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Composition and effects of inhalable size fractions of atmospheric aerosols in the polluted atmosphere. Part II. *In vitro* biological potencies

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ABSTRACT

Exposure to particulate matter (PM) in ambient air has been shown to lead to adverse health consequences. Six size fractions of PM with aerodynamic diameter smaller than 10 μm (PM_{10}) and gas phase were collected at six localities with different major pollution sources. Extracts of samples were assessed for AhR-mediated toxicity, (anti-)estrogenicity, (anti-)androgenicity and genotoxicity. The biological responses were interpreted relative to chemical characterization. Historically, for regulatory purposes, evaluation of air pollution was based mainly on assessment of the sum of PM_{10}. In the case of AhR-mediated activity, PM_1 was responsible for more than 75% of the activity of the particulate fraction from all localities. The assessed effects were correlated with concentrations of polycyclic aromatic hydrocarbons (PAH), organic carbon content and specific surface area of the PM. A significant proportion of biologically active chemicals seems to be present in the gas phase of air. The results suggest that an average daily exposure based just on the concentrations of contaminants contained in PM_{10}, as regulated in EU legislation so far, is not a sufficient indicator of contaminants in air particulates and adoption of standards more similar to other countries and inclusion of other parameters besides mass should be considered.

1. Introduction

Exposure to air pollutants is associated with various diseases such as bronchitis, asthma, lung cancer, respiratory problems or arteriosclerosis — for review see Bernstein et al. (2004). Ambient air can be polluted by a complex mixture of pollutants that are either associated with particulate matter (PM) or present in the gas phase. PM can be subdivided into different fractions according to its aerodynamic diameter. These fractions are coarse (2.5–10 μm), fine $PM_{2.5}$ (<2.5 μm), and ultrafine $PM_{0.1}$ (<0.1 μm; De Kok et al., 2006). While the coarse particles, formed mainly by mechanical processes (Bernstein et al., 2004), are deposited mostly in upper respiratory airways and are eventually expelled by mucociliary clearance, fine and ultrafine particles, originating mainly from combustion sources, pass into the alveoli where they can persist (Lippmann et al., 1980). Moreover, ultrafine particles have been determined to be able to penetrate into the circulatory system and to even produce toxic effects in organs other than the lung (Polichetti et al., 2009). Thus, the toxicological significance of chemicals associated with PM seems to be inversely proportional to particle diameter (de Kok et al., 2006; Englert, 2004; Kampa and Castanas, 2008).

Organic extracts of PM contain substances with the potential to elicit genotoxic, dioxin-like or estrogenic and antiestrogenic activities (Claxton et al., 2004; Clemons et al., 1998; Novák et al., 2009). Previous studies of the toxic potency of ambient air have focused mostly on pollutants associated with a single size fraction of air particles omitting the distribution of the toxic compounds within different size fractions of PM. Some contaminants are present also in the gas phase (Castro-Jiménez et al., 2009; Fernández et al., 2002; Lammel et al., 2009) and thus extracts of the gaseous phase of ambient air can produce specific effects such as dioxin-like activity or modulate estrogen- or androgen-dependent signaling pathways (Klein et al., 2006; Novák et al., 2009).

In this study the biological potency of six subfractions of PM_{10} from six localities has been studied. Samples were extracted with organic solvents and selected biological responses measured and concentrations of several classes of chemicals determined. The sampling sites were chosen to provide samples with broad range of diverse physical–chemical characteristics. A summary of gravimetric, geological and chemical analyses of the PM samples are given elsewhere (Landlová et al., part I) while toxicological characterization of organic extracts of PM and gas phase fractions of the air samples are given here. The *in vitro*

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bioanalytical characterization included dioxin-like activity, (anti-) estrogenicity, (anti-)androgenicity and genotoxicity.

2. Material and methods

2.1. Air sampling

Particulates were collected by using a high volume air sampler PM10 HVS1 (Umwelttechnik MCZ GmbH, Germany), which was equipped with a multi-stage cascade impactor (Andersen Instruments Incorporated, USA, series 230, model 235), which collected six size fractions of particulates. Particles were sampled on slotted glass fiber collection substrata and glass fiber Hi-Vol filters. The collected fractions represent particulates with aerodynamic diameters of 7.2–10 μm (A); 3–7.2 μm (B); 1.5–3 μm (C); 0.95–1.5 μm (D); 0.49–0.95 μm (E) and <0.49 μm (F). In parallel with the cascade impactor, chemicals in the gaseous phase were collected by using a medium volume sampler Leckel MVS6 (Sven Leckel Ingenierbüro, DEU) equipped with quartz and polyurethane foam filters in tandem (fraction G).

Halves of the filters from the cascade impactor were used for gravimetric, mineralogical and heavy metal assessments (Landlová et al., part I). The other filter halves, as well as polyurethane foam filters from the Leckel sampler, were extracted with dichloromethane in a Büchi System B-811 automatic extractor. These extracts were used for both instrumental and bioanalytical characterizations. Composites of four one-week subsamples were used for bioanalytical characterization.

Samples were collected over a 28 day period at six localities in the south-east of the Czech Republic differing mainly in the type of dominant pollution sources from July 2007 to February 2008 (Table 1). Locality I was a cement mill near Brno. This facility was located in a relatively clean locality so the main source of pollution was cement production. Locality II was a stone quarry that is located near locality I and served as a source of limestone for the cement mill. The quarry is located in woods not far from locality I, so the main source of air pollution was the quarrying and transportation of the limestone. Locality III was a small airport in rural countryside about 2 km south-east from the Brno metropolitan area. The air traffic at the airport was not intense so the main source of air contamination was probably agriculture and pollution-transported from the Brno metropolitan area. Locality IV was an important traffic junction in the center of Brno. The main source of pollution was probably vehicle traffic. Site V was located in a small village 25 km south of Brno. The village is in a rural area relatively far from larger urban areas. Households in this village mainly heat with coal that probably represented the main source of pollution. Sampling site VI was located in the industrial zone of a small town 50 km south-east of Brno. The locality was in the vicinity of glassworks, machine works and a tile factory. More detailed descriptions of the localities and sampling procedures and conditions including methodology and QA/QC and results of chemical and mineralogical analyses are published elsewhere (Landlová et al., part I).

2.2. Bioassay procedures

Four individual bioassays were used to assess biological effects of air samples. The H4IIE-*luc*, rat hepatocarcinoma cells stably transfected with the luciferase gene under control of the AhR were used to quantify the dioxin-like activity of the samples. This bioassay is a well-established model for evaluation of AhR-mediated activities of pure substances as well as environmental samples and the activity is reported as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (bioTEQ; Villeneuve et al., 2000). popTEQ, a portion of the overall bioTEQ produced by persistent organic pollutants (POPs), was assessed by removing non-persistent chemicals such as PAHs by treating samples with sulfuric acid using method described previously (Novák et al., 2007). Estrogenic effects were examined using a cell model MVLN; human breast carcinoma cells transfected with a luciferase gene under control of estrogen receptor activation (Demirpence et al., 1993; Freyberger and Schmuck, 2005; Villeneuve et al., 2002). Effects of extracts on MVLN cells were assessed in the presence or absence of competing endogenous ligand. Antiestrogenicity was assessed by simultaneous exposure of the sample extract and 17β-estradiol (11 pM). The bioluminescent yeast assay was used for detecting anti/androgenic activity. The assay was based on a yeast (*Saccharomyces cerevisiae*) strain stably transfected with genes for human androgen receptor along with firefly luciferase under transcriptional control of androgen-responsive element. Another yeast strain constitutively expressing luciferase served for assessment of cytotoxicity (Leskinen et al., 2005). Yeast cells were exposed to the samples alone or in combination with testosterone (10 nM) to assess the effect in interaction with physiological ligand of the AR. Genotoxic potency of extracts was determined by use of the microplate version of the SOS chromotest with *Escherichia coli* PQ 37 as a bacterial test strain (Quillardet and Hofnung, 1985), which has been described previously (Škarek et al., 2007a). More detailed information on cultivation and experimental conditions of the bioassays is included in online Supporting Information.

Table 1
Sampling sites and their main pollution sources.

Label	Locality	Pollution source
I	Cement mill	Cement production, waste incineration
II	Stone quarry	Quarrying, stone transport
III	Small airport	Agriculture, air traffic
IV	Traffic junction	Traffic, local heating
V	Village	Local heating
VI	Town	Industry, local heating

2.3. Data analysis

Correlations were calculated using the nonparametric Kendall tau procedure because there was insufficient data to confirm if it was normally distributed, which is one of the assumptions of parametric testing. Kendall tau was chosen because it is more robust than the Spearman correlation based on ranks. Statistical significance was defined as Type I (p) errors of less than 0.05. Results of the H4IIE-*luc* assay were reported as toxic equivalents (bioTEQ) expressed as fg of tetrachlorodibenzo-*p*-dioxin per m^3 of air or ng per g of particulate matter in the respective fraction. The calculation was based on EC_{25} values as described previously (Villeneuve et al., 2000). The calculated TEQ (pahTEQ) values were derived from analytical data on 12 PAHs with available relative potency values, which were assessed with the same model cell line as in our study (Machala et al., 2001), using toxic equivalency factor approach described previously (Safe, 1998).

IC_{25} values (m^3 of air or μg of particulate matter per ml of exposure medium) for antiestrogenicity and antiandrogenicity, were calculated from dose–response curves compared to signal of competitive concentration of added natural ligand 11 pM estradiol and 10 nM testosterone, respectively, which was considered to be the maximum (100%) response. The values in graphs and statistical analyses are expressed as an index of antiestrogenicity (AE) or antiandrogenicity (AA), respectively, which correspond to reciprocal value of IC_{25}. Thus greater antiestrogenicity and antiandrogenicity are expressed as the decrease in activity of the signal given by a specified amount of competing estrogen or testosterone in the medium, respectively. In the case of the yeast model, results from the AR-specific yeast strain were normalized to the results from a constitutively luminescent strain to take into account the effects of the samples on yeast propagation (Leskinen et al., 2005). Genotoxicity is expressed as relative genotoxic units (RGTU), a reciprocal value of minimum genotoxic concentration × 100 (Čupr et al., 2006).

3. Results and discussion

The study was designed to examine diverse samples of air particulate matter (PM) to address a variety of possible scenarios that can occur in the environment (Table 1). To at least partly cover the factor of season variation and because it was not possible to sample all localities simultaneously, sampling started in summer at localities with sources of pollution that do not change seasonally (cement mill, quarry) and ended at sites that were presumed to be most polluted in cold season due to local heating (village and industrial town) in winter. The diversity of PM types has been demonstrated based on gravimetric, mineralogical and chemical characterization (Landlová et al., part I). In order to determine relative abundance and the toxic potential of various PM size fractions, inhalable PM_{10} was aerodynamically classified to subfractions of coarse PM (A, B), accumulation size PM (C, D, E) and mostly ultrafine PM (F) (see Supporting Information Table S3). This relatively high resolution PM classification could provide an insight into size-specific distribution of chemicals with toxic potential within inhalable PM. At localities I and II, PM contained a relatively greater proportion of coarse PM (fractions A, B) when compared with the other sites. This was caused by the characteristics of the main pollution sources at these sites, which was cement production at locality I (cement mill) and quarrying and transport of limestone in case of locality II (stone quarry). At the other sites, the ratio was shifted toward fine and ultrafine particulate fractions (Landlová et al., part I). Particulates from localities III (airport-rural countryside) and IV (traffic junction) contained, beside maximum in the finest fraction of air particulates, relatively high proportions of the B fraction (3–7.2 μm) of PM that probably came from the traffic (dust whirling; Bernstein et al., 2004). At the other two localities, there were relatively small amounts of particle fractions A, B, C (>1.5 μm) and the main portion of particulate matter consisted of ultra-fine particles that probably originated from combustion of coal for local heating in case of locality V (village) and industry such as glass works, machinery plant and tilery at locality VI (small town-industrial locality). More discussion of non-toxicological characterization of the PM samples can be found in Landlová et al. (part I). Toxic potencies are based on organic extracts of the filters to describe the worst-case scenario of organic compound exposure but it does not take into account effects of particles *per se* as it is not clear how to use them directly in the submerged cell exposure scenario in a well-defined way (Paur et al., 2011). Toxic potencies of PM extracts, were expressed per weight of PM (Fig 1A, C, E), to describe differences among particulate matter in the different size fractions, as well as per volume of air (Fig 1B, D, F) to account for amounts of PM fractions in the samples of air and to compare the toxic potencies of PM fractions with the toxicity of compounds present in the gas phase fraction (G).

3.1. Dioxin-like activity of PM extracts

The H4IIE-*luc* assay is useful for detecting the potency of mixtures of chemicals that activate the aryl hydrocarbon receptor (AhR). These compounds have been shown to be involved in numerous health effects such as impairment of the reproduction, immune and nervous systems (Mukerjee, 1998). Moreover, the AhR interacts with hormonal signaling pathways and thus compounds activating AhR might indirectly act as endocrine disruptors as has been shown in the case of estrogen receptors (Safe and Wormke, 2003). There have been several studies describing AhR-mediated effects of PM_{10} (Brown et al., 2005; Cigánek et al., 2004; Clemons et al., 1998), PM_1 (Wenger et al., 2009a) or total particulate matter (Cigánek et al., 2004; Hamers et al., 2000; Klein et al., 2006).

AhR-mediated activity was observed in samples from all localities. However, in some fractions, the potency was insufficient to allow calculation of an EC_{25}-derived bioTEQ (Fig 1A, B). The potency of air sample extracts exhibited a spatial gradient gradually increasing from locality

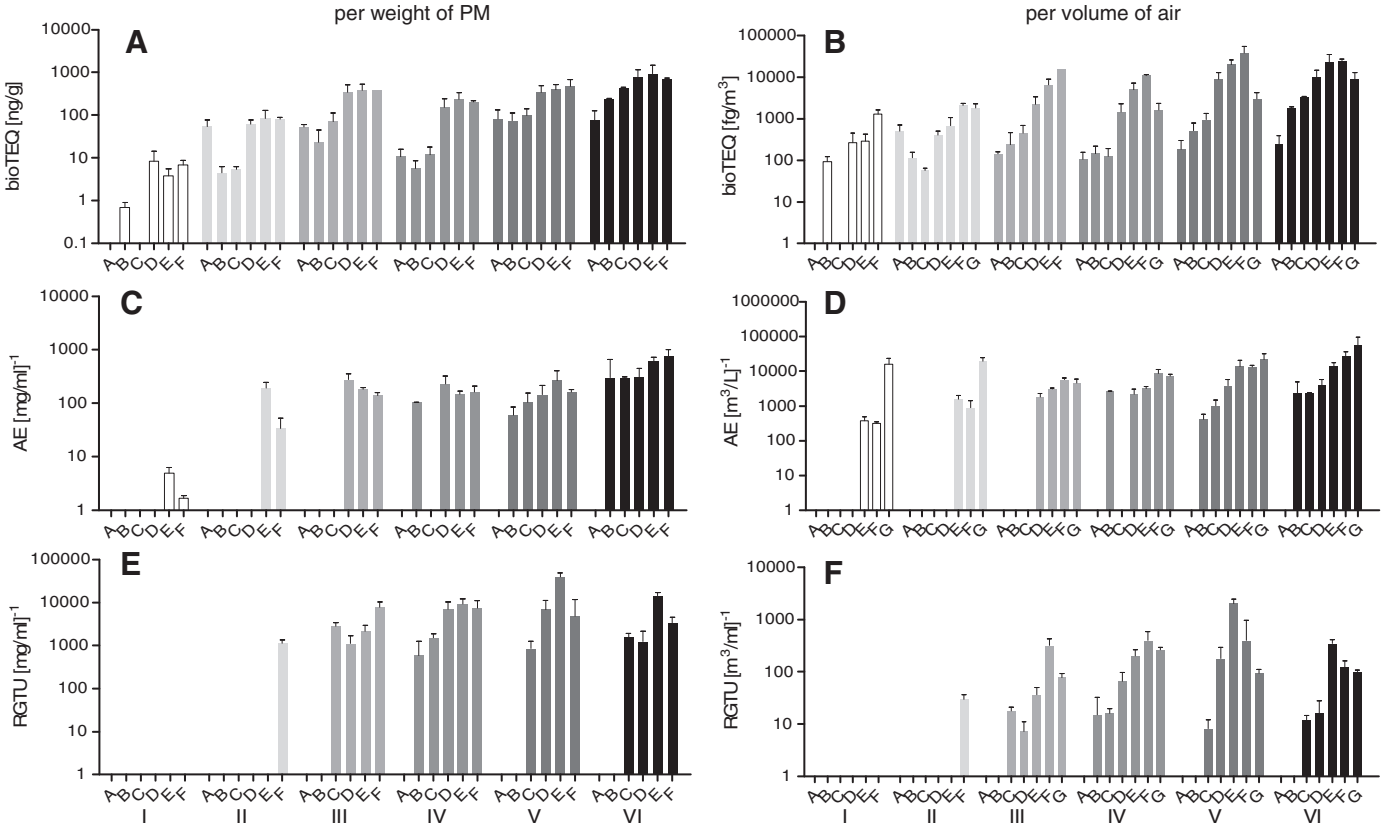

Fig 1. Toxicological characterization of PM size fraction extracts (mean ± SD); bioTEQ — AhR mediated activity; AE — antiestrogenic index (reciprocal value of IC_{25}); RGTU — relative genotoxic units (reciprocal value of minimal genotoxicity concentration × 100); A–F: size fractions of PM (A:7.2–10; B: 3–7.2; C 1.5–3; D: 0.95–1.5; E: 0.49–0.95 and F: <0.49 μm); G: gas phase fraction; I–VI: sampling sites.

I to locality VI with a potency inversely proportional to the aerodynamic diameter of the particles. However, in the case of locality II, a significant portion of the dioxin-like compounds were present in the coarsest fractions of PM (A). The concentration of bioTEQ in this fraction was 10-fold greater than those of the successive PM fractions (B, C) from the same locality. The trend was similar when the concentrations of bioTEQ were expressed per cubic meter of air. The B fraction accounted for more than 30% of PM_{10} at this locality (Landlová et al., part I). This is consistent with the concentrations of PAHs (Landlová et al., part I). PAHs can also be strongly associated with organic matter in PM (Fernández et al., 2002). However, the amount of organic matter in fraction A was not significantly greater than that in the other PM fractions from the same locality (Landlová et al., part I). Thus, the A fraction from the locality near the quarry has unique characteristics that were not described by the mineralogical characteristics measured.

AhR-mediated activity of persistent compounds (popTEQ) was responsible for 3–8% of the concentration of bioTEQ at localities II, III and V; 18 and 12% at sites I and VI, respectively, and 32% at locality IV. For more details see the Supporting Information. The greater proportion of popTEQ in samples from locality I might be explained by the fact that the cement kiln incinerated also fuel containing chlorinated compounds that could contribute to bioTEQ at this locality. However, the absolute values are less than in the case of samples from localities IV, V and VI. At locality VI, the mean contribution of POPs to bioTEQ was elevated only in the A size fraction, which had a relatively great popTEQ concentration that was responsible for 71% of bioTEQ. The rest of the PM fractions had TEQ concentrations that were similar to those of other localities where the popTEQ contribution was less (see Supporting Info). The relatively great popTEQ concentration in the PM fraction at this locality probably originated from industrial sources. The primary presumptive source of pollution at locality IV was traffic but it is not likely that traffic could produce such large amounts of dioxin-like POPs. The absolute concentrations of popTEQ at locality IV were similar to those at locality V but their relative contribution was more significant due to the fact that the overall bioTEQ concentrations were about four times less at locality IV. It is likely, at least at locality V, that persistent compounds originated from local heating because this locality is situated in a rural area without significant sources of pollution besides heating with coal. Moreover, occasional burning of household wastes along with coal occurred in the village and thus a significant amount of dioxins and other dioxin-like compounds could be generated (Gullett et al., 2001). Although locality IV is situated in the city and most households have gas heating, it might be possible that waste burning could contribute to the type of pollution as in the case of locality V.

AhR-mediated responses in the trans-activation assay were correlated with concentrations of PAHs (both sum of 16 PAHs and sum of 8 genotoxic PAHs), total organic carbon content in PM and less distinctively with calculated specific surface area of PM (Fig 2). However, the toxic equivalents calculated from PAHs levels (pahTEQ; see Supporting Info Table S1) contributed on average 17% and 3−6% of overall bioTEQ in PM extracts at site IV and the rest of the localities, respectively (Table S2). The relative contributions of PAHs are generally similar to those in the study of Wenger et al. (2009a) or less than those observed in other studies (Brown et al., 2005; Cavanagh et al., 2009; Novák et al., 2013). The rest of the TEQ concentrations could be attributed to both non-assessed PAH representatives or, more likely, PAH derivatives such as polycyclic aromatic ketones and quinones present in ambient air that have been shown to possess dioxin-like potency (Bekki et al., 2009; Misaki et al., 2007).

3.2. Antiestrogenic activity assessment of PM extracts

Xenoestrogenicity, i.e. affecting of estrogen receptor by xenobiotics, is one of the best described modes of action of endocrine disruptors and

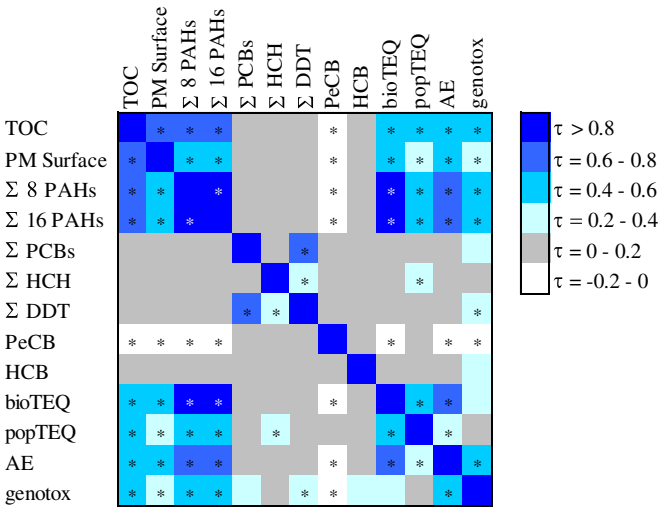

Fig 2. Correlation of assessed parameters in PM (Kendall tau; significant values are labeled with asterisk); TOC — total organic carbon, PM surface — calculated PM surface area, 8 PAHs — Σ 8 carcinogenic PAHs, 16 PAHs — Σ US EPA indicator PAHs, Σ PCBs — polychlorinated biphenyls, Σ HCH — hexachlorcyclohexan, PeCB — pentachlorbenzen, HCB — hexachlorbenzen, bioTEQ — total AhR-mediated activity, popTEQ — AhR-mediated activity of persistent compounds, AE — antiestrogenic index, genotox — genotoxic potential.

there is general agreement that defects in estrogenic signaling are important in mediating reproductive and developmental toxicity and carcinogenesis (Borgert et al., 2003; Combes, 2000). While weak estrogenic response was observed in three fractions of the largest air particulates (A, B, C) from locality I (see Fig S1 in the Supplementary Information) the other extracts of air particulates displayed antiestrogenic or no effect (Fig 1B, C). Some previous studies reported estrogenic effects associated with air PM (Clemons et al., 1998; Wang et al., 2004; Klein et al., 2006; Wenger et al., 2009b) and the gas phase of ambient air (Klein et al., 2006). On the other hand, antiestrogenic properties of PM and gas phase air extracts were observed in two different regions in our previous study (Novák et al., 2009). This discrepancy was probably caused by the fact that, due to different characteristics of pollution sources, the composition of environmental pollutant mixtures is site-specific. Some air pollutants such as some PAHs are estrogenic (Villeneuve et al., 2002), while other PAHs or compounds in diesel exhausts, are reported to be antiestrogenic (Arcaro et al., 1999; Okamura et al., 2002). The overall activity of the mixture depends on ratios of the constituents. Thus, although there might be some differences in the bioassay conditions in our study and those of previous studies (Novák et al., 2009), the difference in observed effects is probably due to the specific composition of air pollutant mixtures in the region studied here. This hypothesis could be supported by the fact that PM extracts from locality I was ambiguous. While extracts of the coarser PM fraction elicited estrogenic effects, extracts of the finer fractions were antiestrogenic. This result suggests that PM size fractions from the same locality could contain mixtures of organic compounds that possess different toxicological characteristics. Effects of samples III, IV, V, VI, when expressed as an antiestrogenicity index (AE, inverse value of IC_{25}) in gravimetric mode, were significantly correlated with bioTEQ, sum of PAHs, total organic content and specific surface of the PM (Fig 2). These correlations are consistent with PAHs, and dioxin-like and antiestrogenic compounds concur in PM samples. This result is also consistent with specific interactions i.e. antiestrogenic effect of dioxin-like compounds via AhR-dependent mechanism as has been described previously (Okamura et al., 2002; Ueng et al., 2004). The link between dioxin-like activity and antiestrogenicity has been thoroughly described by mechanistic studies reviewed by Safe and Wormke (2003).

3.3. Genotoxicity assessment of PM extracts

Genotoxicity is a well-documented effect of air pollutants both *in vivo* and *in vitro* (Lewtas, 2007). It is known to be closely related to oxidative properties of PM that are often associated with metal content (Wessels et al., 2010). Thus the organic air sample extracts used in our study do not describe overall genotoxic potential of the ambient air and they indicate mainly genotoxic potential of organic pollutants within the samples. To minimize consumption of samples microplate modification of SOS chromotest has been used. The study focused only on direct genotoxicity (without metabolic activation). Genotoxic effects were produced by PM extracts from localities II–VI (Fig 1E, F). The most genotoxic samples came from locality IV. Again, the greatest effects were produced mainly by compounds present in the small size fractions of air particulates. This is consistent with the studies on genotoxicity of size fractions of PM_{10} (Buschini et al., 2001; Čupr et al., 2013; Funasaka et al., 2003). On the other hand, in total suspended particles, Škarek et al. (2007b) observed greater genotoxic potency associated with fractions with particle diameters greater than $PM_{2.5}$. Interestingly, there was a decrease of genotoxicity of F fraction at localities V and VI. A similar trend has been described previously and it has been proposed that it is caused by lower levels of POPs in the finest PM fraction (Čupr et al., 2013; Topinka et al., 2013). However, the dioxin-like potential profile does not support this hypothesis with our samples (Fig 1). Genotoxic potency was correlated with antiestrogenicity, the concentration of the sum of PAHs and DDT as well as total organic carbon (Fig 2). This is in agreement with data from the literature because many PAHs are known for their genotoxic properties (Topinka et al., 2013) and it has been described that exposure to DDT, which interferes with estrogenic signaling, is associated also with genotoxic effects in mussels as well as in mammals *in vivo* (Binelli et al., 2008; Canales-Aguirre et al., 2011).

3.4. Anti-androgenicity of PM extracts

Anti-androgenicity has been shown to play a role in mediating sexual disorders caused by xenobiotics in males (Sultan et al., 2001). Previously, antiandrogenic effects had been associated with some mixtures of air pollutants such as diesel exhausts (Okamura et al., 2004; Taneda et al., 2004). In our previous work, we have shown anti-androgenic properties of extracts from PM_{10} samples (Novák et al., 2009, 2013). In the present study, PM-associated antiandrogenic effects were produced only by extracts from the largest diameter particulates (A) and finest (F) fractions from locality I with 0.14 and 0.12 of volumetric antiandrogenic index $[(m^3/ml)^{-1}]$, respectively. Some compounds in diesel emissions, such as 3-methyl-4-nitrophenol, have been described as antiandrogenic (Li et al., 2006; Owens et al., 2006) and the cement kiln close to locality I also burns fuel oils and other low quality fuels, so it might be possible that the antiandrogenic activity could be due to compounds from the kiln emissions. Alternatively, the burning processes in the kiln are carefully controlled so there should be a minimum of un-burned compounds in the exhaust.

3.5. Gas fraction effects

Most previous studies concerned with *in vitro* effects have focused on compounds associated with PM in air. However, there have been some studies in which a considerable portion of the observed biologically active compounds can be present also in the gas phase (Klein et al., 2006; Novák et al., 2013, 2009). In this study similar results were observed for all four bioassays. Significant activity was observed in the extracts of the gaseous fraction (G) from most localities.

AhR-active chemicals were detected in extracts of the gas fraction from all localities, however only extracts from localities II, IV, V and VI produced enough activity to describe an EC_{25} and quantify the concentrations of bioTEQ (Fig 1B). AhR-mediated activity was produced mainly by non-persistent compounds because popTEQ concentrations accounted for less than 4% of the concentration of bioTEQ at localities II, V, VI. The greatest contribution from POPs was 17% at locality IV which is equivalent to concentrations observed in the PM fractions. Alternatively, when comparing the calculated contribution of PAHs (pahTEQ) to the total concentration of bioTEQ, the situation is not significantly different from that in PM fractions and the assessed PAHs did not account for more than 4% of bioTEQ (Supporting Info). Similar results have been reported previously (Novák et al., 2013). This indicates that also in the gas phase routinely assessed PAHs do not account for most of the AhR-mediated activity and other non-persistent chemicals such as their derivatives are involved.

Anti-estrogenic effects have been produced by gas fraction extracts from all localities and the activities were comparable to or greater than the effects of the PM fraction extracts. The data are comparable with antiestrogenicity of the gas phase extracts from the industrial region in our previous study (Novák et al., 2009). Greater effects were observed in the samples from localities V and VI where they could be increased by compounds from combustion sources that were probably less significant in the studied regions in the previous work.

Significant genotoxic effects were produced by the gas phase fraction extracts from localities III, IV, V and VI (Fig 1E, F). The greatest genotoxic potential was observed in the air from locality IV while the others contained less than half as much genotoxic activity. This was probably due to the intensive traffic at locality IV, which was presumably the principal source of pollutants there and traffic has been suggested as an important source of genotoxic compounds before (De Kok et al., 2006; Škarek et al., 2007a). However, direct comparison of the data is not possible because the previous studies do not provide any absolute genotoxicity units (Du Four et al., 2005; Škarek et al., 2007a,b).

Antiandrogenicity was almost exclusively associated with gas phase fractions of samples and it was detected in samples from all six localities (0.28; 0.58; 2.3; 0.19; 0.49 and 1.8 IC_{25}^{-1} $[m^3/ml]^{-1}$, respectively). The detected potentials seem to be less than those reported in previously studied industrial regions (Novák et al., 2009) but similar to potentials of air from Banja Luka that were assessed with mammalian cell-based bioassay (Novák et al., 2013).

3.6. Implications for air quality assessment

The present data describe toxic potential of ambient air pollutants and so they cannot be directly applied to a situation *in vivo*, because they do not cover important processes such as metabolisation or differences in bioavailability among the fractions in the study (Elad et al., 2008), which would play important roles *in vivo*. However, the results indicate that there can be significant differences in toxicological characteristics of PM from different pollution sources (Grahame and Schlesinger, 2007) and also in different size fractions of PM. The observed biological effects were mostly in the fine and ultrafine fractions. Although it does not have to be a common rule for other toxic effects such as endotoxins that were described to be associated mostly with the coarse fractions of PM (Traversi et al., 2011) or oxidant capacity and toxicity that was shown to be connected to the pollution source rather than to the PM size (Wessels et al., 2010). While in the US ambient air quality standards focus mainly on $PM_{2.5}$ levels assessment (US-EPA, 2007), EU guidelines for assessment of air pollution focus so far on evaluation of daily average of PM_{10} exposure (EU-Commission, 1999) without considering the distribution among size-spectra of PM including ultrafine fraction of particles that seems to be the most harmful (Araujo and Nel, 2009; Sioutas et al., 2005). In this study, PM_1 accounted for more than 90% of the dioxin-like equivalents of PM_{10} at all localities besides the stone quarry (II) and the industrial locality (VI) and even there PM_1 accounted for more than 75% of the bioTEQ of all PM extracts (Fig 3). Greater contribution of the finer fractions was also observed in the results of the other bioassays. Moreover, the fine and ultrafine PM are the most effective in penetrating into the respiratory and circulatory tracts (Lippmann et al.,

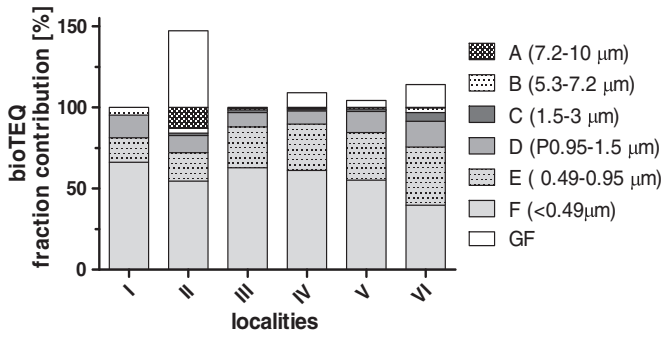

Fig 3. Percentage contribution of PM size fractions to overall assessed toxicity equivalent of TCDD (bioTEQ) of PM_{10} from localities I–VI with bioTEQ of gas phase; PM — particulate matter size fractions [μm]; GF — gas phase fraction.

1980; Polichetti et al., 2009). Thus, evaluation of air pollution should not be limited just to gravimetric assessment of PM_{10} but should consider also the size spectrum of more dangerous fine and ultrafine PM fractions as well as results from bio-analytical methods to integrate toxic potencies of samples including unidentified as well as identified chemicals. Since a significant portion of toxic pollutants was present in the gas phase (Fig 1B, D, F) with 50% of bioTEQ of fraction G approximately half as great as that of PM_{10} at locality II (Fig 3) more attention should be given to the gaseous phase. Thus, fraction G could be also involved in mediating toxic effects of ambient air pollutants and should be also taken into account in ambient air contamination evaluation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2013.10.013.

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