TITLE PAGE

Size-fractionated PM10 monitoring in relation to the contribution of endotoxins in different polluted areas.

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ABSTRACT:

Particulate pollution is an environmental concern that is widespread and difficult to resolve. Recently various regulatory improvements around the world have been agreed upon to tackle this problem, especially as related to the fine fraction of particulates, which more closely correlates to human health effects than other fractions. The size-fractionation of inhalable particles and their organic composition represent a new area of research that has been poorly explored thus far. Endotoxins are a type of natural organic compound that can be found in particulate matter. They are correlated with Gram-negative bacterial contamination. Health outcomes associated with exposure to these toxins are not specific and often overlap with the health effects of PM (Particulate Matter) exposure, including asthma, bronchitis, acute respiratory distress syndrome and organic dust toxic syndrome. Very little information is available on the endotoxin distribution in different PM10 size fractions. This study examined PM10 size fractions and their endotoxin content. Sampling was conducted at five different locations: one urban, two rural and two rural sites that were highly influenced by large-scale farm animal production facilities. For each location, six different PM10 fractions were evaluated. PM10 sub-fractions were categorised as follows: $PM_{10-7.2}$ (1.15-31.30 µg/m^3); $PM_{7.2-3.0}$ (1.86-30.73 µg/m^3); $PM_{3.0-1.5}$ (1.74-13.90 µg/m^3); $PM_{1.5-0.95}$ (0.24-10.57 µg/m^3); $PM_{0.95-0.49}$ (1.22-14.33 µg/m^3) and $PM_{<0.49}$ (13.15-85.49 µg/m^3). The ranges of endotoxin levels determined were: $PM_{10-7.2}$ (0.051-5.401 endotoxin units (EU)/m^3); $PM_{7.2-3.0}$ (0.123-7.801 EU/m^3); $PM_{3.0-1.5}$ (0.057-1.635 EU/m^3); $PM_{1.5-0.95}$ (0.040-2.477 EU/m^3); $PM_{0.95-0.49}$ (0.007-3.159 EU/m^3) and $PM_{<0.49}$ (0.039-3.975 EU/m^3). Our results indicated consistency of the PM1 fraction at all of the sites and the predominant presence of endotoxins in the coarse fraction. The observed abatement of the PM10 and endotoxin levels was very high (above 1:10) as little as 50 meters from the pollution source. This kind of model is useful to both improve our knowledge about PM10 endotoxin distribution and to evaluate the potential risks for the health of neighbouring populations.

KEY WORDS: endotoxin, lipopolysaccharide, particulate matter, PM10, air pollution

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MAIN TEXT

INTRODUCTION

The health impact of PM10 inhalation is a major concern in the field of air pollution research (Schwarze et al. 2006). In the last twenty years, the main findings of wide-ranging research activities on this topic have demonstrated that PM10 pollution is particularly related to urban environments (Pelucchi et al. 2009); is correlated with primary emissions (such as traffic pollution) (Querol et al. 2008) but is also the result of nucleation and condensation processes in the atmosphere (Perez et al. 2010); is more dangerous when the particles are of the finest aerodynamic diameters (Polichetti et al. 2009; Schmidt 2009); and its quality, in addition to the aerodispersed quantity, could be a fundamental factor in the assessment of its harmful effects on human health (Valavanidis et al. 2006). Epidemiological studies have repeatedly suggested that enhanced ambient PM levels result in increased morbidity and mortality (Brook et al. 2010; Pelucchi et al. 2009). Morbidity related to respiratory diseases, chronic obstructive pulmonary diseases (COPDs) and cardiovascular diseases increases, especially for respiratory diseases and COPDs, in the range of 1-20% for *coarse* particles and 9-1% for *fine* particles (Brunekreef and Forsberg 2005).

The chemical composition of these particles is variable depending on sampling area characteristics (kerbside, urban, industrial or rural) and meteorological and orographic conditions (Perez et al. 2008). The organic fraction percentage in the total mass is about 20% in the coarse fraction, but not in the fine or ultrafine particulate fractions (Cho et al. 2009). Endotoxins may be among the substances in the organic fraction (Ramgolam et al. 2009). This type of pollutant, having a biological origin, is more abundant in rural rather than urban environments (Cho et al. 2009), especially when there is a specific source of biomass, such as composting plants, a waste water treatment plant or animal farming (Liebers et al.

2007). However, a contribution of endotoxins to particulates has also been observed at urban sites (Traversi et al. 2010). Aerodispersed endotoxins are associated with various health effects, both negative and supposedly positive, depending on the degree of exposure dose and time. Negative effects have been observed in a number of studies, especially in work environments characterised by high exposure doses (>10 ng/m^3). These work environments include a spectrum of activities ranging from animal farming to composting plants, all of which present a high level of bacterial contamination and an abundance of organic substrates (Deacon et al. 2009; Liebers et al. 2007). Positive health effects have been suggested with low levels of exposure in children living in rural, as opposed to urban, environments in the form of a low incidence of allergic cases (Lundin and Checkoway 2009; Zhu et al. 2010). The endotoxin component is more abundant in the coarse than in the fine particulate fraction (Morgenstern et al. 2005). Concentrated animal-feeding operations represent a major source of airborne endotoxins (Ko et al. 2010). Globally, this large-scale activity is confined to small areas, where in areas with a high demographic rate especially (Greger 2010), the numbers of animals reach levels of 40 U.B.A./km^2 nationally, and can be higher in certain regional district (ISTAT 2010). Swine and poultry farming activities represent two environments in which organic particles exhibit the most critical degree of contamination (Charavaryamath and Singh 2006), and this kind of pollution can generate adverse health effects for both on-site farmers and their neighbours (Ko et al. 2010; Omland 2002; Rimac et al. 2009). In working environments, the "*threshold limit value time-weighted average*" (TLV-TWA) proposed by the ACGIH is 10 ng/m^3 (Liebers et al. 2006). Adverse effects in resident populations near farming activities are also been recorded (Liao et al. 2010; Smit et al. 2008). The "no observable adverse effect levels" (NOAEL) are considered to be those <50 EU/m^3 (Delery and Cicolella 2009). Additionally, recent studies have discussed the protective effects of endotoxin exposure with respect to asthma symptoms and allergic sensitisation (Pakarinen et al. 2008; Priftis et al. 2007) and lung cancer (Lenters et al. 2010). This kind of positive effect has been recorded for endotoxin levels above 38.6 EU/mg in dust in the home (Sordillo et al. 2010).

Few studies have described the distribution of the inhalable fraction of particulates (Chen and Hildemann 2009). The aim of this study was to determine the endotoxin contribution in six PM10 sub-fractions from various polluted areas (animal farms and background rural and urban environments) and to observe the air dispersion of this particulate component. Additionally, it may be possible to estimate the persistence of this type of biological pollutant in the air within a distance of 50 m from the source in order to supply useful data in the evaluation process of the associated risk for resident human health.

MATERIALS AND METHODS:

Sampling sites and collection of PM10 sub-fractions

Size-fractionated samples of ambient PM were collected in the summer of 2009. Five sampling locations were chosen to represent considerably different situations of endotoxin exposure. The sites, chosen based on the prevailing wind direction, included an urban background site and two rural sites two different distances from a significant endotoxin source (at 50 meters distance and near the fan shutters of a barn of an intensive farming operation).

Table 1 summarises the main features of the sampling sites. Two rural background sites were included, at which a sampler was placed 50 m from shed particle sources. Riva is a small town (~3.600 inhabitants) near Turin within a plain, while Fiano (~2.800 inhabitants) is located near a hilly region northwest of Turin. Both towns are located approximately 30 km from Turin (~910.000 inhabitants). PM samples were collected using a Sierra-Andersen high volume cascade impactor (Air Flow PM10-HVS sampler with a multi-stage cascade impactor with preselector complies with EN-12341 norm by Analitica Strumenti) at a flow electronically controlled of 1.27 m^3/min. Samplers with the same drawing are recently validated (Furuuchi et al. 2010). We collected 15 consecutive half-daily samples of airborne PM in 6 different size ranges. Another different particle size fractions range (only 5 fractions) has been sampled, with the same equipment, modifying the sampling flow at 0.63 m^3/min, but we set

the above detailed conditions in order to collect a higher quantity of PM and to split better the course fraction.

Firstly the PM10 was selected by preselector, then the multi-stage impactor determined the division of different particle sizes of sampled particles by differentiation of the aerodynamic diameter able to identify the type of trajectory which particles take inside the suction flow related to the three main aerodynamic factors of the particles themselves: dimension, shape and density. Particle size fractions (10.0-7.2, 7.2-3.0, 3.0-1.5, 1.5-0.95, 0.95-0.49, and <0.49 µm) can be sampled with a micron cut-off efficiency at 50% per spherical particles with uniform mass carried out at 25°C and 1013 mBar. Glass microfibre filters with ten splits (Type A/E, 8'' x 10'', Gelman Sciences, Michigan, USA) were used to collect particles on each impactor plates, at the end a glass microfibre filters as back-up filters is present to collect the finest particles (<0.49 µm) didn't stop before during the inlet path. Approximately 100 filters were pre- and post-conditioned by placing them in a dry, dark environment for 48 h, and most were adapted for use in sub-fraction selection equipment. They were then weighed in a room with controlled temperature and humidity. Each sampling session (a day of sampling) was carried out for a total of about 8 hours, every day from 8:00 a.m. to 4:00 p.m. In each session we collected samples at two different sites: for 4 hours locating the sampler at a point 50 meters from the relevant source and for 4 hours locating the sampler near the farm shed. The PM10 concentration in the air volume sampled was calculated as previously described (Traversi et al. 2010).

Particle extraction:

Each filter was treated individually. Different portions of the filters were used for extraction: one-half (51.75 cm^2) of the impactor plate filters and one-sixteenth (51.75 cm^2) of the back-up filters. Each portion was placed in a 50 ml sterile polypropylene pyrogen-free tube with 20 ml of RPMI-1640 medium supplemented with 0.1 mg/ml penicillin-streptomycin, 4 mM L-glutamine and 0.025% Tween-20. The tubes were placed in an ultrasonic water bath for 10 minutes, and then vortexed for one minute. This procedure was repeated three times. The

samples were centrifuged at 5000 rpm for 10 minutes to remove the glass fibre, and the supernatant was collected. The resulting clear supernatant was assayed for endotoxin evaluation. If not otherwise specified, all chemicals were purchased from Sigma, USA.

Endotoxin assay:

Endotoxins were assayed using the endpoint chromogenic *Limulus* amebocyte lysate (LAL) method (QLC-1000 n° 50-648U, Cambrex, Walkersville, MD, USA) at 37°C with an automated microplate reader (ELX 800 UV, Bio-Tek Instruments, Inc.) following the manufacturer's instructions. *Escherichia coli* 0111:B4 endotoxin was used as a standard endotoxin. The reference curve was obtained as specified by the Cambrex kit, using 6 different dilution points and a regression coefficient of at least 0.98. The limit of detection (LOD) was 0.01 EU/ml. Only the quantified values falling between the first and the last point of the curve was considered acceptable. The performance characteristics of the certified method were respected both for linearity and reproducibility. In particular for the precision is required a coefficient variation of the absorbance in the replicate <10%, generally values of 3-4% are obtained in the determination sessions of our data. The degree of conformity of a measured quantity to its true value and the absence of affecting factors was assured by the use of different reference blank and standards and then by including spiked aliquots of the sample to control the non-inhibitory dilutions. All test samples were spiked with a known amount of endotoxin (0.4 EU/ml). The spiked solution was assayed along with the unspiked samples, and their respective endotoxin concentrations were determined. The difference between the two calculated endotoxin values was equal to the known concentration of the spike ± 25%. About the selectivity recently it has been demonstrated that there isn't difference in the use of recombinant factor C (rFC), that theoretically excludes interferences, from the *Limulus* pathway in a fluorometric assay (Thorne et al. 2010). Sample concentrations were reported as endotoxin units (EU) per millilitre of eluant, EU per milligram of PM10, and EU per cubic meter of air collected. A total of 90 filters were analysed, and all samples yielded quantifiable concentrations of endotoxins and particulates.

Statistical analysis:

Statistical analyses were performed using the SPSS Package, version 17.0 for Windows. A T-test for independent variables and a one-way multivariate ANOVA were used (we used the Tukey test for post-hoc multiple comparison) to compare the means. A Spearman correlation coefficient was used to assess the relationships between the variables. The mean differences and correlations were considered significant at $p<0.05$, highly significant at $p<0.01$.

RESULTS AND DISCUSSION

Sampling PM10 for each sites

Table 2 shows the data (mean and standard deviation) obtained for each parameter measured. The concentration of total PM10 (calculated from the quantity and volume data) are presented first, then the endotoxin contribution to the total mass of PM10 and its concentration in the air, which are discussed in the next section and lastly the temperatures recorded during the samplings. The temperatures were fairly constant during the sampling period (standard deviation of the total sampling period below 2.7°C). The ANOVA, which was performed assuming the PM10 concentration as the dependent variable and the different sampling sites as the independent variables, indicated a significant difference ($F= 51.523$; $p<0.01$) between farming sites versus the others (urban and rural). A significant difference was also found between the two total PM10 mean levels recorded by the different farm activities. No significant differences were found among the urban and rural background sites, Riva presented background particle levels between those of Torino and Fiano probably due to the proximity of a busy highway. Fiano shows the lowest levels but with an high variation coefficient among the different sample days (~35%)(table 2). The PM10 levels measured in rural and farming place during this work is comparable to other data in the in literature (table 3).

224 Size-fractionated PM10

225 Figure 1 shows the split size composition of PM10 in each sampling site, urban or rural
226 (figure 1A) and farming (figure 1B). The Anova analysis reported a significant results in
227 which the finest fraction (<0.49 µm) is significantly higher than the others ($F= 6.816$, $p<0.01$)
228 (figure 1C). This is confirmed in various studies in which the finest particulate pollution is
229 homogeneously diffused in the atmosphere (Perez et al., 2010). An aggregated cut-off, such
230 as 3 µm, didn't produce significant difference considering all the data (figure 1C, grey part).
231 Moreover on the figure 1A we can see that PM3 represented over 80% of the total PM10 at
232 the background sites (80% in Torino, 88% in Riva and 89% in Fiano), and interestingly, the
233 amount of PM1 was the same in the three cities (approximately 30 µg/m^3).
234 In Riva and Fiano, PM10 consisted almost exclusively of PM1, while in Turin, a greater
235 amount of the largest fractions was composed of coagulation and condensation nuclei from
236 the smaller fractions. In the urban site, both the major presence of emission sites and the
237 interaction of a more abundant number of particles in a limited air volume, probably explain
238 the PM>1 levels recorded in Turin. This kind of secondary generation in urban sites has
239 been widely observed and discussed (Pey et al. 2009).
240 Generally the finest fraction is more abundant in the urban sites and in another season,
241 when the combustion emissions are wider diffused (Cuccia et al. 2010), we are probably
242 able to observe this trend but it is necessary to consider that the samplings were carried out
243 in summer and many urban fine particulate sources in that period weren't present such as
244 domestic heating then there was a limited traffic and in general less antrophic pressure due
245 to the holiday period (no school, ect.). Moreover in the area, just around Torino, there is a
246 wide and diffused PM2.5 pollution level common to the whole Padana plain (Traversi et al.
247 2009) but, at the moment, there are only few and very recently data published on the finest
248 fractions (Caggiano et al. 2010; Cuccia et al. 2010). Table 3 shows a comparison with other
249 published data even if there are many heterogeneities mainly in the environmental sample
250 site characteristics and PM size cut offs. The high maximum level recorded for the finest

fraction is mainly influenced by the swine farming samples, where was recorded a high PM pollution for all the fractions.

With respect to the farm sampling sites, PM3 represented 72% of the total PM10 for the pig farm, while it constituted only 40% of the PM10 at the poultry farm (figure 1B). However, considering the increase in each fraction observed, a several-fold increase in the amount of all fractions was found between the corresponding background and farm sampling sites, with the greatest increases being recorded for the two coarse fractions (3-7.2 µm and 7.2-10 µm). Moreover the coarse particles generated from farm activities appeared to be equally distributed in the two coarse fractions (3-7.2 µm and 7.2-10 µm). The poultry farm mainly produced PM with an aerodynamic diameter greater than 3 µm. The fine particles sampled 50 m from the shed in Fiano represented almost the same quantity as those sampled near the shutter of the fan system so the particles, generated from the poultry shed, had aerodynamic diameters greater than 3 µm. The hog farm produced also a considerable amount of particles with an aerodynamic diameter <0.49 µm, it could be due to the presence of numerous farm machineries.

Endotoxin determination

Total endotoxin in PM10 for each sites

In Table 2, the endotoxin concentration is expressed as EU (endotoxin units) per one mg of PM or one m^3 of sampled air. The ANOVA performed assuming the endotoxin concentration as a dependent variable and the different sampling sites as the independent variables showed a significant difference ($F=6.721$; $p<0.001$) between endotoxin levels at the background sites and at the farms sites.

Higher endotoxin concentrations were found in Fiano compared to the other background sites, probably due to the proximity of a grove of trees near the sampler (within 10 m). No significant differences were found in endotoxin concentrations between the two animal farms, in spite of a considerable difference in the amount of sampled particles at these sites. Pigs disturb more ground and were found to produce more biologically inactive particles,

279 which may explain the difference observed in the amount of PM10, but not in its biological
280 components at this site. On the table 3 other published data are reported, of course there
281 are many confounding factors in the applied methods making the comparison with our
282 results only indicative.
283
284 Endotoxin content in size-fractionated PM10
285 Figure 2 shows the distribution of endotoxin levels in the PM10 fractions. At the background
286 sampling sites, more than 50% of the endotoxin content was found in the 3-10 µm fraction
287 (66% in Turin, 52% in Riva, 62% in Fiano) (figure 2A). Moreover the endotoxin content of the
288 PM1,5 fraction (background mean 0.133±0.082 EU/m^3) is comparable with other data
289 reported in the literature (den Hartigh et al. 2010).
290 The endotoxin mean difference is significantly higher in the coarse fractions considering all
291 the data (cut-off 3 µm, $F = 8.674$; $p<0.01$) (figure 2C). The endotoxin levels contained in the
292 PM0.49 fractions were quite constant, measuring only approximately 0.1 EU/m^3 (less than
293 20% of the total measured in Turin and Riva and less than 10% of that in Fiano). The
294 endotoxin pollution was relatively constant between the two types of farming activities, and
295 the endotoxin distribution in the PM10 fractions followed the abundance of particle sizes. At
296 the poultry farm, endotoxins were mainly present in the coarse fraction, while at the pig farm,
297 they were distributed more evenly (figure 2B). On the table 3 other literature data are
298 showed, even if the comparison is hard for various heterogeneities among which the applied
299 PM size cut off, the samplings (personal or environmental, indoor or outdoor, high or low
300 flow rate), and in the EU determination methods.
301 As showed on the figure 3 the endotoxins levels increase directly and significantly with the
302 inhalable fractionated particulate pollution. This evidence is true, considering all the collected
303 data, and it is better considering only the data collected near the farming activities. On the
304 other hands an opposite correlation exists between endotoxins levels and particles
305 considering only the urban or rural data. This evidence described the relevance of the farm
306 sources in the endotoxin content modulation. Moreover the reduction of the total endotoxin

levels at 50 m far from the animal sheds is above one fold. This data seems to demonstrate a weak persistence of this kind of pollutant in the atmosphere due to its coarse fractions correlation.

CONCLUSION

An evaluation of size-fractionated PM10 was successfully conducted at five different locations within the Padana plain. Each location represents specific conditions of exposure to both particulate matter and adsorbed endotoxins, and the conclusions reached regarding the conditions observed at the background and farming sites are:

- This sampling method was proven useful in determining the PM10 mass and endotoxin content among the 6 sub fractions of different aerodynamic diameters determined. There were two fractions referred to as *coarse* fractions (10-7.2 and 7.2-3 µm), two *fine* fractions (3-1.5 and 1.5-0.95 µm) and two *ultrafine* fraction (0.95-0.49 and <0.49 µm).
- PM in the background contained a high percentage of fine particles and, particularly, ultrafine ones, while the coarse components were more abundant at the farm sampling sites, with a similar distribution of the two coarse fractions, moreover the size distribution of the PM10 generated near the animals shed is influenced by the kind of farming animals.
- The endotoxin distribution in the PM10 samples showed that there were no significant differences in the amount of endotoxins at the two farm shed sampling sites. In fact, it seemed to suggest that the difference of approximately 70 µg/m^3 in particles found between the two farms was not associated with biological activity or at least did not have a Gram-negative origin.
- At the background sites, the endotoxins were also present mostly in the coarse fraction, and this was in agreement with results presented in the literature (Morgenstern et al. 2005; Wang et al. 2007).
- There was almost an order of magnitude difference between the endotoxin levels measured at the background sites (just above 100 EU/mg PM10) and the farm sites

(just above 1000 EU/mg PM10), which was in accord with data presented in the literature (Liebers et al. 2006). Considering the PM10 aerodispersed concentration, these levels are considerably below toxicologically relevant levels, both in the air of the background sites and sampled near the farm shed.

- Nevertheless, this evaluation could be far from indicative of the shed's indoor particulate and endotoxin pollution at these sites, as typically, indoor levels of PTS, PM10 and endotoxins at farms are greater than 25 mg/m^3 and 6000 EU/m^3, respectively (Oppliger et al. 2005).

Finally, we observed that endotoxin pollution is strictly correlated with the coarse particle fractions, and the abatement of this kind of pollution generated from a farm applying current Italian regulations is nearly complete at short distances. At 50 meters from the shed, without the influence of other natural sources, the endotoxin levels were comparable to those found at an urban background site. The evaluation of health risks for people who live alongside farmsheds is an evolving issue, in which more consideration must to be given to biological contamination due to air pollution from the perspectives of both infectivity and toxicity.

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Figure 1.

Composition of size-fractionated PM10 at each sampling site. **A)** It shows the concentration of particles and their size-related distribution at the three background sites (urban site in Torino and rural ones in Fiano and Riva). **B)** It refers to the PM10 and its sub-fractions levels in the sites near the two farms. **C)** Mean fractioned PM10 concentrations, with relative

standard deviation; circles are used for each sub fractions, while squares are used, in the grey part, for the data assembled by a 3 µm cut-off. On the last figure the statistical analysis results are reported.

Figure 2.

Endotoxin levels detected with the LAL assay on PM filters after extraction with RPMI-1640 medium supplemented with 0.025% Tween-20. **A)** It shows the concentration of endotoxin and their distribution in the PM10 fractions at the three background sites (urban site in Torino and rural ones in Fiano and Riva). **B)** It refers to the endotoxins levels in the PM10 sub-fractions in the sites near the two farm-sheds. **C)** Mean endotoxin concentrations in each PM10 sub-fractions, with relative standard deviation; circles are used for each sub fractions, squares are used, in the grey part, for the data assembled by a 3 µm cut-off. On the last figure the statistical analysis results are reported.

Figure 3.

Regression analysis between the size fractioned PM10 levels and their endotoxin content. The linear regression is reported for both the total data and selecting only background or farm data.

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