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Characterization of Estrogenic Activity of Riverine Sediments from the Czech Republic

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Abstract. Extracts of sediments from rivers in an industrialized area in the Czech Republic were used to evaluate suitability of a simple *in vitro* bioassay system to detect estrogen receptor (ER)-mediated activity in the complex mixture. Total estrogenic activity was detected by measuring luciferase activity in a stably transfected cell line containing an estrogen-responsive element linked to a luciferase reporter gene. For appropriate interpretation of ER-mediated activity, the effect of sediment extracts on the cell cytotoxicity was assessed at the same time. All sediment samples elicited considerable estrogenic activity. Fractionation of the extracts along with bioassay testing and subsequent instrumental analysis allowed the estrogenic fractions to be identified. The Florisil fraction, which was intermediate in polarity, was the most estrogenic. Instrumental analysis documented that the concentration of the degradation products of alkylphenol ethoxylates did not occur at sufficient concentrations to account for the estrogenic activity. Mass-balance calculations and testing of fractions confirmed that certain polycyclic aromatic hydrocarbons (PAHs) or their metabolites were the most likely compounds contributing to estrogenicity. Some other compounds, such as PCNs and PAH derivatives, that were present in the first and second fraction were tested for their potential estrogenic activity. Their ER-mediated activity and contribution to the overall responses of the complex extracts were very low. The concentrations of 17β-estradiol present in the bioassay media was an important factor for the evaluation of (anti)estrogenicity of single compound(s) or complex mixtures.

A number of compounds present in the environment have been reported to elicit disrupting effects on normal physiological function of the endocrine system of mammals, fish, birds, reptiles, as well as invertebrates (Ankley *et al.* 1998). Most studies of such effects have focused on individual chemicals at relatively high concentrations. However, in environmental matrices, these chemicals are present as complex mixtures with other compounds, often in low concentrations. Thus, humans and wildlife are exposed to complex mixtures of both artificial and natural chemicals, which may interact to produce additive, greater than additive, or antagonistic effect (Safe *et al.* 1997). Effects of endocrine-disrupting chemicals on animals in the aquatic environment, especially river ecosystems, have been documented (Sumpter and Jobling 1995; Bortone and Davis 1994).

Aquatic sediments serve as a sink for a number of contaminants and thus as an integrative measure of exposure of the aquatic ecosystem. Sediment can contain mixtures of biologically active compounds with different mechanism of action. The bottom-dwelling animals are directly exposed to these chemicals and through them the pollutants can enter aquatic food chains. In addition, contaminants in sediments can directly affect micro- and meiobenthic communities.

Endocrine disruptors (EDs) present in sediments can have a variety of structures, and thus, their analytical determination would be daunting. Moreover, for a number of compounds, the endocrine-disrupting potency is unknown. Thus, the analytical determination of total ED activity of the complex mixture is not possible at this point. Some integrative measures of exposure are needed to determine endocrine-disrupting potential of complex mixtures. Several basic mechanisms exist for endocrine disruption, including receptor-mediated mechanism (ligand agonists and antagonists), inhibition of synthesis, inhibition or acceleration of metabolism of endogenous hormones. Despite their various structures, a number of chemicals can elicit effects via a mode of action similar to estrogen. *In vitro* recombinant cell bioassays, in which a reporter gene is under the control of receptor binding, enable estimation of the total receptor-mediated activity of samples and also account for possible interactions between compounds in the mixture (Joyeux *et al.* 1997). In this way estrogenic compounds, which are defined as compounds producing effects that are mediated through the estrogen receptor, can be characterized (Gillesby and Zacharewski 1998; Zacharewski 1997). The complex mixture of contaminants present in environmental matrices includes both estrogenic and antiestrogenic components. Effects of such mixtures can be determined by the relative contribution

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of each type of estrogen receptor (ER)-active compound and the nature of their interactions (Kramer and Giesy 1995).

Estrogenic activity has been previously detected in complex extracts from environmental samples, such as pulp and paper mill sludge and effluents (Koistinen *et al.* 1998) or particulate matter in air (Clemons *et al.* 1998). In most studies, the active agents have not been identified. Identification of causative agents is complicated due to complex composition of the samples. A useful strategy for determining the causative agents is the toxicant identification and evaluation (TIE) approach, including fractionation of the active extract (Hilscherova *et al.* 2000). Fractionation enables to separate groups of compounds with different characteristics. In the active fractions the causative agents can be identified by more specific chemical analysis.

There are no previous records of the endocrine-disrupting potential of contaminants present as complex mixtures in sediments of Czech rivers. The objectives of this study were (1) to determine potential estrogenic chemicals in sediments from an industrial area, (2) to examine the utility of *in vitro* recombinant cell line system for screening sediments, and (3) to estimate estrogenic or antiestrogenic potency of sediments. Other goals include comparison of the responses of the whole extracts at different concentrations of 17β-estradiol in the medium and assessment of the effects of sediment extracts on cytotoxicity and protein content of the cells. Sediment extracts were fractionated based on the polarity and tested on bioassays and instrumental analysis to determine the classes of compounds responsible for the (anti)estrogenic activity. Limited mass-balance calculations were performed to determine the proportion of the estrogenicity accounted for by the analyzed compounds of known potency. Relative potencies of some of the chemicals present in the active fractions were determined.

Materials and Methods

Complete details of the sample collection, processing, extraction, and fractionation procedure have been described in a previous study (Hilscherova *et al.* 2001). Surface sediments (top 5 cm layer) were collected in the Czech Republic from Rivers Morava, Drevnice, and Drevnice's tributaries in an industrial region of the Czech Republic (Figure 1). Seven sediments were collected in October 1996 (samples B = before floods), and six were collected in October 1997 (samples A = after floods). Dry sediments were homogenized, and 20 g of the sediment fraction < 2 mm were Soxhlet extracted for 16 h with dichlormethane (DCM; Burdick & Jackson, Muskegon, MI), and the extracts were fractionated into three fractions of different polarity by use of a Florisil column (Khim *et al.* 1999). The first fraction (F1), eluted with 90 ml high-purity hexane (Burdick & Jackson), contained polychlorinated biphenyls (PCBs), a portion of polychlorinated dioxins/furans (PCDD/DFs), and n-alkanes. The second fraction (F2) containing polycyclic aromatic hydrocarbons (PAHs), organochlorine (OC) pesticides, alkylphenols (APs), and rest of PCDD/DFs was eluted with 100 ml 20% DCM in hexane. Polychlorinated naphthalenes (PCNs) eluted in both F1 and F2. The third fraction eluted with 100 ml 100% DCM contained polar metabolites and sterols.

Instrumental Analysis

Analysis of PCDD/DFs, PCBs, and PAHs has been described in detail previously (Hilscherova *et al.* 2001). The concentrations of 16 U.S.EPA priority pollutant PAHs were determined. Alkylphenols and OC pesticides in F2 were determined following the method described (Khim *et al.* 1999). Reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection was used to quantify nonylphenol (NP) and octylphenol (OP). Samples and standards were injected (10 μl) by a Perkin Elmer Series 200 autosampler (Perkin Elmer, Norwalk, CT) onto an analytical column, Prodigy™ ODS (3), 250 × 4.6 mm column (Phenomenex, Torrance, CA), which was connected to a guard column Prodigy ODS (3), 30 × 4.6 mm and eluted with a flow of acetonitril (ACN) and water at a gradient from 50% ACN in water to 98% ACN in water delivered by Perkin Elmer Series 200 pump for 20 min. Detection was accomplished using a Hewlett Packard 1046A fluorescence detector (Hewlett-Packard, Wilmington, DE) with an excitation wavelength of 229 nm and an emission wavelength of 310 nm. NP and OP detection limits for the analytical method were 1 ng/g on a dry weight basis (DW).

Concentrations of OC pesticides were determined using a Hewlett Packard 5890 series II gas chromatograph equipped with a capillary column HP-5 (Hewlett Packard; 50 m length × 0.2 mm ID) coated at a film thickness of 0.33 μm and with an electron capture detector (GC/ECD). Hydrogen was used as the carrier gas with a constant flow (1.3 ml/min). Injection volume of 1 μl was made splitless. Injector and detector temperatures were set at 280°C and 310°C. The column oven temperature was programmed as described previously (Khim *et al.* 1999). Detection limits were 0.02 ng/g DW for HCB and HCH congeners and 0.1 ng/g DW for other compounds.

Cell Line and Cell Culture Conditions

A bioassay based on a human breast cancer cell line MCF-7 stably transfected with a reporter gene, allowing expression of the firefly luciferase enzyme under control of the estrogen-regulatory element was used (Pons *et al.* 1990). The cells were obtained from Dr. Michel Pons, Institut National de la Sante et la Recherche Medicale, Montpelier, France. MCF-7-luc cells (MVLN) were grown in Dulbecco's modified Eagle medium with Hams F-12 nutrient mixture (Sigma D-2906) supplemented with $NaHCO_3$, 1 mM sodium pyruvate (Sigma), 1 μg/ml insulin (Sigma I-1882). For culturing the cells on 100-mm plates 10% of defined fetal bovine serum (FBS; Hyclone, Logan, UT) was added to media. For bioassays in 96-well plates 5% charcoal-stripped FBS (Hyclone) with lesser background for 17β-estradiol ($E_2 < 5$ pg/ml) was used. The cells were cultivated until almost confluent with 10 ml media at 37°C in humidified CO_2 incubator, 5/95% CO_2/air, $> 90\%$ humidity. For bioassays cells were plated in 96-well culture ViewPlates (Packard Instruments, Meriden, CT) at a density of 15,000 cells in 250 μl media. Cells were dosed 24 h after plating in triplicate with 1.25 μl extract solution; the final concentration of solvent (DCM) was 0.5%. At least three separate standard calibrations with concentrations of 0.15 to 500 pM 17β-estradiol (E_2) were used. There were always at least three replicates of blank without any treatment and solvent control on every plate. The exposure time for all bioassays was 72 h. Each sample was dosed in six serial dilutions (1:3 diluting step) with three or four replicates per dilution. Two concentrations of the separated fractions (1:1 and 1:3 dilution) were tested on the bioassay with charcoal-stripped media and also in the media with addition of competing endogenous substrate. To examine the antiestrogenic potency of the extracts, 10 pM of E_2 (EC_{20} concentration) was added as a competitive inhibitor of ER binding. The responses were compared to solvent plus 10 pM E_2 as positive control that was run in parallel with the samples. Luciferase activity was determined by measurement of substrate-induced luminescence as described in previous studies (Koistinen *et al.* 1998; Hilscherova *et al.* 2001).

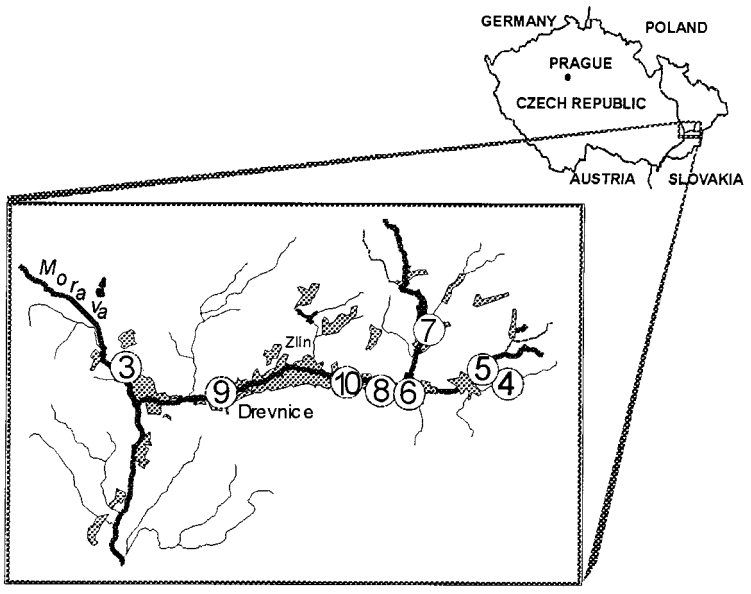

Fig. 1. Location of sampling sites on rivers in the Czech Republic.

Cell Viability Assay

A cell viability index was calculated as a ratio of fluorescence of viable and nonviable cells (Kramer and Giesy 1995; Richter *et al.* 1997). In viable cells the substrate calcein-AM (Molecular Probes, Eugene, OR) was hydrolyzed by esterases to a green fluorescent product, which was detected by fluorescence with excitation and emission wavelengths of 485 and 530 nm, respectively. In dead cells, ethidium bromide (Sigma) can enter cells with damaged membranes and forms a fluorescent product by binding to DNA (excitation 530 nm, emission 645 nm). Ethidium bromide and calcein-AM were added to the incubation media at final concentration of 0.5 μM, plates were incubated at room temperature for 15 min. Fluorescence was measured with a microplate scanning fluorometer, Cytofluor 2300 (Millipore, Bedford, MA).

The protein content was determined by Fluorescamine assay as described previously (Lorenzen and Kennedy 1993; Sanderson *et al.* 1996). The amount of protein per well was calculated based on calibration with standard bovine serum albumin.

Standard Compounds

In addition to the target compounds analyzed in sediments in this study, derivatives of PAHs (methyl and hydroxy PAHs) and PCNs were expected to occur in sediment extracts. Although these compounds were not quantified in sediments, their estrogenic potency was tested in the bioassay to predict their possible contribution to estrogenicity observed in sediments. Because estrogenic potential of PAH derivatives and PCNs have not been reported earlier, this study provided additional information by testing these compounds. The following derivatives of PAHs were tested for their (anti)estrogenic activity with the MCF-7-luc cells and AhR-mediated with the H4IIE-luc cells: 1-methyl-naphthalene (1 CH_3-NAPT), 1,2-dimethyl-napthalene (1,2 CH_3-NAPT), 3,6-dimethyl-phenanthrene (3,6 CH_3-PHE), 9-methyl-anthracene (9 CH_3-ANT), 9,10-dimethylanthracene (9,10 CH_3-ANT), 3,9-dimethyl-benzo(a)anthracene (3,9 CH_3-BaA), 1-methyl-benzo(c-)phenanthrene (1 CH_3-BcPHE), 6-hydroxy-chrysene (6 OH-CHR), 1-hydroxy-pyrene (1 OH-PYR). The standards were obtained from AccuStandard (New Haven, CT) and were greater than 99% purity. All compounds were tested at six different dilutions, with a range of concentrations of 2.5–500 μg/L for hydroxylated PAHs and 0.75 to 250 μg/L for methylated PAHs. The role of competing E_2 in ER-mediated activity of PAHs derivatives was examined by testing at three different levels of E_2: in charcoal-stripped media in which E_2 had been reduced ($E_2 < 0.9$ pM), at the ED_{20} concentration of 10 pM and at the ED_{90} concentration of 170 pM of E_2 concentration. Dilutions of PAH derivatives as well as appropriate E_2 calibrations were prepared in toluene.

Twenty PCNs and six technical mixtures of PCNs (Halowaxes) were screened in MCF-7-luc cells to determine ER-mediated activity (Table 1). For screening purposes two different concentrations were used, the maximum concentration as reported in Table 1 and one-third of this concentration. PCN standards were all high purity (> 93% up to > 99% purity), obtained from different sources (Blankenship *et al.* 2000). Both PCN congeners and E_2 standards were prepared in isooctane and the response was evaluated in charcoal-stripped medium as well as in the presence of 10 pM of E_2.

Data Analysis

Luciferase activity responses in samples were expressed as relative luminescence units (RLU). The viability index, protein content, and microscopic examination were used to evaluate cell condition. When cytotoxicity was observed, those data points were assessed by analyzing the data two ways. First, these values were excluded from the calculations of E_2-EQs. Also, in an attempt to make use of these values where cytotoxicity was observed and extend the linear working range of the data set, the response (estrogenicity) was normalized to the viability index. Nonnormalized data were compared with data normalized to the viability index. Protein normalization was not used for ER-mediated activity, because response induction is correlated with estrogen-induced protein synthesis (Villeneuve *et al.* 1998). The mean solvent control response was subtracted from both standard and sample responses. The significance of response relative to solvent control was evaluated by Student's *t*-test and nonparametric Mann-Whitney test ($\alpha = 0.05$). The EC_{20}, EC_{30}, and EC_{50} concentrations from standard (E_2) dose-response curves were calculated by probit analysis. The dose-

Table 1. ER-mediated activities of polychlorinated napthalenes (PCNs) and Hallowax mixtures tested at two different concentrations of E_2: in charcoal-stripped medium deprived of E_2 (< 0.9 pM) and in medium with addition of 10 pM E_2 (= EC_{20})

PCN Substitution	PCN Congener No.	Highest Tested Dose (ng/well)	Effect	% Solvent Control[a]	
				Stripped Media	10 pM E_2
2,3	10	625	A	80*	89*
1,2,5,6	36	1.25	A	82*	105
2,3,6,7	48	12.5	E	94	116*
1,2,3,5,8	53	12.5	E	113*	117*
1,2,3,4,6,7	66	12.5	A	79*	78*
1,2,3,5,6,8	68	12.5	A	74*	89*
1,2,3,6,7,8	70	12.5	E	94	114*
1,2,3,4,5,6,7	73	1.25	A	85*	63.5*
1,2,3,4,5,6,8	74	12.5	E	105	120*
Halowax 1013		1250	A	80*	78*
Halowax 1014		1250	A	69*	92*
Halowax 1051		1250	A	78*	82*
Halowax 1099		12.5	E	97	128*
Halowax 1001		12.5	A	88*	107

[a] Solvent control = 100%.

Effect: A = antiestrogenic, E = estrogenic. Listed percents of solvent control at the highest tested concentration (*marks significant effects, Mann-Whitney, *t* test, $p < 0.05$).

The following PCN congeners were also tested but did not elicit significant (anti)estrogenic activity: 2-CN; 1,4-DiCN; 1,5-DiCN; 1,2,7-TriCN; 1,2,3,4-TetraCN; 1,2,4,6-TetraCN; 1,2,6,8-TetraCN; 1,2,3,6,7-PentaCN; 1,2,3,5,6,7-HexaCN; 1,2,4,5,6,8-HexaCN; 1,2,3,4,5,6,7,8-OctaCN and Hallowax 1000.

response curves of sediment extracts did not meet the criteria for applying probit analysis, which are equal slope and equal efficacy (maximal induction). Thus, the multiple point estimates method (Villeneueve *et al.* 2000), which enables to account for the nonparallel slopes of the samples dose-response curves, was used for calculations of the estrogenic equivalents per g (ng E_2-EQ/g) sample. Sample responses were converted to a percentage of the mean maximum response observed for the E_2 standard and plotted as a function of log μl sample. Linear regression was applied to the linear part of the log-transformed dose response curve. The concentration producing a response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response of the E_2 standard was calculated and used to determine relative potency.

Simple mass-balance calculation was conducted based on a limited number of compounds to compare the estradiol equivalents (E_2-EQ) from bioassay and analytical results (EEq). An equivalency factor approach was applied where the measured concentrations of individual compounds were multiplied by the appropriate E_2-relative potency values (ERPs) to calculate the analytical estrogenic equivalent (EEq) (Safe 1995). ERPs were previously determined with MCF-7-luc cells for some alkylphenols (Villeneueve *et al.* 1998) and PAHs (Clemons *et al.* 1998). Nonparametric (Spearman) correlation analysis was performed to characterize relationship between E_2-EQ determined from bioassays and those calculated from analytical results. Statistical calculations were conducted by use of the STATISTICA/w 5.0 program (StatSoft, Tulsa, OK).

Results and Discussion

Estrogenic Activity of Sediment Extracts

Complete dose-response relationship was obtained with 0.15–500 pM of 17β-estradiol (E_2) standard. Reproducibility of the replicate standard calibrations exhibited a coefficient of variation (CV) of between 5 and 25%. Overall, the EC_{50} for E_2 was 44.3 ± 11.9 pM (n = 26, CV = 0.27).

Significant induction of luciferase activity was observed with total extracts of all sediments. The DCM extracts represent total extractable organic contaminants present in sediments. As little as 0.1 mg of sediment was sufficient in some samples (5A, 9A, 3B, 6B, 9B) to elicit a significant response. The maximal induction (% E_2-max) caused by extracts was between 30% and 126% of the maximal induction elicited by E_2. Most samples did not reach the maximal efficacy (Figure 2A). Cytotoxicity, as determined by the viability test, was observed at the two greatest concentrations of some sediment extracts tested. Other studies testing environmental extracts, such as air particulate or black liquor from pulp mills also reported dose-dependent induction in ER-mediated activity with maximal induction less than the E_2 maximum (Clemons *et al.* 1998; Balaguer *et al.* 1996). Even though they did not evaluate cytotoxicity, they reported apparent distress of the cells (*i.e.*, spherical morphology) at the greatest concentrations (Balaguer *et al.* 1996).

Multiple point estimates of E_2-EQs were calculated to account for the low level of induction and nonparallel slopes observed for some sample. ER-mediated potency was expressed as the amount of sample causing the same level of response as the EC_{20}, EC_{30}, and EC_{50} of E_2. Due to the unequal slopes and efficacies, point estimates based on different levels of response can vary (Figure 3; Villeneuve *et al.* 1998). However, the shapes of the dose-response curves were similar and the E_2-EQ values based on the point estimates were correlated (Table 2). The E_2-EQs of the whole extracts varied significantly among sites ranging from 10 to 1,200 pg E_2/g sample.

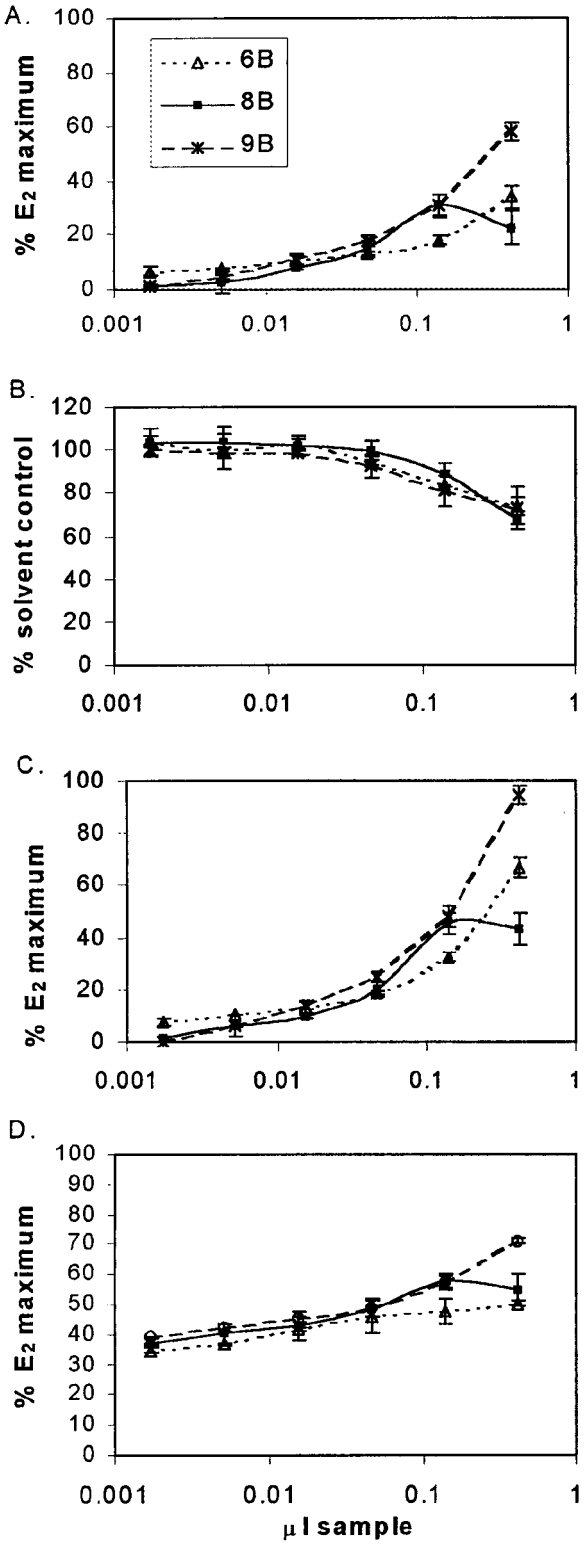

Fig. 2. Examples of dose-response curves for A: luciferase activity, B: viability index, C: luciferase activity normalized to viability index, D: luciferase activity after addition of 10 pM in the media for complex organic sediment extracts. The responses are expressed as % E_2 maximum = percent of induction caused by sample extract relative to maximal induction obtained with E_2 calibration (A, C, D) and as percent of solvent control (for B, solvent control = 100%)

Addition of E_2

A common practice in *in vitro* estrogenicity testing is to perform the assay in media deprived of available steroid hormones. This can maximize the sensitivity for detection of weak estrogens and evaluate the maximal ability of the sample to bind to the ER (Kramer *et al.* 1997). However, under natural body conditions, a certain level of E_2 is always present in exposed animals. To determine the estrogenic potential of the sediment extracts in the presence of competing E_2, the dose-response study was conducted in a charcoal-stripped media (deprived of $E_2 < 0.9$ pM) and also with E_2 addition. In our study, E_2 at a concentration of 10 pM, which was equivalent to the EC_{20}, was added to all samples. The addition of natural ligand did not change the character of response of the complex sediment extracts, all samples still elicited pronounced estrogenic effects even in the presence of E_2 (Figure 2D). The responses at lower sample concentrations were increased by addition of E_2 (Figure 2D), however the maximal fold induction was similar to that without E_2, reaching a maximum between 50% (sample 6B) and 134% of E_2-max (sample 9A). These result document additive effect of the samples and E_2 added at the 10 pM level. The role of E_2 concentrations in the testing media for complex mixtures was documented in a study where treatment of the cells with black liquor from pulp and paper production plus E_2 caused significantly higher induction than any component alone or even higher than the maximal induction caused by E_2 (E_2-max) (Zacharewski *et al.* 1995). The authors suggested that black liquor can potentiate the inducing activity of E_2. Alternatively, as in our results, neither synergistic nor antagonistic interactions were observed in studies of estrogenic activity of organic extract from air particulate matter or methanol-extracted pulp and paper mill effluent fraction after cotreatment with E_2 (Clemons *et al.* 1998; Zacharewski *et al.* 1995).

Cytotoxicity

As mentioned, cytotoxicity could be a significant confounding factor when evaluating (anti)estrogenic effects of complex environmental mixtures. In our study, cytotoxicity was assessed as a viability index, which significantly decreased at the greatest or two greatest concentrations of all extracts (Figure 2B). This observation confirmed the morphological damages to cells observed by microscopic examination. Despite the decrease in the viability index, there was a significant dose-dependent increase in ER-mediated activity in all samples (Figure 2A). To account for cytotoxicity, the results were normalized to the viability index. In samples collected after floods (A), the cytotoxicity was measured separately from the luciferase assay. Averaging of both luciferase activity and viability index and calculation of the ratio of the averages resulted in great variation and did not enable reasonable calculation. Further optimization of the assay enabled sensitive simultaneous measurement of viability index, protein content and luciferase activity in each of the 96 wells. This approach was applied for the B-type (before flood) samples and for these the luciferase responses from each well could be normalized separately for the specific viability index (Figure 2C). E_2-EQs

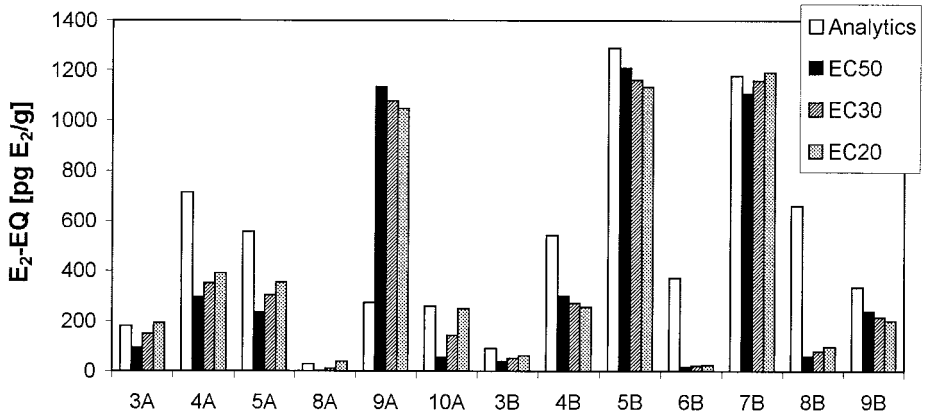

Fig. 3. Estrogenic equivalents (E_2-EQs) in DCM extracts of sediment samples (pg E_2 equivalents/g dry weight) were calculated from the analytical results and from the bioassays as described in the data analysis section. For the bioassays the estrogenic equivalents values were determined as multiple point estimates at levels of response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response produced by the standard (E_2max). For samples 6B and 8B the values based on EC_{50} are approximations because maximal response for these sample did not reach 50% E_2max. The analytical EEq was calculated on limited number of compounds for which E_2-relative potency values (ERPs) are known (some PAHs, alkylphenols) by multiplying the ERP by concentration of the compound. A = sediments sampled after floods (October 1997), B = sediments sampled before floods (October 1996). Numbers refer to sample site

Table 2. Spearman rank order correlations of estrogenic equivalents determined from analytical results and calculated from bioassays (all samples, not normalized for viability index) at levels of response equivalent to 20% (EC_{20}), 30% (EC_{30}), and 50% (EC_{50}) of the maximal response produced by the standard (E_2); *p*-level in parentheses

		Bioassays		
		EC_{20}	EC_{30}	EC_{50}
Analytic	EQ	0.654 (0.015)	0.698 (0.008)	0.654 (0.015)
Bioassays	EC_{20}		0.972 (< 0.001)	0.912 (< 0.001)
	EC_{30}			0.962 (< 0.001)

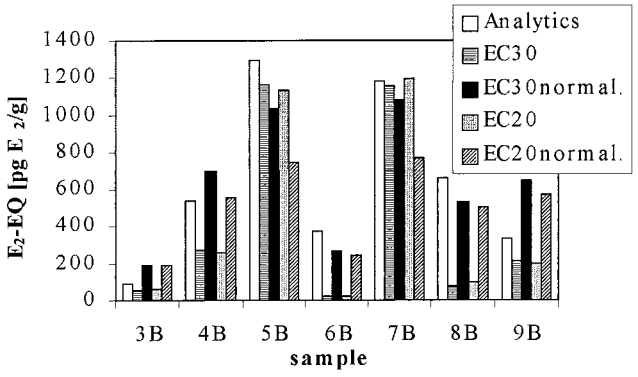

Fig. 4. Effect of normalization of luciferase induction value to viability index. Comparison of E_2-EQs determined from the bioassays raw data and the same data normalized to viability index. The results for two levels of response are compared: 20% (EC_{20}) and 30% (EC_{30}) of the maximal response produced by the standard (E_2max). Sample labels as in Figure 1

for the B samples were compared before and after normalization of the response to viability index (Figure 4). After normalization, the efficacy ranged from 50% to 115% of E_2-max. The E_2-EQs estimated from the normalized data are generally greater than those that were estimated before normalization. Protein content was determined in cells after 72 h of treatment with the extracts (data not shown) as another measure of cell condition. Protein content was correlated with viability index. Decreases of both were observed at the greatest concentrations of extracts. But protein content is a less sensitive measure and more variable than the viability index.

Fractions

Sample extracts were separated on Florisil column into three fractions based on polarity. The effect of concentration of competing E_2 was examined by testing the fractions at two different levels, at 10 pM of E_2 and in charcoal stripped medium (E_2 < 0.9 pM) (Figure 5).

Some of the compounds separated to the first fraction, including PCDDs, PCDFs, and PCBs, are known to elicit effects mediated by the aryl hydrocarbon receptor (AhR). Modulation of endocrine pathways by AhR agonists, such as AhR-mediated antiestrogenicity, has been reported along with complex interactions between ER and AhR signal transduction (Safe 1995; Navas and Segner 1998; Kharat and Saatcioglu 1996). No significant antiestrogenic activity was detected in the first fraction when tested in the E_2 stripped media. However, after addition of E_2 (10 pM) to the media, antiestrogenic activity was observed in the first fraction of some samples (3A, 4A, 9A, 5B). The small effect reflects the low concentrations of OC compounds in samples (Hilscherova *et al.* 2001). However,

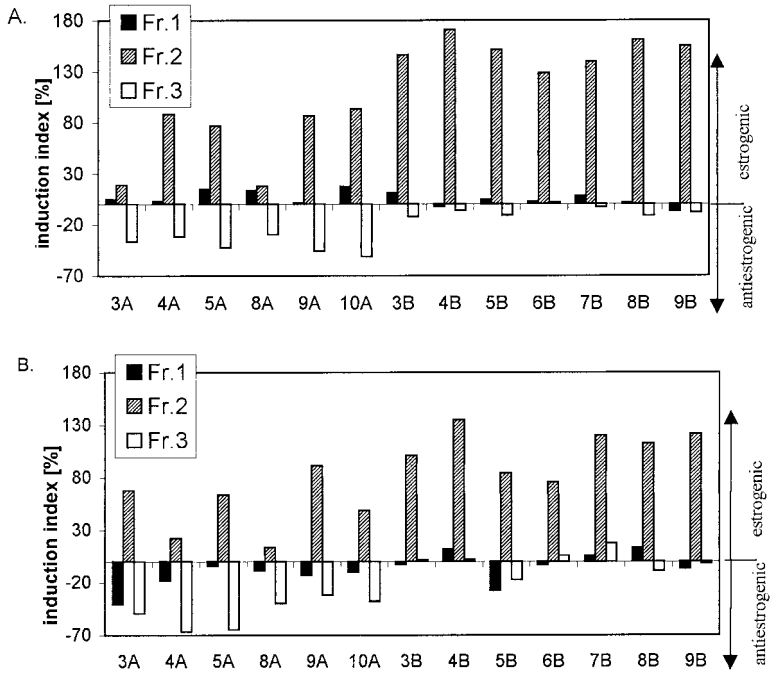

Fig. 5. (Anti)estrogenic activity in the sediment extract fractions. Induction index is defined as the % of sample induction over solvent control minus 100%. Thus, zero value corresponds to the solvent control level, positive values show estrogenic effects, and negative values antiestrogenic effects. A: tested in charcoal-stripped medium deprived of E_2. B: tested in addition of 10 pM E_2 (compared to solvent control with 10 pM E_2). Sample labels as in Figures 1 and 3

some PCBs and their hydroxylated metabolites may act as weak estrogens (Gierty *et al.* 1997; Soto *et al.* 1995; Waller *et al.* 1995). They can contribute slightly to the estrogenic activity in the first fraction and partly compensate the antiestrogenic effects of PCDD/DFs and coplanar PCBs. Mono-*ortho* PCB congeners such as PCB28 or PCB118 and di-*ortho*-congeners PCB52, 101, 138, 153, and 180 were analyzed in the sediment samples (Hilscherova *et al.* 2001). Concentrations of these compounds were relatively low (sum between 14 and 114 ng/g DW).

Significant estrogenic activity was observed in fraction 2, both before and after the addition of E_2. Pesticides that have been shown to elicit weak estrogenic activity, such as *o,p'*-DDT, endosulfan, toxaphene, and chlordecone, elute in this fraction (Khim *et al.* 1999). Another major group of compounds found in this fraction was PAHs. The studies of (anti)estrogenicity of PAHs are equivocal (Navas and Segner 1998). Affinity of certain PAHs for the ER and estrogenic activity has been reported in a study with MCF-7-luc cells (Clemons *et al.* 1998). Other studies documented only antiestrogenic effects of PAHs (Arcaro *et al.* 1999). Alkylphenols, such as NP, which elicit weak estrogenic activity, are also eluted in this fraction.

Antiestrogenicity was apparent in the third fraction, especially in the sediments sampled after floods. The antiestrogenicity was confirmed after the addition of 10 pM of E_2, when some samples caused a decrease of induction to about 35% of the solvent control. The contributors to antiestrogenic effects in the third fraction of A-type samples are probably some polar compounds, which remain to be identified. The antiestrogenic effects may be related to high AhR-mediated (dioxin-like) activity observed in this fraction (Hilscherova *et al.* 2001). An important confounding factor in the detection of antiestrogenicity could be potential cytotoxicity at greater extract concentrations, because reductions in luciferase expression could have been caused by decreased cell viability and inhibition of ER-mediated activities (Kramer *et al.* 1997). However, no obvious decrease in the viability of cells was detected in the third fraction.

To confirm the estrogenic effect of the second fraction and antiestrogenic effects of third fraction, six dilutions of these fractions were tested on bioassay (Figure 6). Both estrogenic activity in fraction 2 and antiestrogenic activity in fraction 3 were observed to be dose-dependent.

Mass-Balance Calculations

Estrogenic equivalents in the mixture are calculated as the sum of the products of the concentrations of individual compounds multiplied by their potency relative to E_2 (ERPs) (Safe 1995). Because not all of the compounds in the mixture could be quantified and ERPs were not available for all of the compounds that were quantified, only limited mass-balance calculations could be performed to determine the relative contribution of the estrogenic compounds analyzed to the total E_2 equivalents. The second fraction that elicited the greatest activity contained, among other compounds, alkylphenols, PAHs, and OC pesticides. For some of these compounds, specific ERPs were available from previous *in vitro* studies with MCF-7-luc cells (Villeneuve *et al.* 1998; Clemons *et al.* 1998).

Alkylphenols, such as NP and OP, have been reported to be estrogenic in both *in vitro* and *in vivo* laboratory studies (Nimrod and Benson 1996; Routledge and Sumpter 1997; Servos 1999). In the river sediments were detected OP and NP, which are degradation products of their corresponding ethoxylates. This is the first report of concentrations of alkylphenols in Czech sediments. The concentrations ranged from 1.7 to 154 ng/g DW (Table 3). All three PAHs for which ERPs are known

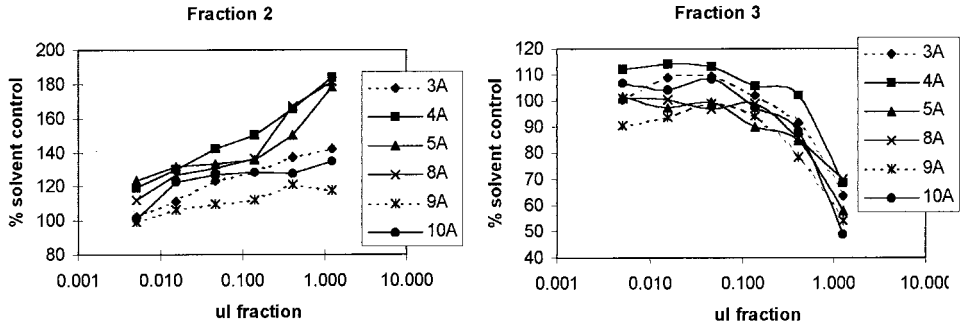

Fig. 6. Dose-response curves of the luciferase activity of the second and third fraction of the sediment extract tested in the stripped media ($E_2 < 0.9$ pM). Sample labels as in Figure 1

Table 3. Alkylphenol and PAH concentrations (ng/g DW) in river sediments and estrogenic equivalents (E_2-EQ, expressed as pg E_2/g DW) contributed by these classes of chemicals (OP = octyphenol, NP = nonylphenol).

	Alkylphenols			PAHs	
Sample	OP	NP	E_2-EQ (pg E_2/g)	Σ PAHs	E_2-EQ (pg E_2/g)
3A	7	43.2	1.12	6,172	177.7
4A	6.8	22.5	0.64	20,060	712.2
5A	8.8	82.9	2.07	16,725	556
8A	1.8	6.4	0.18	1,132	27.4
9A	5.2	137.1	3.27	8,396	269.5
10A	2.2	23.6	0.58	8,778	258.2
3B	4.9	61.6	1.51	3,530	87.9
4B	2	26.5	0.65	16,463	541.3
5B	1.8	9.4	0.25	33,998	1,291.1
6B	2.8	7.1	0.21	11,885	371.7
7B	2.1	23.9	0.59	39,951	1,177.4
8B	5.3	94	2.27	20,395	658.2
9B	3	154.1	3.63	10,530	329.4

(benzo(a)pyrene, benzo(a)anthracene, chrysene) were relatively abundant in the sediment samples. Their concentrations ranged from 21 to 4,260 ng/g DW. ERPs for PAHs were one order of magnitude greater than those for alkylphenols. The concentrations of PAHs were also greater. Therefore, the contribution of PAHs to the calculated EEq was about 98% compared to 2% contributed by alkylphenols (Table 3). F2 also contained small concentrations of some OC pesticides. Studies using multiple assays for assessing estrogenic activity have identified estrogenic potential of some DDT metabolites (*o,p′*-DDT, *o,p′*-DDD, *o,p′*-DDE, *p,p′*-DDE, *p,p′*-DDT) (Soto *et al.* 1995; Klotz *et al.* 1996; Shelby *et al.* 1996; Gaido *et al.* 1997), with ER-affinity approximately 1,000-fold less than 17β-estradiol. The concentration of *p,p*'-DDT was less than the detection limit of 0.1 ng/g in most sediment samples. Concentrations of other OC pesticides were less than 5 ng/gDW. Previous studies have documented the estrogenic activity of some of these OC pesticides at concentrations greater than 1 μg/g (Soto *et al.* 1994). Thus, the relatively small concentrations of these pesticides present in the sediments suggest their contribution to estrogenic potency is insignificant. The mass-balance calculations suggest that PAHs are the primary source of estrogenic activity in the sediments. However, recent studies document that not the parent PAH compounds but their hydroxylated metabolites that are formed during incubation of the cells are probably responsible for big part of the observed estrogenicity (Charles *et al.* 2000). Also, other compounds with unknown estrogenic potency could be present in the complex environmental mixture and contribute to the overall (anti)estrogenic activity.

E_2-EQs calculated from bioassays based on the EC_{20}, EC_{30}, and EC_{50} from the E_2 dose-response and EEqs from analytical results (based on PAHs and alkylphenols) are shown (Figure 3). Concentrations of EEqs were between 28 and 1,178 pg E_2/g DW. E_2-EQs were in good agreement with calculated EEq ($r_{Sp} > 0.65$, $p < 0.05$, see Table 2). In some cases (6B, 8B) the E_2-EQs were significantly less than EEq. This could have been the result of overestimating the relative potencies or due to the antagonistic interactions of other constituents of the mixture. Strong antiestrogenic effects were observed especially in fraction 3 of A samples.

The E_2-EQs for the B samples were also calculated for the data normalized to the viability index. The differences in analytical and bioassay-derived E_2-EQs are much less after normalization to viability index (Figure 4). The absolute values of E_2-EQs were more similar to the EEqs, and the correlation between the estrogenic equivalents derived from bioassays and those that were analytically determined was greater after normalizing the data for viability index. These results suggest that data from bioassays need to be interpreted with caution and cytotoxicity measurement should always be included.

Table 4. Estrogen receptor (ER-)mediated activities of PAHs derivatives

Compound	Abbreviation	ER-mediated activity		
		Stripped Media	10 pM E_2	E_2max
1-methyl-naphthalene	1 CH_3-NAPT	—	E 2.5–75	E 2.5–250
1,2-dimethyl-napthalene	1,2 CH_3-NAPT	A 75–250	—	E 7.5–250
3,6-dimethyl-phenanthrene	3,6 CH_3-PHE	—	E 250	E 7.5–250
9-methyl-anthracene	9 CH_3-ANT	A 25–250	A 250	E 25–250
9,10-dimethylanthracene	9,10 CH_3-ANT	—	E 2.5–75	E 7.5–75
3,9-dimethyl-benzo(a)anthracene	3,9 CH_3-BaA	A 2.5–250	A 75–250	A 25
1-methyl-benzo(c)phenanthrene	1 CH_3-BcPHE	E 7.5–250	E 2.5–75	E 2.5–75
6-hydroxy-chrysene	6 OH-CHR	A 500	—	E2.5–75
1-hydroxy-pyrene	1 OH-PYR	A 500	—	E 7.5–250

ER-mediated activity was tested at three different levels of endogenous substrate E_2: in stripped media deprived of E_2, at 10 pM, and at 170 pM (E_2 max). Observed effects: A = antiestrogenic, E = estrogenic. Listed are only those concentrations (μg/L) where the effects were significant (Mann-Whitney, *t* test, $p < 0.05$)

Model Compounds Tested

As reported, PAHs were the dominant residues in the most active fraction. The results concerning (anti)estrogenic activity of PAHs are ambiguous. There are structural similarities between PAHs and some steroids. It has been documented that, depending on dose and employed assay system, the same chemical may elicit both estrogenic and antiestrogenic effects (Santodonato 1997). In most studies only carcinogenic PAH congeners with four or more rings (four-plus congeners) have been assessed. These PAHs have been reported to be either weakly estrogenic or antiestrogenic. Some studies have reported antiestrogenicity of some PAHs with AhR-mediated activity (Arcaro *et al.* 1999; Chaloupka *et al.* 1992; Tran *et al.* 1996). A study of PAHs with MCF-7-luc cells demonstrated that some PAHs, namely benzo(a)pyrene, benzo(a)anthracene, and chrysene, are capable of interacting *in vitro* with the ER and inducing ER-mediated response (Clemons *et al.* 1998). Detailed studies suggested that estrogen-like activity exhibited by benzo(a)pyrene is predominantly produced by its hydroxylated metabolites (Charles *et al.* 2000) and this could possibly apply for other PAHs as well.

In our study, we tested some PAH derivatives with four or fewer rings and with methyl or hydroxyl substitution. These compounds were found in the second fraction of sediment extracts (qualitative data, not shown). Two hydroxylated and seven methylated PAH-derivatives that were examined for their potential (anti)estrogenic activity are listed (Table 4). The results of these studies demonstrated that the concentration of E_2 in the medium is important parameter in the assay (Table 4). All (anti)estrogenic effects caused by studied compounds were small and close to the limit of significance. These effects were not always completely dose-dependent. Significant dose-dependent antiestrogenicity was found only for 3,9 CH_3-BaA at all three levels of E_2. However, the antiestrogenicity was more pronounced without E_2 addition and decreased at greater E_2 concentrations.

Antiestrogenicity was not significant for most dilutions in the presence of 170 pM E_2. A similar trend was observed for other compounds where estrogenic effects were observed at the greater E_2 concentration, even though the compounds were slightly antiestrogenic in the absence of E_2. The (anti)estrogenic potency of the studied compounds is dependent on the compound concentration and concentrations of ER ligands, if any are present. Probably not only E_2 but also other ER ligands within the complex mixtures can influence the (anti)estrogenic potential of PAH derivatives in environmental samples. Previous findings indicated that the endocrine effect of PAHs may be dependent on the concentration ratio of exo- and endoestrogens (Navas and Segner 1998). Also for hydroxylated PCBs tested on MCF-7-luc cells (Kramer *et al.* 1997) the effect depended on the concentration of E_2 in the media, and the antiestrogenicity decreased at higher E_2 concentrations. *In vitro* studies with MCF-7 cells have documented that only those PAHs that bind to the AhR are antiestrogenic (Chaloupka *et al.* 1992). This observation agrees with our results because only 3,9-CH_3-BaA caused greater AhR-mediated effect (results not shown) as well as significant antiestrogenicity.

Other compounds tested for potential (anti)estrogenicity were PCNs (Table 1). PCNs would have eluted in F1 and F2 of the Florisil column fraction. However, we did not quantify these compounds by instrumental techniques. The primary goal in this study is to test for their potential (anti)estrogenicity. The results for active congeners and technical PCN mixtures that elicited significant response on the bioassays are reported (Table 1). The results are expressed as percentage induction relative to solvent control. Slight estrogenic effect was observed for PCN congeners 48, 53, and 74. Compounds that elicited significant antiestrogenic effect were mostly AhR agonists (congeners 66, 68, 73) (Blankenship *et al.* 2000). However, PCN congener 10 elicited response in MCF-7-luc cells but did not elicit AhR activity. All the other tested congeners elicited no significant ER-mediated activity at tested concentrations. Three of the technical PCN preparations (Halowaxes 1013, 1014, 1051) were found to be antiestrogenic. These mixtures also elicited significant AhR-mediated activity (Blankenship *et al.* 2000). Halowax 1001 also exhibited some antiestrogenicity in media from which most of the E_2 had been removed, but it was not confirmed in the addition of E_2. Halowax 1099 caused induction of luciferase only in presence of E_2. Halowaxes 1000, 1001, and 1099 with no or very little activity contained primarily mono-through tetra-CNs. Alternatively, the active preparations consist primarily of higher chlorinated PCNs (tetra-through octa-CNs). The ER-mediated effects of studied

PAH derivatives and PCNs are relatively weak, and because their concentrations in tested sediments are expected to be much lower that those of other active compounds (such as not substituted PAHs), they would not be expected to contribute significantly to the overall estrogenic responses of the complex sediment extracts.

Conclusions

In vitro cell bioassays can serve as sensitive, specific, and rapid bioanalytical tools to characterize receptor-mediated responses in complex environmental mixtures. While examining receptor mediated responses for the environmental samples, it is important to test the effect of extracts on cell condition to avoid misrepresentation of the results due to cytotoxicity. All studied river sediment extracts elicited estrogenic activity. Bioassays of the total extract coupled with the results of assays on individual fractions can account for interactions within complex mixtures that are not possible to consider in conventional chemical residue analysis and also to account for compounds for which the ER-mediated activity is not known. Fractionation of extracts enables to separate classes of compounds based on their different polarities and thus different characteristics. Thus, fractionation assists in characterization of the complex mixtures while assisting in determining the most active classes of compounds. Fractionation along with limited mass-balance calculations suggested an important contribution of PAHs and/or their metabolites to the overall estrogenic activity. The contribution of alkylphenolic compounds was relatively small. The polar compounds causing antiestrogenic activity in F3 have not yet been identified.

The concentrations of E_2 in the assay medium is an important factor in determining (anti)estrogenicity of single compounds and complex mixtures. Substituted PAHs and some PCNs were relatively less potent to ER-mediated effects, and the effects were dependent on the E_2 concentration in the media.

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