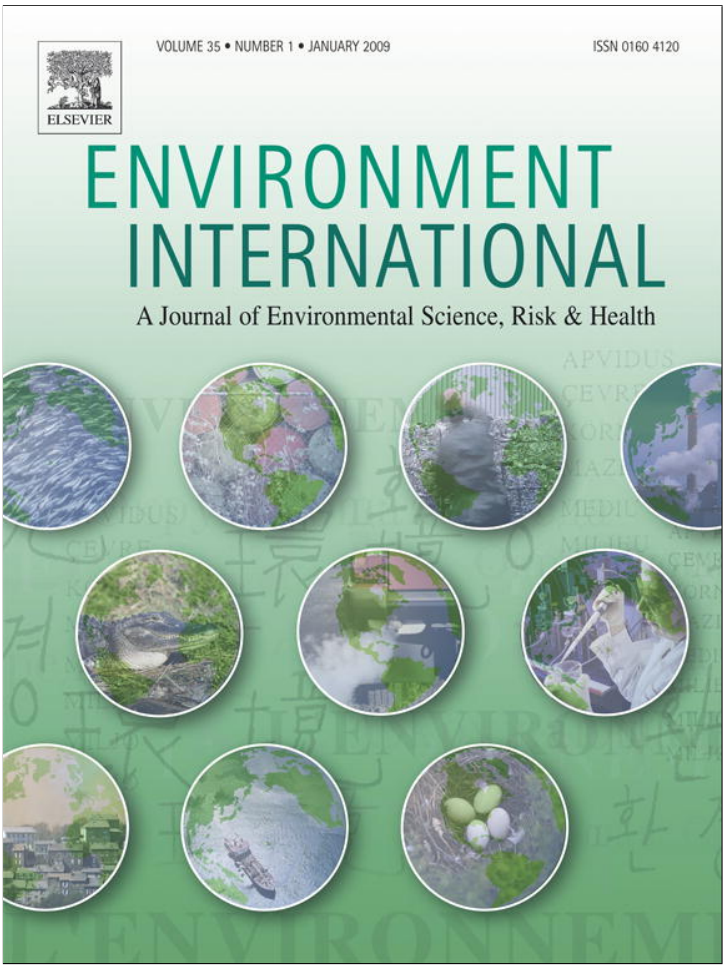

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Pollutants in particulate and gaseous fractions of ambient air interfere with multiple signaling pathways *in vitro*

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ABSTRACT

Traditionally, contamination of air has been evaluated primarily by chemical analyses of indicator contaminants and these studies have focused mainly on compounds associated with particulates. Some reports have shown that air contaminants can produce specific biological effects such as toxicity mediated by the aryl hydrocarbon receptor (AhR) or modulation of the endocrine system. This study assessed the dioxin-like toxicity, anti-/estrogenicity, anti-/androgenicity and anti-/retinoic activity of both the particulate and gas phase fractions of air in two regions with different types of pollution sources and a background locality situated in an agricultural area of Central Europe. The first region (A) is known to be significantly contaminated by organochlorine pesticides and chemical industry. The other region (B) has been polluted by historical releases of PCBs, but the major current sources of contamination are probably combustion sources from local traffic and heating. Samples of both particle and gas fractions produced dioxin-like (AhR-mediated) activity, anti-estrogenic and antiandrogenic effects, but none had any effect on retinoid signaling. AhR-mediated activities were observed in all samples and the TEQ values were comparable in both fractions in region A, but significantly greater in the particulate fraction in region B. The greater AhR-mediated activity corresponded to a greater coincident antiestrogenicity of both phases in region B. Our study is the first report of antiestrogenicity and antiandrogenicity in ambient air. Anti-androgenicity was observed in the gas phase of all regions, while in the particulate phase only in one region due to the specific type of pollution in that area. Even though based on concentrations of individual compounds, except for the OCPs, the level of contamination of the two regions was similar, there were strong differences in responses in the bioassays between the two regions. Moreover, AhR-mediated activity and antiestrogenic potencies were greater in region B, where the pollution level according to the chemical analysis was similar or less than in the other region, which indicates the presence of other atmospheric pollutants with specific effects. The results document the advantage and utility of the simultaneous use of bioassays and chemical analysis in risk assessment of complex environmental samples.

1. Introduction

Ambient air in urban and industrial areas often contains complex mixtures of environmental contaminants that are present in the particulate and/or gas phases of air (Englert, 2004). Traditionally, air pollution monitoring has focused on assessment of concentrations of particles, inorganic oxides, and ozone. Pollution of air by organic compounds is mostly evaluated by analysis of indicator classes of contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or organochlorine pesticides (OCPs). However, air contamination consists of very complex mixtures of chemicals, some of which are unidentified, and these mixtures could potentially produce toxic effects that would not be expected, based on the available analytical data. Specific effects of air pollutants can be assessed by bioassays, which unlike chemical analyses can integrate the effects of all chemicals in the complex mixture, also taking in account interactions such as additivity, antagonism, or synergism.

Air pollution has been linked with adverse health consequences in exposed animals (Bernstein et al., 2004; Kotwal et al., 2005) and humans (for review see Curtis et al., 2006; de Kok et al., 2006). Polluted air has been associated mainly with lung and heart diseases. However, recent studies indicate that air pollutants can possibly impair reproduction. Inhalation of diesel exhaust has been reported to cause reproductive effects in rodents (Li et al., 2006a; Watanabe and Kurita, 2001; Yoshida et al., 1999) and birds (Li et al., 2006b). The effects of air pollution on reproduction have been validated in

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experiments with mice raised either in filtered or non-filtered city air (Lichtenfels et al., 2007; Mohallem et al., 2005). A statistically significant negative correlation between concentrations of particulate matter in air and male to female ratio was observed in newborn children in Sao Paulo, Brazil (Lichtenfels et al., 2007). These authors also observed significantly poorer semen quality in men occupationally exposed to traffic (De Rosa et al., 2003). Some of these effects could possibly be related to endocrine disruptive properties of air pollutants. This hypothesis is supported by the fact that pollutants which are known as potential endocrine disruptors have been detected in indoor air and dust (Rudel et al., 2003) and that the contaminants in extracts of ambient air have been shown to interact with receptors for steroid hormones *in vitro*. Some authors have observed *in vitro* estrogenic (Clemons et al., 1998; Klein et al., 2006; Ueng et al., 2004) and antiprogesteronic (Wang et al., 2005b) effects of air extracts or antiestrogenic and antiandrogenic *in vitro* and *in vivo* effects of traffic exhaust particles (Kizu et al., 2003; Okamura et al., 2004). Another potential mode of action that is widely used in the toxicity evaluation of complex samples is the dioxin-like toxicity and there are several works describing this type of effect in ambient air samples (Arrieta et al., 2003; Ciganek et al., 2004; Clemons et al., 1998; Mason, 1994).

Previous studies dealing with effects of ambient air pollution at the molecular level have focused almost exclusively on pollutants associated with air particles. Nevertheless, a large number of air contaminants is present at least partly in the gas phase (Fernandez et al., 2002; Mandalakis et al., 2002). Moreover, Klein et al. (2006) have shown that a portion of compounds with dioxin-like or estrogenic activity is present in the gas phase of ambient air. This finding, together with the fact that the gas fraction of the air contaminants is readily bioavailable, document that the gas phase of ambient air can be a potentially important source of biologically active contaminants.

In this study, bioassays were used to examine interactions of extracts of both the gaseous and particulate phases of air with intracellular receptors for estrogens (estrogen receptor; ER), androgens (androgen receptor; AR), compounds with dioxin-like activity (AhR-mediated), and retinoids (retinoic acid receptor; RAR). These receptors are known to be involved in mediating some endocrine disruptive effects of xenobiotics. While ER and AR are considered potential targets for endocrine disruptors, AhR- and RAR-mediated effects are usually neglected in the study of this phenomenon (Janosek et al., 2006). AhR-mediated effects are considered a valuable marker of contamination by dioxin-like compounds (Whyte et al., 2004) that can negatively affect liver functions as well as immunity, endocrine and nervous system (Mukerjee, 1998). Although there is no known endogenous physiological ligand for the AhR (Hahn, 2002) and thus it is not considered a part of hormonal signaling pathways, it has been described to cross-talk with estrogen receptor signaling (Ohtake et al., 2003; Safe and Wormke, 2003). Moreover, there is evidence for cross-talk between AhR-dependent signaling pathways and other hormone pathways (Puga et al., 2005; Safe and McDougal, 2002; Widerak et al., 2006).

The RAR is a part of a signaling pathway of retinoids that controls processes such as morphogenesis, development, reproduction, or apoptosis. Although retinoids are not strictly endogenous since they are derived from dietary sources of vitamin A or its precursors, the levels of their active forms are regulated in a hormone-like way such that they are sometimes referred to as 'dietary' hormones (Ross and Zolfaghari, 2004; Simms and Ross, 2000). Moreover, the involvement of retinoid signaling as a target of endocrine disruptive environmental pollutants has been proposed in several studies (Nishikawa et al., 2004; Novak et al., 2007; Smith et al., 2003).

This study evaluated the potential of air pollutants to act through several modes of action. The hypothesis that pollutants from different regions with different profiles of contaminants could modulate endocrine function was tested by collecting air samples in two regions with different sources of pollution and a background locality. Bioassays were used in conjunction with instrumental identification and quantification of residues to assess the specific activities of pollutants in both particulate and gas phases of air.

2. Materials and methods

2.1. Collection and preparation of samples

Air samples were collected in July and August 2005 from three regions (Fig. 1). The first region (A) was represented by 6 discrete sampling locations in a region that is known to be contaminated by organochlorine pesticides and chemical industry. The second region (B), represented by 8 sampling stations, has been polluted by historical releases of PCBs, but the current major sources of contamination are probably combustion sources from local traffic and heating. The third region was a reference area where a composite sample (REF) was prepared from four one-day samples from a background locality at the Košetice observatory, Czech Republic. The reference location is a part of long-term monitoring project EMEP (UN-ECE's European Monitoring and Evaluation Programme) and the level of contamination at that location has been measured continuously since 1988 (Holoubek et al., 2007). The reference location represents a rural region devoid of larger settlements or major sources of contamination. Another set of 6 composite samples, each composed of five 24-hour sub-samples,

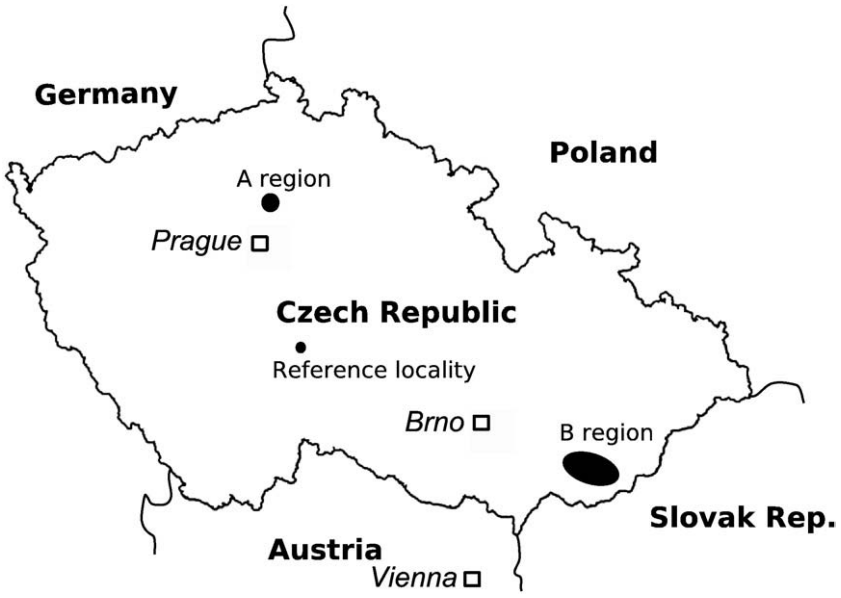

Fig. 1. Locations of studied regions within the Czech Republic.

was collected in the small town of Neratovice in the vicinity of the Spolana chemical plant (region A) in the North-eastern part of the Czech Republic. This plant produces mainly polyvinyl chloride, linear alpha olefins, caprolactam, and inorganic compounds such as sodium hydroxide, liquid chlorine, hydrochloric acid, sulfuric acid, sodium hypochlorite, or ammonium sulfate. However, historically the Spolana plant was a major producer of pesticides including DDT between 1958 and 1969 and lindane until 1975. A total of 60,000 tons of technical HCH (more than 3000 tons of pure lindane) was produced from 1961 until 1975. This region does not contain any large towns or intensive traffic, so the most important sources of contamination represent chemical industry and agriculture.

Region B, located in the South-eastern portion of the Czech Republic (city of Uherské Hradiště and surroundings) was represented by a composite of seven 24-hour subsamples for each of the 8 locations. This region contains important traffic lines, intensive agriculture, several medium-size towns, and paint producing chemical plant, which used to be a significant source of PCBs contamination in the past. Thus, the primary sources of pollution are traffic, local heating, chemical industry, and agriculture.

Since a substantial portion of particulate matter in air comes from combustion processes we also assessed the contamination pattern and specific biological activities of a model sample of fly ash and a domestic ash sample. The sample of fly ash was obtained from municipal incinerator and the domestic ash sample from local heating burning coal together with domestic waste.

Air was collected in midsummer 2005 (end of July, beginning of August) using hi-vol air samplers PS-1 (Graseby Anderson, USA) with Whatmann quartz filter (fraction $d_{ae}<50$ μm) and polyurethane foam filter (Gumotex, Břeclav, Czech Republic) with a density of 0.022 g cm^{-3} in tandem. Sampling volumes were about 400 m^3 d^{-1}. A particulate phase (PP) and gas phase (GP) was collected for each location.

Exposed filters and both ash samples were extracted with dichloromethane in a Büchi System B-811 automatic extractor. One half of the composite extracts was assessed for residues of polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (OCPs) and the other was used for assessment of the specific effects in cell culture bioassays. The volume of the dichloromethane extracts was reduced after extraction under a gentle nitrogen stream at ambient temperature. The part of the extracts for bioassays was transferred to DMSO.

2.2. Identification and quantification of residues

Laboratory blank and reference material were analyzed with each set of air samples. Fractionation of the raw extracts was achieved on silica gel column; sulfuric acid modified silica gel column was used for PCB/OCP samples. 16 US EPA polycyclic aromatic hydrocarbons were measured using GC-MS (HP 6890–HP 5973) with a J&W Scientific fused silica column DB-5MS. GC-ECD (HP 5890) supplied with a Quadrex fused silica column 5% Ph was used to analyze for seven indicator PCB congeners and OCPs (α-HCH, β-HCH, γ-HCH, δ-HCH, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, HCH). Concentrations of residues were calculated by external calibration by use of Pesticide Mix 13 (Dr. Ehrenstorfer) and PAH Mix 27 (Promochem) standard mixtures. PAHs were expressed either as ∑PAHs including the 16 US EPA PAHs or as LW PAHs (sum of PAHs with MW equal or less than 178) or HW PAHs (sum of PAHs with MW equal or greater than 202). Terfenyl and PCB 121 were used as internal standards for PAHs and PCBs analyses, respectively.

2.3. Cell cultures

Four individual bioassays were used to measure the total integrated activities of the air samples. The H4IIE-*luc*, rat hepato-carcinoma cells stably transfected with the luciferase gene under control of the AhR were used for analysis of 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 activity is reported as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (bioTEQ; Villeneuve et al., 2000). The cells were cultured in tissue culture flasks (TPP, Austria) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum Mycoplex (PAA, Austria). The 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), was cultivated in medium DMEM/F12 (Sigma-Aldrich) supplemented with 10% fetal calf serum Mycoplex (PAA, Austria). The murine embryonic carcinoma cell line P19/A15 was used to measure total RAR-mediated activities. P19 cells were purchased from the European Collection of Cell Cultures, Wiltshire, UK. Stable transfectants (P19/A15) were prepared by electroporation as described previously (Pachernik et al., 2005). Cells were transfected with the mixture of 10 μg luciferase reporter pRAREβ2-TK-luc plasmid (provided by Christopher Glass, University of California, USA) and 2 μg selection vector pSV2Neo (Clontech, USA). Transfected cells were then selected in medium containing 400 μg ml^{-1} of G418 (Sigma-Aldrich, Czech Republic), cloned and screened for the response to all-trans retinoic acid (ATRA) by determining the amount of luciferase expression by luminometry. Positive clones that retained the phenotype and *in vitro* differentiation potential of maternal cells were used for further tests. The cells were grown under the same conditions as H4IIE-*luc* cells. The bioluminescent yeast assay was used for detecting anti-/androgenic activity of the air sample extracts. The assay was based on *Saccharomyces cerevisiae* strain stably transfected with human androgen receptor along with firefly luciferase under transcriptional control of ARE (androgen-responsive element). Another strain constitutively expressing luciferase served for assessment of cytotoxicity as described in Leskinen et al. (2005).

2.4. Bioassay procedures

All cell culture bioassays were performed in 96 well microplates with final volume 200 μl of exposure medium per well. H4IIE-*luc*, MVLN and P19/A15 cells were seeded in densities of 15,000; 20,000 and 7000 cells/well, respectively. H4IIE-*luc* cells were exposed in the cultivations medium, P19/A15 cells in medium DMEM/F12 (Sigma-Aldrich) supplemented with 10% fetal calf serum Mycoplex (PAA, Austria) and MVLN cell line was exposed in DMEM/F12 (Sigma-Aldrich) supplemented with 5% dialyzed fetal calf serum (PAA, Austria), which was additionally dextran/charcoal treated to further decrease background concentrations of estradiol. Approximately 24 h after plating, cells were exposed to the extracts dissolved in DMSO and calibration reference 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Ultra Scientific, USA; dilution series 0–500 pM) in case of H4IIE-*luc*, 17β estradiol (E2; Sigma-Aldrich; Czech Republic; dilution series 0–500 pM) for MVLN and all-trans retinoic acid (ATRA; Sigma-Aldrich; Czech Republic; dilution series 0–10,000 nM) for P19/A15. Effects of extracts on MVLN and P19/A15 cells were assessed either singly or in combination with competing endogenous ligand. The anti-/estrogenicity was assessed by simultaneous exposure of the sample extract and 17β estradiol (11 pM), and the RAR-mediated response was tested also in addition of 32 nM ATRA (concentrations near their EC_{50}). The final concentration of the solvent was less than 0.5% v/v in the exposure media and appropriate solvent controls were tested. Several dilutions of each extract were tested to provide dose–response curve for each sample. Every dilution was tested in triplicate. At least three independent assays were conducted for each sample in all test systems. After 24 h of exposure, the intensity of luciferase luminescence corresponding to the respective receptor activation was measured using the Promega Steady Glo Kit (Promega, USA). Cytotoxicity of tested dilutions of the samples was assessed using neutral red uptake assay (Freyberger and Schmuck, 2005) in all cell line bioassays and data from cytotoxic sample dilutions were excluded from calculations.

Experiments with the androgen-responsive yeast model were performed according to Leskinen et al. (2005) with final concentration of the solvent not exceeding 1% v/v in a well. Yeast cells were exposed to calibration standard testosterone (Sigma-Aldrich; Czech Republic; dilution series 0–10^{-5} M), the sample alone or in combination with testosterone (10^{-8} M) to assess also the effect in interaction with physiological ligand of the AR. Yeast cells were incubated for 2.5 h and then the signal was detected after addition of D-luciferin substrate. To assess possible cytotoxicity, the control assay with constitutively luminescent strain was conducted along with the assay with AR-specific strain.

2.5. Data analysis

To determine the significance of the response to treatments relative to controls, statistical analyses were performed using a one-way ANOVA from at least three independent experiments ($p<0.05$). Results of the H4IIE-*luc* assay were reported as bioTEQ expressed as ng of TCDD per m^3 of air based on EC_{25} values because some samples did not reach EC_{50} responses (Villeneuve et al., 2000). IC_{25} values (m^3 of air per ml of exposure medium) for antiestrogenicity and anti-androgenicity 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 as 100% response. The values in graphs and statistical analyses are expressed as an index of antiestrogenicity (AE) or anti-androgenicity (AA) respectively, which correspond to reciprocal value of IC_{25}, thus greater antiestrogenicity and anti-androgenicity are expressed as the decrease in activity of the signal given by a specified amount of competing estrogen or testosterone in the medium. In the case of the yeast model, the results from AR-specific yeast strain have been normalized to the results from

Table 1
Concentrations of contaminants in air sample extracts (ng m^{-3}) from background locality (REF) and two regions of interests (A, B); ∑PAHs—sum of 16 US EPA polycyclic aromatic hydrocarbons; LW PAHs—sum of polycyclic aromatic hydrocarbons with MW ≤ 178; HW PAHs—sum of polycyclic aromatic hydrocarbons with MW ≥ 202; ∑PCBs—sum of polychlorinated biphenyls; ∑OCPs—sum of organochlorinated pesticides; GP—gas phase; PP—particulate phase

Localities	∑PAHs		LW PAHs		HW PAHs		∑PCBs		∑OCPs	
	PP	GP	PP	GP	PP	GP	PP	GP	PP	GP
REF	0.49	3.0	0.14	2.6	0.35	0.43	1×10^{-3}	0.034	0.68×10^{-2}	0.35
A1	1.5	24	0.45	20	1.1	4.3	22×10^{-3}	0.17	0.31	8.9
A2	1.8	30	0.58	25	1.3	4.9	34×10^{-3}	0.25	1.0	19
A3	2.1	28	0.72	23	1.4	5.9	78×10^{-3}	0.37	6.4	116
A4	1.8	30	0.69	24	1.1	6.8	39×10^{-3}	0.37	0.4	2.0
A5	1.5	24	0.46	20	1.1	4.2	43×10^{-3}	0.20	1.8	32.8
A6	3.1	30	1.1	26	2.0	4.7	46×10^{-3}	0.18	1.4	24.9
B1	4.1	42	0.88	34	3.2	8.2	20×10^{-3}	0.12	1.9×10^{-2}	0.55
B2	2.3	23	0.59	18	1.7	4.9	17×10^{-3}	0.10	1.6×10^{-2}	0.45
B3	2.9	35	0.70	27	2.2	8.1	24×10^{-3}	0.15	1.7×10^{-2}	0.47
B4	3.4	34	0.74	26	2.6	8.1	34×10^{-3}	0.92	2.0×10^{-2}	0.52
B5	2.9	20	0.52	15	2.3	4.7	21×10^{-3}	0.18	2.1×10^{-2}	0.62
B6	3.9	107	1.53	91	2.4	17	28×10^{-3}	0.32	2.2×10^{-2}	0.46
B7	3.2	31	0.78	24	2.4	6.3	16×10^{-3}	0.18	1.2×10^{-2}	0.47
B8	4.8	89	1.3	70	3.5	19	52×10^{-3}	1.0	4.2×10^{-2}	0.65

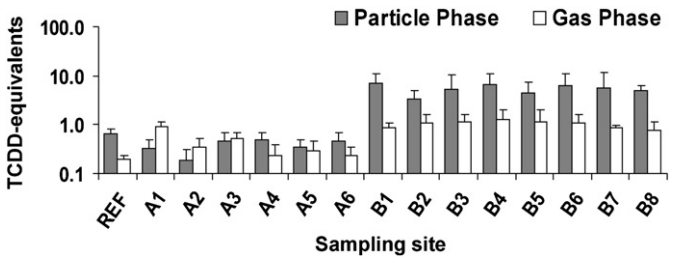

Fig. 2. TCDD-equivalents (ng bioTEQ/m^3; mean+SEM) of the particle and gas phases of air samples from background locality (REF) and two regions of interest (A, B).

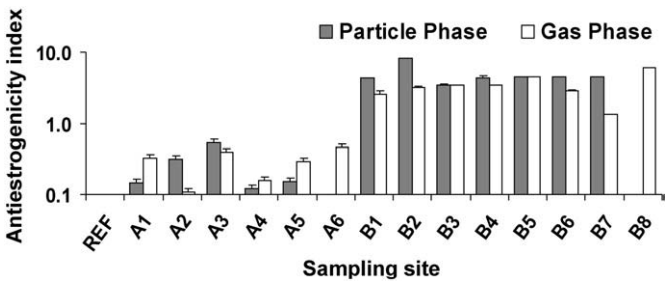

Fig. 3. Index of antiestrogenicity (reciprocal value of IC_{25}, $[m^3/ml]^{-1}$ mean+SEM) of the particle and gas phases of air samples from the background locality (REF) and two regions of interest (A, B) assessed in co-exposure with 11 pM 17β Estradiol; no column = no significant activity.

constitutively luminescent strain to cover the effects of the samples on yeast propagation (Leskinen et al., 2005). However, the results from sample dilutions that were considered cytotoxic were discarded from the data analyses. Spearman rank correlation coefficient (r_s) was applied as a measure of relations among the compared parameters in logarithmic form. Multivariate variation of performed bioassays as well as chemical parameters was further summarized in the principal component analysis (PCA) as an effective technique simplifying the correlation structure through linear transformation of the original variables. Principle components analysis (PCA) was conducted to provide component loading vectors explaining the relationships among the bioassays and chemical contamination and component score vectors as pair-wise uncorrelated variables that were used for the final exploratory survey of the data from the examined regions and the two air fractions. The most informative bilinear projections showing the relation between objects (examined samples) and variables (bioassays and chemical parameters) was obtained in PCA based on a correlation matrix of the $\log_{10}$-transformed variables. Component weight vectors were scaled to the unit length one. Biplot was used as a common graphical tool representing not only projections on extracted principal components but also the 2D loadings of original variables by lines. All statistical analyses were performed with the software STATISTICA for Windows 8.0 (StatSoft, Inc. USA).

3. Results

3.1. Characterization of the samples according to chemical analyses

Concentrations of major classes of organic contaminants including 16 US EPA indicator PAHs, 7 indicator PCBs, and organochlorine pesticides DDTs, HCHs and HCB are reported in Table 1. Concentrations of the indicator pollutants expressed per cubic meter of air were approximately 10-fold greater in the gas fraction than in the particulate fraction in most samples. Concentrations of all of the indicator pollutants in air from the reference location were approximately 10-fold less than those from the other two regions in both the particulate and gas phases, except of OCPs, whose concentrations in region B were only slightly greater than the concentrations in the reference locality (Table 1). Overall, concentrations of the individual and total PAHs and PCBs, as well as concentrations in the separate fractions, did not differ significantly between regions A and B. However, there were some differences in concentrations among the individual sites within regions. The greatest concentrations of PCBs in gas phase were observed in samples B4 and B8, while concentrations in most of the other samples from region B were similar to those from region A. Similarly, concentrations of PAHs were comparable among most sites of both regions A and B except for greater values at sites B6 and B8. The only statistically significant difference between the two regions was found for concentrations of OCPs in both air phases, which were significantly greater in region A compared to region B (Table 1). Concentrations of OCPs in region B were homogenous across the region, while there were significant differences among the samples from region A. The greater concentrations of OCP observed in this region was primarily due to HCHs, which comprised more than 90% of the total OCPs concentration. The dominant congener was α-HCH with about 80% and 40–60% contribution to the sum of HCHs in the gas and particulate fractions, respectively. The greatest concentrations of HCHs were found at site A3 located near the Spolana chemical plant reaching 1.1×10^2 ng m^{-3} in the gas phase and 6.2 ng m^{-3} in the particulate one, while other sites in region A contained 3- to 50-fold lesser concentrations.

Table 2
Contaminant concentrations and bioassay responses of ash sample extracts (∑PAHs, ∑PCBs, ∑OCPs see Table1; bioTEQ—toxic equivalents of TCDD assessed by bioassay; AE—index of antiestrogenicity; AA—index of anti-androgenicity (reciprocal value of IC_{25}, $[\mu g/ml]^{-1}$); n.d.—not detected)

	Domestic ash	Fly ash
(ng/g)		
∑PAHs	65×10^2	73×10^2
∑PCBs	12×10^2	4.4
∑OCPs	28	11
bioTEQ	28	4.1
$(\mu g/ml)^{-1}$		
AE	n.d.	32×10^{-3}
AA	2.7	2.5

3.2. AhR-mediated activity

AhR-mediated (dioxin-like) activity expressed as bioTEQ determined directly in the H4IIE-*luc* bioassay was observed in all samples of both gas and particulate fractions including those from the reference site. Concentrations of bioTEQ differed among regions (Fig. 2), with greater bioTEQ concentrations in both phases in region B. Concentrations of bioTEQ in the particulate fraction from region B were at least 10-fold greater than those in samples from region A and the reference locality ($p<0.001$). Also concentrations of bioTEQ in the gas phase of region B samples were greater that those of region A and REF. While concentrations of bioTEQ in the gas fractions from region A samples seem to be comparable to or greater than those from the reference location, concentrations of bioTEQ in the particulate fractions for some samples of region A were less than those of from the reference location. BioTEQ were associated predominantly with the particulate fraction of air in region B and the reference location, while concentrations of bioTEQ in both fractions in region A were comparable. Both ash samples displayed measurable concentrations of bioTEQ but the domestic ash sample extract contained seven-time greater bioTEQ than the fly ash (Table 2).

3.3. Anti-estrogenic activity assessment

There was no significant estrogenic activity observed in any of the samples. There were statistically significant differences in antiestrogenic activities among the two regions of interest as well as the reference location. While no statistically significant antiestrogenicity was observed in either fraction of the reference sample, extracts from regions A and B exhibited measurable antiestrogenicity when co-exposed with estradiol (Fig. 3) as well as when exposed alone (data not shown). Anti-estrogenicity was detected in both fractions of most of the samples except samples A6 and B8. Anti-estrogenic activities of samples from region B were at least 10-fold greater than those from region A. This difference was statistically significant for both particulate and gas fractions. The antiestrogenicity was comparable for both fractions of most samples from region B. A positive, statistically significant correlation between bioTEQ and AE in gas phase fractions ($r_s=0.78$; $p<0.001$) and particle fraction ($r_s=0.53$; $p=0.04$) was observed. While domestic ash extract did not produce any significant anti-/estrogenicity, the fly ash sample extract was clearly antiestrogenic (Table 2).

3.4. Anti-androgenic activity assessment

While there were no obvious anti-/androgenic effects of any of the samples in the absence of androgen, some of them caused statistically significant anti-androgenicity in the presence of testosterone. The gas fractions from all sites exhibited anti-androgenicity (Fig. 4).

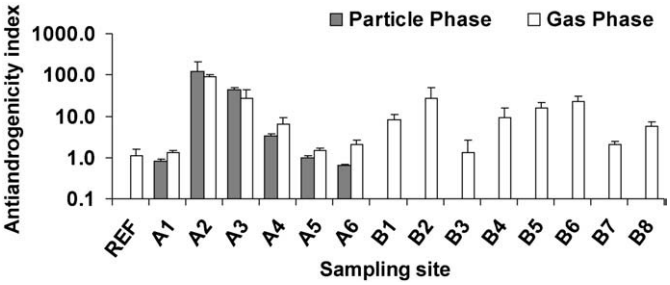

Fig. 4. Index of anti-androgenicity (reciprocal value of IC_{25}, $[m^3/ml]^{-1}$ mean+SEM) of the particle and gas phases of air samples from background locality (REF) and two regions of interest (A, B) assessed in co-exposure with 10 pM testosterone; no column = no significant activity.

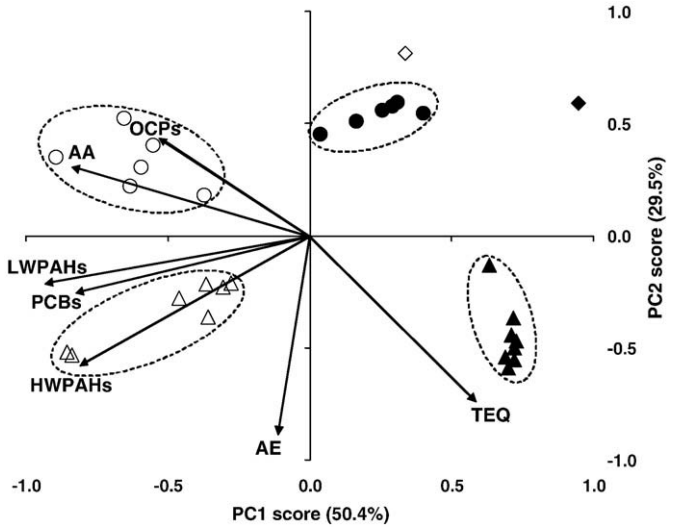

Fig. 5. Biplot presentation of the regional- and air phase-specific pattern of pollution based on the performed biotests and studied contaminants. The samples from different regions are distinguished by shape of the points: circle (region A), triangle (region B), diamond (reference site). The air phases are distinguished by the point pattern: empty (gas phase), full black (particulate phase).

There was no anti-/androgenic activity in extracts of the pre-cleaned polyurethane foam filters (results not shown). There was a region-specific pattern in anti-androgenicity of the particulate phase. Statistically significant effects were observed for the particulate fractions from all locations within region A, while there was no anti-/androgenic activity in any of the particulate fractions from region B or the reference locality. In region A there was more activity in the gas phase than the particulate phase for all samples except for samples 2 and 3, where the activities were similar in both fractions. There were statistically significant intra-region differences. The greatest anti-androgenicity was observed in both fractions of samples A2 and A3 that were nearly 100-fold greater than those of samples A1, A5 and A6. About ten-fold differences in anti-androgenicity were also found among the gas phase samples for region B, with greatest effects caused by samples 2, 5 and 6. Statistically significant anti-androgenicity was observed in both ash samples with similar IC_{25} values (Table 2).

3.5. Interaction with the retinoic signaling pathway

There were no statistically significant effects of either fraction from any of the locations on the RAR activity with or without the addition of 32 nM ATRA (data not shown).

3.6. Multivariate analysis

The PCA analysis accounted for 80% of the total variance and simplified the multivariate pattern and allowed the variables and samples to be projected onto a two dimensional space. When the outcomes of specific bioassays were used together with concentrations of the measured pollutants as master variables, the response patterns separated the samples from the various regions as well as the gas and particulate phases (Fig. 5). The results of bioassays (bioTEQ, AE), which were significantly correlated with PC2 clearly separated the regions, while they did not distinguish between the two phases from region A. Conversely, concentrations of individual residues, primarily PAHs and PCBs and partly anti-androgenic index, which were strongly correlated with PC1, contributed significantly to the separation of the air fractions. Thus, the best characterization and classification of locations and sample types was derived when using combination of instrumental and bio-analytical techniques.

4. Discussion

The reference locality has been very well characterized because it has been monitored since 1988 (Holoubek et al., 2001, 2007) and the results of the previous reports as well as our study indicate that the reference location is relatively clean. The concentrations of the studied contaminants have been established as the general background for the Czech Republic and thus it has been chosen to represent the Central Europe regional background in EMEP (UN-ECE's European Monitoring and Evaluation Programme; Holoubek et al., 2000, 2001). The somewhat greater concentrations of PCBs are due to their historical production, use and releases and are typical for Central Europe (Holoubek et al., 2000). Concentrations of PCBs in both regions A and B are comparable to urban and industrial areas of other European regions (Klanova et al., 2007; Menichini et al., 2007). However, concentrations of PCBs in the gas phase from stations B4 and B8 were relatively great. This is probably because these localities are situated within an industrial area with greater masses of PCBs in soil from the past releases. Concentrations of PAHs in samples from regions A and B were comparable with PAHs concentration in other residential areas of the Czech Republic (Ciganek et al., 2004) and other urbanized regions of Europe, such as Belgium (Du Four et al., 2005) or Sweden (Bostrom et al., 2002). Both target regions as well as the area surrounding the reference location contain relatively intense agriculture, which could explain the concentrations of historically used organochlorine pesticides (OCPs). However, the concentrations of OCPs in region A were approximately 100-fold greater than those in the other areas, which indicates that this region has been contaminated by historical production of OCPs at the Spolana chemical plant. Overall, the chemical analyses suggest that, besides OCPs concentrations in region A, both A and B regions have similar patterns of relative concentrations of the residues quantified in this study and that the concentrations are similar to those observed in other urbanized localities of Central Europe (Ciganek et al., 2004; Holoubek et al., 2000). Concentrations of all of the classes of contaminants were approximately 10-fold greater in the gas phase than in the particulate phase when reported on a volumetric basis. This finding is in agreement with work of Klein et al. (2006) who observed the same trend for PAHs and OCPs and the reverse trend for PCDD/Fs, which were not determined in our study.

Although the relationship between exposure to some air pollutants and non-specific adverse effects such as genotoxicity, oxidative stress or cytotoxicity has been known for some time (for review see de Kok et al., 2006), there is limited information on specific effects of air pollution such as endocrine disruption. There have been several studies describing AhR-mediated effects of the PM_{10} particulate phase fraction of ambient air (Brown et al., 2005; Ciganek et al., 2004; Clemons et al., 1998) or total particulate matter (Ciganek et al., 2004; Hamers et al., 2000; Klein et al., 2006). This type of effect is a useful marker of contamination by dioxin-like compounds, which have been shown to be involved in numerous health effects such as impairment of immunity and nervous system or reproduction (Mukerjee, 1998). The predominant portion of TEQ has been shown to be associated with the PM_{10} fraction and so there were no big differences between dioxin-like activity of PM_{10} or total air extract (both particulate and gas fraction; Ciganek et al., 2004). The study of Klein et al. (2006), which is to our knowledge the only one that has assessed concentrations of TEQ in both the particulate and gas phases, confirmed that the AhR-mediated activity of ambient air is greater in the particulate phase. We observed the same result for samples from the reference locality and region B. However, the AhR-mediated activity, expressed as bioTEQ was comparable in both fractions of region A, which had generally lesser concentrations of bioTEQ.

Interaction of air pollutants with estrogen receptors has been reported previously. Estrogenic activity was found in extracts of the particulate phase of air from several urban localities (Clemons et al., 1998; Klein et al., 2006; Matsumoto et al., 2005; Wang et al., 2004), while there is only one study describing estrogenicity in air gas phase so far (Klein et al., 2006). Because some PAHs have been shown to be estrogenic it has been proposed that the estrogenic activity of extracts was due to the presence of these compounds (Clemons et al., 1998). Alternatively, another report suggested that the estrogenicity of the particulate phase of air could be caused by bisphenol A, which was relatively abundant in the air on the studied urban locality (Matsumoto et al., 2005).

Our results document strong antiestrogenic effects produced by extracts from both particulate and gas fraction of air samples. This divergence in observed effects from above-mentioned studies with ambient air might be explained by differences in contamination patterns at studied localities, type of bioassays applied, or exposure conditions. We used comparable conditions and the observed EC_{50} values (42.7 ± 13.6 pM) for E2 were similar (57 pM; Matsumoto et al., 2005) or even 10-fold less (580 pM; Clemons et al., 1998) as those reported in studies that observed estrogenicity of air particulates

extracts using cell growth and reporter gene assays, respectively, based on the same cell line as our bioassay.

It has been described that extracts from motorcycle exhaust particles, which should at least partly represent traffic-derived air particle contamination, were antiestrogenic both *in vitro* in MCF-7 cell line and *in vivo* in immature female rats (Ueng et al., 2004). Those authors have shown that antiestrogenicity is probably produced by AhR-dependent cytochrome induction, because it can be eliminated by co-treatment with AhR and cytochrome inhibitor α-naphthoflavone. This finding is in agreement with the correlation of bioTEQ and antiestrogenicity observed in our study. The link between dioxin-like activity and antiestrogenicity has been thoroughly described by mechanistic studies reviewed in Safe and Wormke (2003). However, Ohtake et al. (2003) showed that activated AhR can associate with unliganded ER and cause recruitment of co-activators that leads to activation of ER-dependent gene transcription. However, the activity of liganded ER has been inhibited by activated AhR. Thus, it seems that dioxin-like compounds might act as estrogens in absence of ER ligands but they are antiestrogenic in the presence of estrogens (Ohtake et al., 2003). This might play role in the observed differences between our results and results from the previous studies because the other authors did not assess estrogenic effects in presence of estradiol as we did. It might be also possible that even after dialysis and charcoal-stripping the background estradiol concentration in our exposure medium was greater than in the previous studies. Thus, it seems that it is important not to focus only on interaction of potential endocrine disruptors with hormone receptors in basal conditions but also in co-exposure with physiological levels of the respective hormones to obtain results that could be more representative. Moreover, the co-exposure of the model cell line by studied compounds and respective hormones could better simulate situation *in vivo*.

Even though the different levels of estradiol in exposure medium could contribute to the differences in observed anti-/estrogenicity among studies, the major reason is probably the specific contamination pattern in studied localities. There are significant differences in history of use of chemicals (e.g. pesticides) in central Europe compared to the regions of interest that were studied in the other discussed studies (Clemons et al., 1998; Klein et al., 2006; Matsumoto et al., 2005; Wang et al., 2004). Anyway, both the studies describing estrogenicity and/or antiestrogenicity unequivocally show the potential of the air pollutants to interact with ER signaling.

Some studies have shown anti-/androgenicity of diesel exhaust particles (Okamura et al., 2004; Taneda et al., 2004) and emissions from wood combustion (Owens et al., 2006) but to our knowledge, we are the first to observe anti-androgenicity in complex ambient air samples. The anti-androgenicity seems to be due primarily to contaminants present in the gas phase because the gas phase samples from both regions and the reference locality were anti-androgenic (Fig. 4). We have excluded the possibility that this effect could be an artifact caused by some compounds that are part of the filter by exposure of a blank filter extract, thus the observed anti-androgenicity is indeed produced by some compounds present in the gas phase of the air. Some anti-androgenic compounds are present even in the reference locality, which is relatively clean. The anti-androgenic activity of particulate fraction of air has been detected only in samples from region A (Fig. 4). This indicates that the effect could be caused by contaminants specific for the chemical plant, which were not present in the air of the other regions, possibly some OCPs or their degradation products. One potential anti-androgenic compound could be HCH because its α-enantiomer, which is the most persistent form of HCH, has been reported to be anti-androgenic *in vitro* (Schrader and Cooke, 2000).

While there are papers describing effects on retinoid signaling caused by complex extracts from other environmental matrices such as sediments (Novak et al., 2007; Vondracek et al., 2001) or by pulp mill-produced contaminants *in vitro* (Alsop et al., 2003) the fact that no significant effects of air extracts on retinoid signaling were observed makes it probable that the compounds responsible for this effect are not present in sufficient levels in the air.

4.1. Fly ash and domestic ash evaluation

Because air contaminants can originate from combustion processes such as waste burning, indicator pollutant concentrations and bioassay responses for samples from waste burning municipal incinerator and local heating burning mainly coal and some portion of waste were assessed. It has been described previously that soot particles from biomass fuel contain chemicals interacting with ER and progesterone receptor (Wang et al., 2005a). Dioxin-like activity was greater in the domestic ash, while both samples showed comparable antiandrogenic potencies (Table 2) and fly ash sample also weak antiestrogenic effect. Together with results from chemical analyses it indicates that the ashes from domestic furnaces burning also wastes could pose greater danger to the environment than those from incinerators, where the wastes are burned under controlled conditions. However, the data are too limited to make any general conclusion on the topic; they are just indicating the potential of these types of samples to contribute to the specific pollution of the atmosphere.

The bioassay results show differences of the contamination pattern of the two regions of interest (Figs. 2–4). Region A showed distinct anti-androgenic potency in the particulate phase and much greater concentrations of OCPs, which could be potentially related to this effect. On the other hand, the AhR-mediated and antiestrogenic potencies are much more significant in region B, where the pollution level according to the chemical analysis is similar or less than in region A. Thus, bioassays provide additional information that indicates presence of other atmospheric pollutants of possible risk. The specific bioassays were also the major driving forces in separating the samples from different regions in the PCA (Fig. 5). Nonetheless, the best separation of both the regions and phases was in PCA based on the results of both bioassays and chemical analysis, documenting the advantage and necessity of common use of these two approaches in risk assessment of the complex environmental samples.

5. Conclusion

Bioassays are useful tools for assessment of specific effects of airborne pollutants. We have demonstrated that compounds with specific effects are present not only in the particulate phase, but also in the gas phase of air, which could be relatively bioavailable. The study documents the utility of the specific bioassays in indicating the contamination with other pollutants than those that are rutinelly monitored and providing results that clearly separated the regions with different type of pollution. The results emphasize the important role bioassays can play in environmental biomonitoring for the correct risk assessment and the complementarity of the bioassay results with chemical analysis data. The bioassays can monitor for classes of chemicals without identifying each component and can be used in bioassay-directed fractionation and identification schemes and point to previously unidentified compounds that could contribute to the total activity of a class of compounds. However, the data obtained using the *in vitro* bioassays may only indicate the presence of compounds with specific effects and cannot be directly interpolated to the situation *in vivo* because these bioassays do not include the processess such as uptake or metabolisation of the polutants that occur *in vivo*.

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References

Alsop D, Hewitt M, Kohli M, Brown S, Van der Kraak G. Constituents within pulp mill effluent deplete retinoid stores in white sucker and bind to rainbow trout retinoic acid receptors and retinoid X receptors. Environ Toxicol Chem 2003;22:2969–76.

Arrieta DE, Ontiveros CC, Li WW, Garcia JH, Denison MS, McDonald JD, et al. Aryl hydrocarbon receptor-mediated activity of particulate organic matter from the Paso del Norte airshed along the US–Mexico border. Environ Health Persp 2003;111:1299–305.

Bernstein JA, Alexis N, Barnes C, Bernstein IL, Nel A, Peden D, et al. Health effects of air pollution. J Allergy Clin Immunol 2004;114:1116–23.

Bostrom CE, Gerde P, Hanberg A, Jernstrom B, Johansson C, Kyrklund T, et al. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environ Health Persp 2002;110:451–88.

Brown LE, Trought KR, Bailey CI, Clemons JH. 2,3,7,8-TCDD equivalence and mutagenic activity associated with PM10 from three urban locations in New Zealand. Sci Total Environ 2005;349:161–74.

Ciganek M, Neca J, Adamec V, Janosek J, Machala M. A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons. Sci Total Environ 2004;334–35:141–8.

Clemons JH, Allan LM, Marvin CH, Wu Z, McCarry BE, Bryant DW, et al. Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM10 air particulate material using *in vitro* gene expression assays. Environ Sci Technol 1998;32:1853–60.

Curtis L, Rea W, Smith-Willis P, Fenyves E, Pan Y. Adverse health effects of outdoor air pollutants. Environ Int 2006;32:815–30.

de Kok T, Driece HAL, Hogervorst JGF, Briede JJ. Toxicological assessment of ambient and traffic-related particulate matter: a review of recent studies. Mutat Res Rev Mutat Res 2006;613:103–22.

Demirpence E, Duchesne MJ, Badia E, Gagne D, Pons M. MVLN cells — a bioluminescent MCF-7-derived cell-line to study the modulation of estrogenic activity. J Steroid Biochem Mol Biol 1993;46:355–64.

De Rosa M, Zarrilli S, Paesano L, Carbone U, Boggia B, Petretta M, et al. Traffic pollutants affect fertility in men. Hum Reprod 2003;18:1055–61.

Du Four VA, Janssen CR, Brits E, Van Larebeke N. Genotoxic and mutagenic activity of environmental air samples from different rural, urban and industrial sites in Flanders, Belgium. Mutat Res Genet Toxicol Environ Mutagen 2005;588:106–17.

Englert N. Fine particles and human health—a review of epidemiological studies. Toxicol Letters Proceedings of EUROTOX 2003 The XLI European Congress of Toxicology Science for Safety, vol. 149. ; 2004. p. 235–42.

Fernandez P, Grimalt JO, Vilanova RM. Atmospheric gas-particle partitioning of polycyclic aromatic hydrocarbons in high mountain regions of Europe. Environ Sci Technol 2002;36:1162–8.

Freyberger A, Schmuck G. Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol Lett 2005;155:1–13.

Hahn ME. Aryl hydrocarbon receptors: diversity and evolution. Chem Biol Interact 2002;141:131–60.

Hamers T, van Schaardenburg MD, Felzel EC, Murk AJ, Koeman JH. The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution. Sci Total Environ 2000;262:159–74.

Holoubek I, Kočan A, Holoubková I, Hilscherová K, Kohoutek J, Falandyzs J, et al. Persistent, bioacumulative and toxic chemicals in Central and Eastern European Countries. State-of-the-Art Report No. 150a: TOCOEN; 2000.

Holoubek I, Ansorgova A, Shatalov V, Dutchak S, Kohoutek J. Regional background monitoring of PBT compounds — the comparison of the results from measurements and modelling. Environ Sci Pollut Res 2001;8:201–11.

Holoubek I, Klanova J, Jarkovsky J, Kohoutek J. Trends in background levels of persistent organic pollutants at Kosetice observatory, Czech Republic. Part I. Ambient air and wet deposition 1996–2005. J Environ Monit 2007;9:557–63.

Janosek J, Hilscherova K, Blaha L, Holoubek I. Environmental xenobiotics and nuclear receptors—interactions, effects and *in vitro* assessment. Toxicol in Vitro 2006;20:18–37.

Kizu R, Okamura K, Toriba A, Mizokami A, Burnstein KL, Klinge CM, et al. Antiandrogenic activities of diesel exhaust particle extracts in PC3/AR human prostate carcinoma cells. Toxicol Sci 2003;76:299–309.

Klanova J, Kohoutek J, Kostrhounova R, Holoubek I. Are the residents of former Yugoslavia still exposed to elevated PCB levels due to the Balkan wars? Part 1: air sampling in Croatia, Serbia, Bosnia and Herzegovina. Environ Int 2007;33:719–26.

Klein GP, Hodge EM, Diamond ML, Yip A, Dann T, Stem G, et al. Gas-phase ambient air contaminants exhibit significant dioxin-like and estrogen-like activity *in vitro*. Environ Health Persp 2006;114:697–703.

Kotwal SK, Sharma RK, Srivastava AK. Industrial pollutants and animal health — a review. Indian J Anim Sci 2005;75:713–22.

Leskinen P, Michelini E, Picard D, Karp M, Virta M. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 2005;61:259–66.

Li C, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. Anti-androgenic activity of 3-methyl-4-nitrophenol in diesel exhaust particles. Eur J Pharmacol 2006a;543:194–9.

Li CM, Takahashi S, Taneda S, Furuta C, Watanabe G, Suzuki AK, et al. Impairment of testicular function in adult male Japanese quail (*Coturnix japonica*) after a single administration of 3-methyl-4-nitrophenol in diesel exhaust particles. J Endocrinol 2006b;189:555–64.

Lichtenfels A, Gomes JB, Pieri PC, Miraglia SGE, Hallak J, Saldiva PHN. Increased levels of air pollution and a decrease in the human and mouse male-to-female ration in Sao Paulo, Brazil. Fertil Steril 2007;87:230–2.

Mandalakis M, Tsapakis M, Tsoga A, Stephanou EG. Gas-particle concentrations and distribution of aliphatic hydrocarbons, PAHs, PCBs and PCDD/Fs in the atmosphere of Athens (Greece). Atmos Environ 2002;36:4023–35.

Mason GGF. Dioxin-receptor ligands in urban air and vehicle exhaust. Environ Health Persp 1994;102:111–6.

Matsumoto H, Adachi S, Suzuki Y. Bisphenol A in ambient air particulates responsible for the proliferation of MCF-7 human breast cancer cells and its concentration changes over 6 months. Arch Environ Contam Toxicol 2005;48:459–66.

Menichini E, Lacovella N, Monfredini F, Turrio-Baldassarri L. Atmospheric pollution by PAHs, PCDD/Fs and PCBs simultaneously collected at a regional. background site in central Italy and at an urban site in Rome. Chemosphere 2007;69:422–34.

Mohallem SV, Lobo DJD, Pesquero CR, Assuncao JV, de Andre PA, Saldiva PHN, et al. Decreased fertility in mice exposed to environmental air pollution in the city of Sao Paulo. Environ Res 2005;98:196–202.

Mukerjee D. Health impact of polychlorinated dibenzo-*p*-dioxins: a critical review. J Air Waste Manage Assoc 1998;48:157–65.

Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ Sci Technol 2004;38:6271–6.

Novak J, Benisek M, Pachernik J, Janosek J, Sidlova T, Kiviranta H, et al. Interference of contaminated sediment extracts and environmental pollutants with retinoid signaling. Environ Toxicol Chem 2007;26:1591–9.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, et al. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 2003;423:545–50.

Okamura K, Kizu R, Hayakawa K, Toriba A, Mizokami A, Burnstein KL, et al. Variation in the antiandrogenic activity of diesel exhaust particulates emitted under different engine loads. Polycycl Aromat Comp 2004;24:743–57.

Owens CV, Lambright C, Cardon M, Gray LE, Gullett BK, Wilson VS. Detection of androgenic activity in emissions from diesel fuel and biomass combustion. Environ Toxicol Chem 2006;25:2123–31.

Pachernik J, Bryja V, Esner M, Kubala L, Dvorak P, Hampl A. Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: Inhibitory effect of serum. Physiol Res 2005;54:115–22.

Puga A, Tomlinson CR, Xia Y. Ah receptor signals cross-talk with multiple developmental pathways. Biochem Pharmacol 2005;69:199–207.

Ross AC, Zolfaghari R. Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status. J Nutr 2004;134:269S–75S.

Rudel RA, Camann DE, Spengler JD, Korn LR, Brody JG. Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. Environ Sci Technol 2003;37:4543–53.

Safe S, McDougal A. Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers (Review). Int J Oncol 2002;20:1123–8.

Safe S, Wormke M. Inhibitory aryl hydrocarbon receptor-estrogen receptor a cross-talk and mechanisms of action. Chem Res Toxicol 2003;16:807–16.

Schrader TJ, Cooke GM. Examination of selected food additives and organochlorine food contaminants for androgenic activity *in vitro*. Toxicol Sci 2000;53:278–88.

Simms W, Ross PS. Vitamin A physiology and its application as a biomarker of contaminant-related toxicity in marine mammals: a review. Toxicol Ind Health 2000;16:291–302.

Smith GD, Wilburn C, McCarthy RA. Methoprene photolytic compounds disrupt zebrafish development, producing phenocopies of mutants in the sonic hedgehog signaling pathway. Mar Biotechnol 2003;5:201–12.

Taneda S, Mori Y, Kamata K, Hayashi H, Furuta C, Li CM, et al. Estrogenic and anti-androgenic activity of nitrophenols in diesel exhaust particles (DEP). Biol Pharmacol Bull 2004;27:835–7.

Ueng TH, Wang HW, Huang YP, Hung CC. Antiestrogenic effects of motorcycle exhaust particulate in MCF-7 human breast cancer cells and immature female rats. Arch Environ Contam Toxicol 2004;46:454–62.

Villeneuve DL, Blankenship AL, Giesy JP. Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environ Toxicol Chem 2000;19:2835–43.

Villeneuve DL, Khim JS, Kannan K, Giesy JP. Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. Environ Toxicol 2002;17:128–37.

Vondracek J, Machala M, Minksova K, Blaha L, Murk AJ, Kozubik A, et al. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and *in vitro* bioassay techniques. Environ Toxicol Chem 2001;20:1499–506.

Wang JX, Xie P, Xu Y, Kettrup A, Schramm KW. Differing estrogen activities in the organic phase of air particulate matter collected during sunny and foggy weather in a Chinese city detected by a recombinant yeast bioassay. Atmos Environ 2004;38:6157–66.

Wang JX, Xie P, Kettrup A, Schramm KW. Evaluation of soot particles of biomass fuels with endocrine-modulating activity in yeast-based bioassay. Anal Bioanal Chem 2005a;381:1609–18.

Wang JX, Xie P, Kettrup A, Schramm KW. Inhibition of progesterone receptor activity in recombinant yeast by soot from fossil fuel combustion emissions and air particulate materials. Sci Total Environ 2005b;349:120–8.

Watanabe N, Kurita M. The masculinization of the fetus during pregnancy due to inhalation of diesel exhaust. Environ Health Persp 2001;109:111–9.

Whyte JJ, Schmitt CJ, Tillitt DE. The H4IIE cell bioassay as an indicator of dioxin-like chemicals in wildlife and the environment. Crit Rev Toxicol 2004;34:1–83.

Widerak M, Ghoneim C, Dumontier MF, Quesne M, Corvol MT, Savouret JF. The aryl hydrocarbon receptor activates the retinoic acid receptor alpha through SMRT antagonism. Biochimie 2006;88:387–97.

Yoshida S, Sagai M, Oshio S, Umeda T, Ihara T, Sugamata M, et al. Exposure to diesel exhaust affects the male reproductive system of mice. Int J Androl 1999;22:307–15.