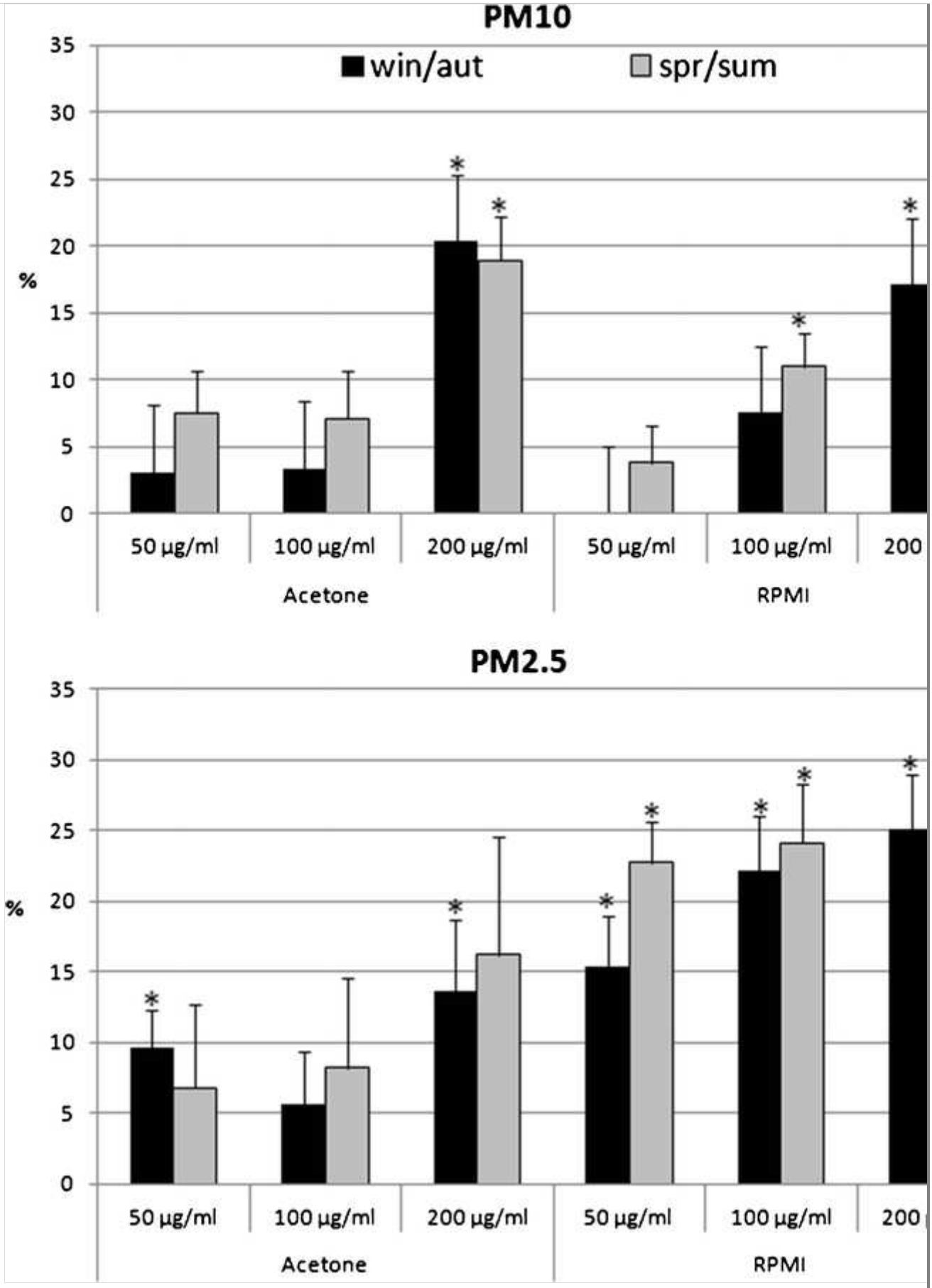
Cell proliferation

Extracts from blank filters had no significant effects on cell proliferation. Figure 2 shows

the effects on cell proliferation of organic and aqueous extracts. The PM10 and PM2.5 extracts inhibited cell proliferation in a dose-dependent manner. The PM10 extracts induced a significant inhibition of cell proliferation only at the highest exposure dose (200 μg/ml), both for the organic and aqueous extracts. However, the PM2.5 extracts also had strong effects on cell proliferation (especially for the aqueous samples) at doses of 50 and 100 μg/ml. No seasonal (autumn/winter vs. spring/summer) differences were observed in the inhibition of proliferation of A549. Moreover, the chemical parameters were not correlated with the inhibition of cell proliferation.

Fig. 2

Inhibition of proliferation of A549 cells after incubation with PM10 (*top*) and PM2.5 (*bottom*) organic solvent extracts (acetone) and aqueous extracts (RPMI) (50, 100 and 200 μg/ml). Inhibition of cell proliferation was calculated by comparing the absorbance of the exposed cultures with the absorbance of non-exposed cultures. *Significant differences from the control, $p < 0.05$ (Mann–Whitney *U* test)



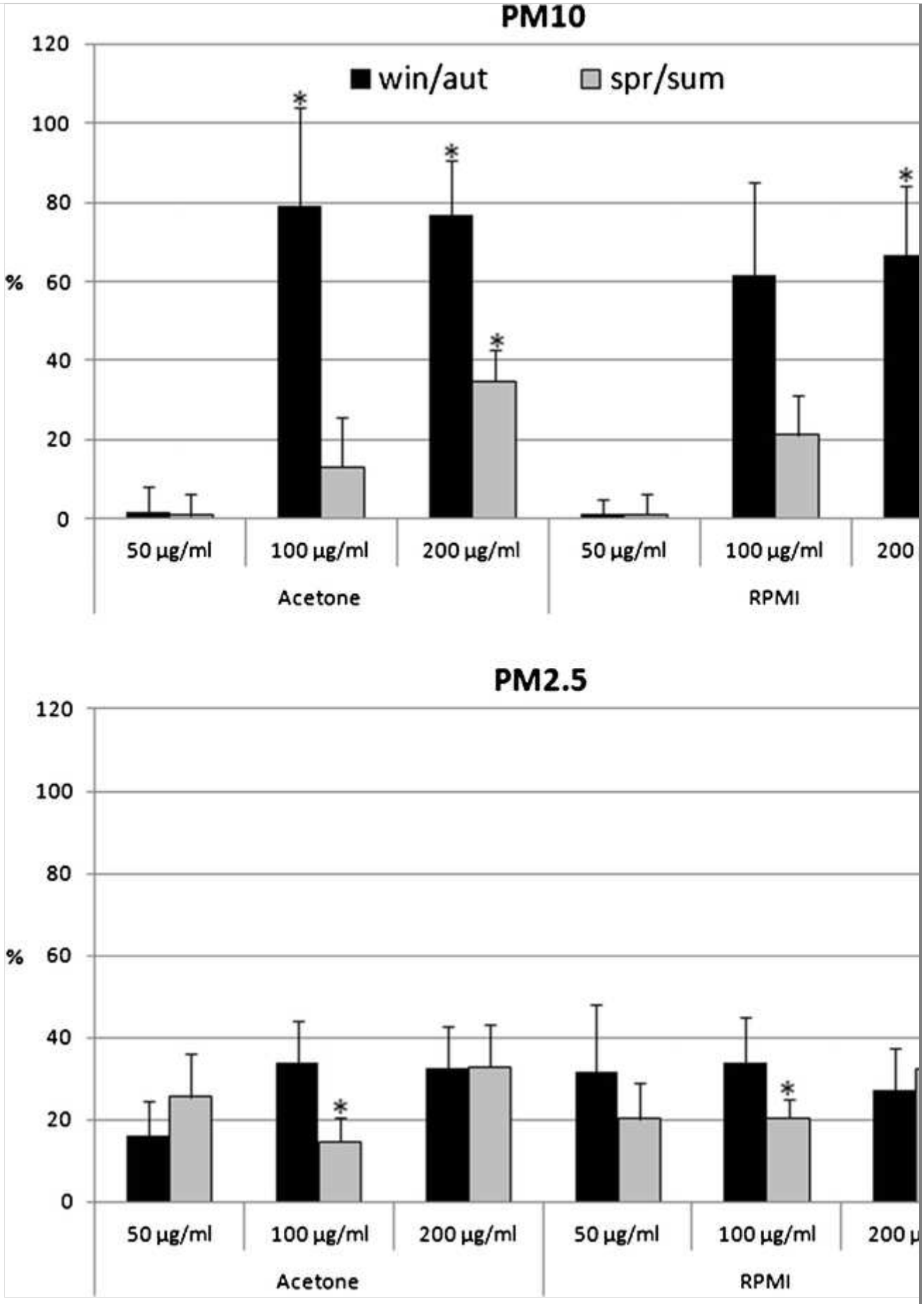
LDH release

An increase in extracellular LDH enzyme activity reflects an increase in the amount of membrane-damaged cells (Lobner **2000**). The release of the cytoplasmic enzyme LDH into

the culture supernatant was used to measure the cytotoxicity of the PM10 extracts. Extracts from blank filters had no significant effect on LDH release. As shown in ~~Fig.~~Figure 3, incubation of A549 cells with PM10 extracts at a concentration of 50 μg/ml had no effect on LDH release compared with control cells.

Fig. 3

LDH release from A549 cells after their incubation with PM10 (*top*) and PM2.5 (*bottom*) organic solvent extracts (acetone) and aqueous extracts (RPMI) (50, 100 and 200 μg/ml). LDH release was calculated by comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures. *Statistically significant differences from the control, $p < 0.05$ (Mann–Whitney *U* test)



Statistically significant increases in extracellular LDH activities were observed after cell exposure at 100 and 200 µg/mL of particle concentration for both the organic and aqueous

extracts. Furthermore, the organic extracts showed seasonal differences (autumn/winter vs. spring/summer) in the induction of LDH release for the two highest concentrations of exposure.

The PM2.5 extracts induced a percentage release of LDH in exposed cells compared with control cells that always ranged between 15 % and 35 %, and these differences were significant only for three cases. No seasonal (autumn/winter vs. spring/summer) differences were found in the LDH release induced by the PM2.5 extracts.

The LDH increase in A549 due to either the organic or aqueous extract was not correlated with the PM10 and PM2.5 concentrations or with any other parameters.

Discussion and conclusions

The current study focused on the chemical characterisation and the cytotoxic effects of particulate matter in the city of Torino. Moreover, in this work, we intended to determine whether the cytotoxic effects produced in A549 cells from the extracts of PM10 and PM2.5 might be related to specific compounds evaluated on the particles.

The north of Italy, and in particular the Padana Plain where Torino is located, is an area of widespread air pollution (Cadum et al. **2009**). The weak dispersion rate observed during winter due to the conformation of the territory represents a relevant factor (Poncino et al. **2009**), and different air pollution indicators exceed the WHO guidelines, especially for particulate matter, i.e. PM10 and PM2.5. In addition, the exposure concentrations of 40 and 25 $\mu g/m^3$ (for PM10 and PM2.5, respectively) defined by the European Directive 2008/50/EC (ECDirective **2008**), which must be met by 2015, were clearly exceeded, and it is difficult to imagine better results for the upcoming years.

In contrast with our previous finding (Gilli et al. **2007a**, **b**) but in agreement with the latest data from another city in the Padana Plain (Camatini et al. **2012**), no significant seasonal differences were observed in total Fe concentrations. A small amount of iron is bio-available (approximately 4 % of total Fe), and this fraction is involved in oxidant generation after PM exposure by directly supporting electron transport, thus producing ROS (Ghio et al. 2012). Bio-available Fe showed a seasonal trend, with higher content of this metal in samples from the cold season compared with those from the warm seasons and was also correlated with the PM10 and PM2.5 concentrations.

The secondary PM components represented approximately 49 % of the total PM10 in term of mass, as confirmed by the findings reported on other urban sites (Lee and Hieu **2013**) in the Italian Po valley, in particular in Gilli et al. (**2007b**) and Marcazzan et al. **2003**). The seasonal trend of both sulphates and nitrates is normally due to the photochemical reactions that occur more frequently in the warm season.

Many other studies have investigated the cytotoxicity of PM in airway epithelial cells (Scapellato and Lotti **2007**), and PM induces different biologic effects depending on the sampling site (Rosas Perez et al. **2007**), size fraction (Alfaro-Moreno et al. **2002**; Hetland

et al. 2004; Osornio-Vargas et al. 2003) sampling time (Frampton et al. 1999) and contaminants adsorbed on the particles (Baulig et al. 2003; Billet et al. 2007; Calcabrini et al. 2004; Frampton et al. 1999; Muller et al. 2006). In this study, PM10 and PM2.5 extracts inhibited cell proliferation and induced LDH release in a dose-dependent manner, similar to the results found in other studies on epithelial cells (Alfaro-Moreno et al. 2002; Osornio-Vargas et al. 2003; Schilirøò et al. 2010). Significant seasonal trends were observed only with the organic PM10 extracts; otherwise, the effects produced by the samples from cold seasons were similar to those induced by the other samples. As previously observed (Schilirøò et al. 2010), aqueous PM extracts seemed to have a greater effect on cell proliferation than organic-solvent (acetone) extracts, whereas organic extracts induced a greater release of LDH. Both types of PM10 extracts have a significant impact on cells, and it is likely that water-soluble components could cross the membranes and induce rapid inflammatory responses; organic-phase compounds still cross the membranes but have a more deleterious effect on the cells (e.g. LDH release). It is important to emphasise that PM10 extracts had a stronger effect on LDH release than PM2.5 extracts (47.38 % vs. 26.77 %, respectively), according to a similar result on the pro-inflammatory potential of PM10 compared with PM2.5 presented by numerous authors (Duvall et al. 2008; Gualtieri et al. 2008; Hetland et al. 2005). As reported in another study (Shang et al. 2013), combined MTT data and LDH data show that PM exposure does not produce cell death by damaging the cell membrane of A549 cells. For the aqueous PM2.5 extract, for example, the discrepancy between LDH release data and the reduction in viability detected by MTT may suggest that the impairment of viability (approximately 25-30 % cell death was observed at concentrations of 200 μg/mL; Fig. 2) may be due to the induction of the apoptotic cell death pathway in which the integrity of the plasma membrane is maintained. In addition, it should be noted that the MTT assay measures cellular metabolic activity, and thus, low MTT values might indicate reduced metabolic activity/mitochondrial damage or reduced cell proliferation.

Finally, no significant correlations were found between the biological effects and PM components (iron, bio-available iron and secondary particulates) evaluated in this study. These results confirm the results of other recent studies in which inconsistent associations were reported between both cell proliferation and LDH release and secondary particulates and metals for in vitro lung cell exposure (Happo et al. 2008, Roig et al. 2013). One possible explanation for the paucity of correlations could be that the biological end-points and the PM components were quantified in chemically separate fractions. Furthermore, the aqueous extracts did not reflect the activity of any particular PM10 chemical component but rather only highlighted the activity of physiologically soluble components. Moreover, the lack of correlations indicates that certain other chemical parameters should be assessed.

The results of the current study supported the assertion that short-term bioassays usinghuman lung cells might be adequate for completing routine chemical analysis and for carrying out preliminary screening of the potential effects of PM-associated airborne pollutants. Our results must be further explored to identify the most dangerous PM fractions, even though the precise components responsible for cytotoxicity following exposure of cells to PM remain to be defined.

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