

CHARACTERIZATION OF DIOXIN-LIKE ACTIVITY OF SEDIMENTS FROM A CZECH RIVER BASIN

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Abstract—Synthetic organic chemicals are present in environmental compartments as complex mixtures and therefore their potential effects are difficult to predict. In this study, *in vitro* bioassays using wild-type fish and rat hepatoma cell lines and their corresponding recombinant cell systems were used to evaluate 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like activity in extracts of sediments collected from rivers of the Czech Republic. All the sediment extracts elicited statistically significant responses in all the cell lines tested. For most sediment extracts, a complete dose–response relationship was obtained. The maximal efficacy of the samples was between 57 and 143% of the maximal induction elicited by TCDD. Greater responsiveness, sensitivity, and reproducibility were observed for recombinant than wild-type cells. Cell line-specific differences in the sensitivity to compounds present in the complex sediment extracts were observed. The TCDD equivalents (TCDD-EQs) determined from the different cell bioassays were correlated. Greater concentrations of TCDD-EQs were obtained with fish cell lines. The TCDD-EQs calculated from the results of chemical analysis of toxic equivalents (TEQs) were in good agreement with those determined by bioassays; the aryl hydrocarbon receptor (AhR)-effects of the identified chemicals appear to be generally additive. This indicates that most of the TCDD-like activity was accounted for by the compounds identified and quantified by instrumental analysis. Fractionation along with mass-balance calculations allowed identification of the active fractions and classes of compounds. Polycyclic aromatic hydrocarbons (PAHs) were found to be responsible for most of the AhR-mediated activity in sediments.

Keywords—*In vitro* bioassays 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Polycyclic aromatic hydrocarbons
Organochlorines Dioxin-like activity

INTRODUCTION

Sediments serve both as a sink and a source for a number of environmental pollutants, especially hydrophobic organic contaminants [1]. Some hydrophobic organic contaminants have slow rates of degradation and can persist in the environment for long periods of time and tend to bioaccumulate and biomagnify in the food chain [2,3]. In rivers, during certain times of year due to floods or human activities, residues associated with sediments can be resuspended and become bioavailable. Classical chemical analysis of complex mixtures of organic residues present in sediments can be both resource and time intensive. Instrumental quantification methods are available for some compounds, whereas other compounds for which neither methods nor standards are available may not be identified or quantified. Chemical analyses provide little information on the biological effects of complex mixtures, and they do not account for possible interactions between or among individual chemicals. *In vitro* bioassays can be used as a specific chemical detector for complex mixtures. They serve as simple, rapid, and sensitive screening systems for presence of and mutual interactions of chemicals with specific modes of action [4]. The application of instrumental analyses to quantify specific compounds in combination with bioassays to quantify the total activity along with specific fractionation techniques

can be applied to assess the potential effects of complex mixtures and determine putative causative agents [5].

The critical mechanism of toxicity for some hydrophobic organic contaminants is their dioxin-like activity. Dioxin-like compounds elicit a variety of toxic effects in animals, including lethality, teratogenicity, embryotoxicity, carcinogenesis, tumor promotion, and others [6,7]. These chemicals bind to the aryl hydrocarbon receptor (AhR) present in the cytosol; their binding affinity has been related to the incidence and intensity of toxic effects [8,9]. Binding of a ligand to the AhR initiates a cascade of actions leading to enhanced transcription of AhR-regulated genes and increases in activities of AhR-responsive enzymes [10]. In addition to the responses of AhR-responsive enzyme activities, expression of specific reporter genes under control of AhR-mediated transcription can be measured [11]. The use of the AhR reporter gene construct often increases sensitivity of the bioassay and eliminates potential interferences and other limitations of endogenous reporters [5].

Organic compounds known to bind to the AhR include, among others, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DFs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). These compounds are often found together in environmental matrices including sediments. The relative potencies of complex mixtures can be expressed as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalent (EQ) units, determined either by bioassays or cal-

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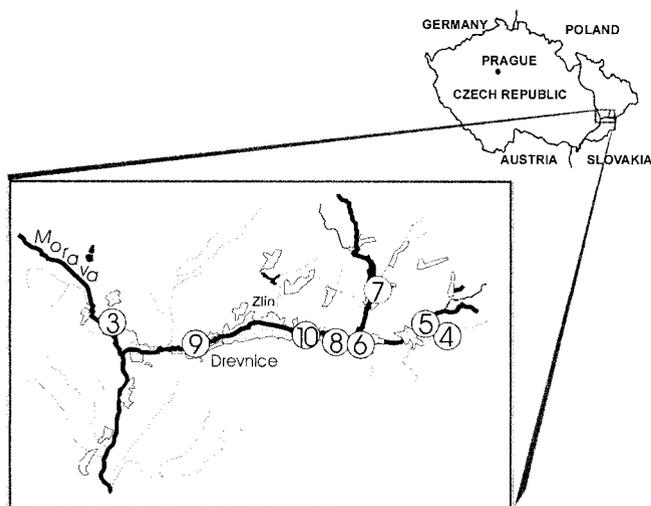


Fig. 1. Map of the Czech Republic, showing sampling locations on rivers.

culated from the concentrations of individual compounds and their relative potency factors (TEFs) [12,13]. For calculations, additivity of the effects of individual chemicals is usually assumed, while bioassays take into account nonadditive interactions such as synergism or antagonism [14].

There are no facilities in the Czech Republic to measure concentrations of dioxins and similar compounds; however, there is a great need to assess contaminated sites and prioritize remediation efforts. The objectives of this study were to test the applicability of *in vitro* cell bioassays for assessment of dioxin-like activity of complex mixture extracts from river sediments. Furthermore, the responsiveness of different cell lines, mammalian versus fish cell lines, and wild-type versus genetically engineered was assessed. After validation of the bioassays by comparison with the results of instrumental analyses, the less expensive but rapid and sensitive bioassay methods could be used to assess sediments for dioxin-like activity. To validate the use of the bioassay, mass-balance calculations were performed by comparison of calculated toxic equivalents based on instrumental analysis and TCDD-EQs based on bioassay results. The TCDD-EQ concentration represents the total AhR-mediated activity as determined in the bioassay. This includes, among other classes of compounds, PCDD/DFs, certain PCB congeners, and PAHs. The assay was used in conjunction with the fractionation to determine the classes of compounds causing the activity, to determine the proportion caused by each fraction, and to account for potential interactions in the complex mixture. The assay was not applied to predict the dioxin-like activity that would be likely to be accumulated into biota and to cause *in vivo* dioxin-like effects. In addition, to evaluate the potential for mobilization of contaminants in sediments, the results of analyses of river sediments collected before and after floods that occurred in the summer of 1997 were compared.

MATERIALS AND METHODS

Sample collection

Surface sediments were collected in the Czech Republic from the Morava River and the Drevnice River and its tributaries (Fig. 1). These rivers are in a narrow valley that contains three industrial cities. Sediments were collected after the floods in October 1997 (denoted by AF). For comparison,

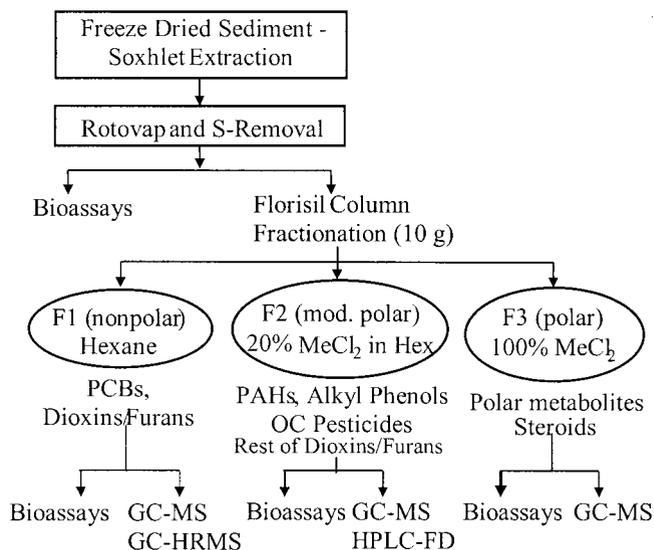


Fig. 2. Sediment extract fractionation and analysis scheme. PCB = polychlorinated biphenyls; PAH = polycyclic aromatic hydrocarbon; GC-MS = gas chromatography-mass spectrometry; GC-HRMS = gas chromatography-high resolution mass spectrometry; HPLC-FD = high-performance liquid chromatography-fluorescence.

sediments collected in October 1996 prior to floods (denoted by BF) were also analyzed. Sediments were collected from the top 5-cm layer by use of a trowel. Large pieces of wood, leaves, and stones, greater than approximately 1 cm, were removed by hand and sediments were freeze dried. Dry sediments were homogenized, ground with a pestle and mortar, and sieved using a 2-mm sieve.

Extraction and fractionation

Twenty grams of sediment were extracted with 400 ml high-purity dichloromethane (DCM; Burdick and Jackson, Muskegon, MI, USA) in a Soxhlet apparatus for 16 h [15]. Sulfur was removed by treatment with acid-activated copper. The extracts were concentrated to approximately 5 ml in a rotary evaporator and then to 1 ml under a gentle stream of nitrogen. The whole extracts were fractionated (Fig. 2) and interferences removed by use of 10 g of activated florisil (60–100 mesh size; Sigma, St. Louis, MO, USA) packed into a glass column (10-mm i.d.). The first fraction (F1), which was eluted with 90 ml of high-purity hexane (Burdick and Jackson), contained PCBs and a portion of PCDD/DFs. Organochlorine (OC) pesticides, PAHs, and a number of their derivatives and the remaining PCDD/DFs and alkylphenolethoxylates were eluted in the second fraction (F2) with 100 ml 20% DCM in hexane. The third fraction (F3) eluted with 100 ml of 100% DCM contained the most polar compounds, including breakdown products of steroids.

Instrumental analysis

The PAHs were quantified by injecting the samples into a Hewlett-Packard 5890 series II gas chromatograph equipped with a 5972 series mass spectrometer detector (GC-MSD) (Avondale, PA, USA). Further details of PAH analysis and quantification are given elsewhere [15]. The PAH standard consisted of 16 components listed by the U.S. Environmental Protection Agency (U.S. EPA, method 8310), including acenaphthene, acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*ghi*]perylene,

benzo[*k*]fluoranthene, chrysene, dibenzo[*a,h*]anthracene, fluoranthene, fluorene, indeno(1,2,3-*cd*)pyrene, naphthalene, phenanthrene, and pyrene. Calibration standards of 0.2 to 5 $\mu\text{g/ml}$ of each PAH were used.

Concentrations of noncoplanar PCBs were determined using a Hewlett-Packard 5890 series II gas chromatograph equipped with a capillary column HP-5 (Hewlett-Packard; 50-m length \times 0.2-mm i.d.), with a film thickness of 0.33 μm and with an electron capture detector (GC-ECD) operated in splitless mode. The temperature program began with an 80°C hold for 1 min, followed by an increase of 20°C/min to 180°C, 3°C/min to 280°C, 20°C/min to 300°C, with a 10-min hold at 300°C. Injector and detector temperatures were 280 and 310°C, respectively. Seven indicator congeners of PCBs (28, 52, 101, 118, 153, 138, 180) were quantified by the internal standard method using calibration standard solutions at concentrations ranging from 0.01 to 4 $\mu\text{g/ml}$. The recoveries of individual PAHs and PCBs were highly consistent among sediment samples from each location.

Concentrations of 2,3,7,8-substituted PCDDs and PCDFs and non-*ortho* coplanar PCBs were measured in F1. The ^{13}C -labeled congeners of TCDD and OCDD were spiked into F1 fractions and treated with concentrated sulfuric acid. The extracts were then passed through 1 g of silica gel impregnated activated carbon to remove interfering compounds. The PCDDs/DFs and non-*ortho* coplanar PCBs were eluted with toluene and analyzed using a high-resolution gas chromatograph and a high-resolution mass spectrometer (HRGC-HRMS). A Hewlett-Packard 6890 series HRGC interfaced with an Autospec Ultima (Vg) HRMS was used. Injection was splitless. A DB-5 (60 m \times 0.25-mm i.d.), at 0.25- μm film thickness, capillary column was used to separate PCDD/DF congeners. The column oven temperature was programmed from 160°C (3 min) to 200°C at a rate of 40°C/min and then to 306°C at a rate of 2°C/min. Injector and ion source temperatures were held at 280 and 250°C, respectively. The mass resolution of the mass spectrometer was greater than 10,000 MU. The mass spectrometer was operated at an electron impact energy of 40 eV. The PCDD/DF congeners and coplanar PCBs were determined by selected ion monitoring (SIM) at the two most intensive ions of the molecular ion cluster. Recoveries of internal standards varied from 55 to 96%. The estimated concentrations were not corrected for recovery. The method detection limit for PCDD/DF congeners was 0.02 $\mu\text{g/g}$ dry weight. A portion of the PCDDs/DFs occurred in F2 of the florisil column fractionation but was not quantified.

Cell lines and cell culture conditions

Exposure to compounds exhibiting aromatic hydrocarbon receptor (AhR)-mediated activity was measured in two ways, i.e., as an increase in 7-ethoxyresorufin-*O*-deethylase (EROD) activity in the wild-type cells [16]; and as an increase in luciferase activity in the recombinant cells. In the wild-type cells, EROD was assessed as a specific measure of the activity of cytochrome P4501A1 that is regulated via the AhR [5]. In the recombinant cell lines, the firefly luciferase gene is under the control of a dioxin-responsive DNA enhancer element. Stimulation of the expression of this gene by AhR agonists was detected by luminiscence. Wild types of the rat hepatoma cells (H4IIE) [16,17] and dessert top minnow (*Poeciliopsis lucida*) hepatoma cells (PHLC-1) [18] were used. Recombinant cell lines derived from the wild-type rat (H4IIE-luc) [17] and rainbow trout hepatoma cell line (RLT2.0) [19,20] by stable trans-

fection with the luciferase gene plasmid under the transcriptional regulation of dioxin-specific enhancers were also used. Cells were cultured by standard procedures developed at the Michigan State University Aquatic Toxicology Laboratory (East Lansing, MI, USA) [16,20]. Cells were cultivated in appropriate media with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at specific temperature in a humidified CO₂ incubator, 5/95% CO₂/air, >90% humidity. The H4IIE-wt and H4IIE-luc cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma D-2902, St. Louis, MO, USA) at 37°C. The PHLC-1 cells were grown in minimal essential medium (α -MEM, Sigma M-3024) and 2 mM L-glutamine (Sigma G-5763) at 30°C. The RLT2.0 cells were grown in Basal Medium Eagle (BME; Life Technologies, Grand Island, NY, USA) with phenol red and 2 mM L-glutamine at 21°C.

For quantification of TCDD-EQ activity, cells were plated in 96-well microplates. RLT2.0 cells were plated at a density of 50,000 cells/well. All other cells were plated at 15,000 cells/well. Cells were preincubated overnight to attach and treated 24 h after plating with standards or extracts in DCM. The final concentration of solvent (dichloromethane) was 0.5%. To determine a dose-response relationship, cells were exposed to six different concentrations of the whole extract (each dilution threefold). Three to four replicates were dosed for each dilution. With every experiment, three separate TCDD calibration standards in DCM (each in three replicates) were measured. Full dose-responses were achieved for standards with final TCDD concentrations between 1.25 and 1,250 pM. After 72 h of exposure, the endpoints measured were EROD activity in H4IIE-wt and PHLC-1 cells and luciferase activity in H4IIE-luc and RLT 2.0 cells. Before measurement, confluent cells were examined microscopically to check for possible cytotoxicity or microbial/fungal contamination. The cell condition was also checked by use of a viability index [19].

Luciferase assay. Culture medium was removed, cells were washed with phosphate buffer saline, and incubated for 20 min with LucLite™ reagent (Packard Instruments, Meriden, CT, USA) at room temperature. Luciferase activity was measured as luminiscence produced at 30°C with a microplate-scanning Dynatech ML 3000 luminometer (Dynatech Laboratories, Chantilly, VA, USA) [16].

EROD assay. CYP1A activity (EROD) was measured by determining the rate of cleavage of 7-ethoxyresorufin to resorufin [16]. Briefly, medium was removed, cells were washed with PBS and lysed by freezing with nanopure water. Cells were then incubated with buffer and 4 μM 7-ethoxyresorufin at 30°C; after 20 min, nicotinamide adenine dinucleotide phosphate (NADPH) was added. After incubating for 1 h at 30°C, the reaction was stopped by addition of fluorescamine in acetonitrile for concurrent determination of protein content [21,22]. The fluorescences of resorufin and fluorescamine-protein adduct were measured simultaneously using a microplate Cytofluor 2300 (Millipore, Bedford, MA, USA) spectrofluorometer at excitation/emission wavelengths of 530/590 nm for resorufin and 400/460 nm for fluorescamine-protein adduct. Bovine serum albumin was used as a standard for quantification of protein.

Data analysis

The EROD activity in wild-type cells was recalculated for the produced resorufin, normalized to protein content, and expressed as mean pmol resorufin/min/mg protein. For recom-

Table 1. Comparison of TCDD-induction of AhR-mediated response in fish and mammalian cell lines ($n = 9$ for every cell line)^a

Cell line	EC50 (pM)	Standard error	CV (%)	Linear working range (pg TCDD/well)	Variability = CV (%) within experiment			Maximal induction factor (max/control)
					TCDD concentration/well (pg)			
					0.3	3	30	
H4IIE-wt	34.5	1.96	9.9	0.1–10	10	7.3	15	4
H4IIE-luc	23.7	1.4	10	0.1–10	11	10	7	15
PHLC-1	52.6	0.95	3.1	0.3–30	7	25	22	5
RLT2.0	74.9	6.3	14.7	1–100	8	24	9	8

^a H4IIE-wt and PHLC-1, measured EROD activity; H4IIE-luc and RLT2.0, measured luciferase activity; CV = coefficient of variation = standard deviation divided by mean, studied at three different TCDD concentrations; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR = aryl hydrocarbon receptor.

binant cell lines, normalization to protein was found to be unnecessary. Wells were inspected to verify that they had approximately the same numbers of cells. Sample responses were expressed as relative luminiscence units (RLU) for the recombinant cell lines. Sensitivities of the cell lines to TCDD were compared by use of TCDD standard dose–response curves; EC50 concentrations were calculated by probit analysis. Due to unequal slopes and efficacies (maximal induction) of the responses, probit analysis could not be used for dose–responses of the sediment extracts. Calculation of multiple point estimates of the TCDD-EQs [23] was used to characterize these responses when slopes and efficacies were not equal. Sample responses were converted to a percentage of the mean maximum response observed for the TCDD standard (TCDD_{max}) and plotted as a function of log μ l sample. Regression equations were derived for the linear portion of each dose–response curve. 2,3,7,8-TCDD equivalents, based on bioassay results (TCDD-EQs), were then calculated from the amount of sample producing a response equivalent to 50% of the maximal response (EC50) produced by the standard (TCDD). To account for the nonparallel slopes, the range of TCDD-EQs based on the level of response produced by EC20 and EC80 of TCDD are presented [23].

For fractions, single point estimates of TCDD-EQs were calculated by projecting the magnitude of induction caused by 1:3 dilutions of the extract fractions on the TCDD standard dose–response curve.

Total concentrations of TEQs based on instrumental analysis were calculated as the sum of TEQs from individual compounds, assuming additive responses to chemicals in the mixture [5]. The TEQs were obtained by multiplying the concentration by appropriate toxic equivalent factor (TEF) or relative potency (RP) values. The human/mammalian World Health Organization (Paris, France) (WHO) toxic equivalency factors (TEF) values were used for PCDD/DFs and PCBs [13]. For PAHs, relative potencies derived from the in vitro H4IIE-wt cell line assays were applied [24].

Because sample sizes were small and in some cases results were not normally distributed, the relationships and/or differences in the EC50 and TCDD-EQ values determined in bioassays were analyzed by nonparametric methods. The statistical significance of differences among EC50 values for TCDD were evaluated by use of the Mann–Whitney test. Relationships among TCDD-EQs derived from different cell lines and TEQs were evaluated by correlation analysis. All statistical analyses were performed with Statgraphics (Rockville, MD, USA).

RESULTS AND DISCUSSION

Comparison of cell lines

Testing conditions. The choice of DCM as the extract vehicle was directed by the extraction and analytical procedure. This solvent was equally as efficient for delivering analytes to cells as other solvents (i.e., isooctane, toluene, hexane) and it did not cause any background response in any of the tested cell lines. During incubation, DCM evaporates quickly, leaving the extracted compounds in the medium. However, due to its relatively great volatility, caution must be taken to keep dosing reproducible. Furthermore, sample dilutions must be stored at low temperature (4°C or less).

The longer exposure time (72 h) was chosen based on our previous studies and for potential metabolization of labile compounds. Time-course studies determined that maximum induction of EROD in H4IIE-wt and of luciferase activity in H4IIE-luc occurred at 72 and 24 h, respectively [16,25]. Induction of luciferase in recombinant fish cells (RLT2.0) is significantly slower than in mammalian cells, with maximal response of RLT2.0 cells to inducing compounds observed after 6 d [19]. The absolute values of EROD induction have been reported to be greater at time periods shorter than 72 h in PHLC-1 cells, with the activity least variable at 72 h [26].

Standard dose–response. The responsiveness of wild-type cells and stably transfected fish or rat hepatoma cells to TCDD standard were compared. The EC50 values in the studied cell lines were calculated from nine replicate measurements and ranged from 23 to 75 pM TCDD as determined by probit analysis (Table 1), with coefficients of variation of up to 15%. Statistically significant differences ($p < 0.05$, Mann–Whitney test) were observed between the EC50 for mammalian and fish cell lines. The EC50 values were greater for fish than for mammalian cell lines. The EC50 value for H4IIE wild-type cells was significantly greater than that for the recombinant H4IIE-luc cells. Other parameters describing the performance of different cell lines are summarized in Table 1. Variability in three different parts of the curve (lower, middle, upper) was less than 25%. The maximal induction factor, defined as the magnitude of induction relative to solvent control, was 1.5-fold to threefold greater for the recombinant cell lines. The linear working range was about 100-fold for all studied cell lines. Earlier studies have reported a threefold greater maximum induction and greater linear working range for recombinant mammalian cells relative to wild-type cells [16,27]. The inhibition of EROD activities by TCDD [11,26] was observed

Table 2. TCDD-like activity of sediment samples 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) determined in *in vitro* bioassays; presented are TCDD-EQs determined by the response equivalency approach at the level of response equivalent to 50% median effective concentration (EC50) of the maximal response produced by the standard TCDD_{max} and the range of TCDD-EQs calculated from responses equivalent to 20% (EC20) and 80% (EC80) of TCDD_{max} (range in parentheses)

Sample	H4IIE-wt	TCDD-EQs in studied cell lines (ng TCDD equivalents/g dry wt)		
		H4IIE-luc	PHLC-1	RLT2.0
3A	2.3 (1.8–3.0 ^a)	4.4 (3.5–5.7)	12.8 (9.3–17)	18.9 (18.5–19.3)
	9.1 (7.2–11.4)	8.6 (7.2–10.4)	27.8 (20.6–30.6)	34.6 (31–38)
4A	2.6 (2.4–2.8 ^a)	7.2 (8.2–6.4)	27 (20.4–30.8)	13.4 (29.7–6.2 ^a)
	1.0 (0.9–1.0 ^a)	1.9 (2.2–1.7 ^a)	6.4 (7.9–5.3 ^a)	2 (1.8–2.3)
8A	5.2 (4.1–6.8 ^a)	6.7 (5.3–8.3)	24.6 (19–31)	14.7 (14.3–15)
	1.9 (1.6–2.2 ^a)	4.6 (4.1–5.1)	19.2 (14.6–24.7)	7.1 (9.0–5.7)
10A	8.7 (6.6–11.5)	16.3 (11.8–22)	35.3 (28.6–35.3)	230 ^b
	17.4 (11.6–26)	23 (15–35.7)	80 (54.6–113.8)	293 ^b

^a Relative potency estimates generated at magnitudes greater than the observed efficacy of sample.

^b Response was over 50% TCDD_{max} at all tested concentrations; the single point estimate is based on 90% TCDD_{max} (EC90).

only at the greatest tested concentration (100 pg/well = 1,250 pM).

Dose-responses for complex mixtures. The bioassays were used as a chemical detector to report TCDD-EQ values for complex mixtures extracted from sediments. The responsiveness of different cell lines to sediment extracts was compared for six sediments sampled after floods in October 1997 (samples AF) and two sediments sampled before floods in October 1996 (samples 5BF, 7BF), which contained greater concentrations of AhR-active residues. All cell types were sufficiently sensitive to determine TCDD-EQs in sediment extracts. Depending on the cell line and sampling site, whole extracts equivalent to as little as 0.03 to 1 mg sediment were sufficient to cause significant induction relative to the solvent control. No significant cytotoxicity was observed except at the greatest dosed concentration (dose equivalent to 25 mg sediment). The wells that exhibited cytotoxicity were excluded from calculations.

Complete dose-response curves were obtained with most whole extracts in all cell lines. However, the efficacy (maximal level of induction) varied among cell lines. Some whole extracts reached efficacies greater than the maximal induction caused by the TCDD standard (TCDD_{max}), whereas other samples did not reach the maximal standard efficacy. In H4IIE-luc cells, all the whole extracts caused approximately the same maximal induction as TCDD. The greatest differences were observed for H4IIE-wt cells, where efficacies ranged from 53% (sample 8AF) to 154% (sample 7BF). This violates the assumption of equal efficacy required for the use of linearized functions to calculate TCDD-EQs [23]. Thus, the values calculated for the samples with variable efficacies are considered to be semiquantitative approximations [28].

Variations of the replicate measurements for samples were relatively small for both recombinant cell lines' coefficients of variation ([CV] generally <20%), but greater variations and some outlier values occurred for wild-type cells. The results

from H4IIE-luc cells were most reproducible with least variability. Greater concentrations of some extracts caused inhibition of EROD activity in both mammalian and fish wild-type cells, suggesting that some compounds in the mixture can act as competitive inhibitors for the induced enzyme [26,11]. Estimation of TCDD-EQs was based on the calculation of the amount of sample needed to produce a response equivalent to EC20 to EC80 of TCDD. The estimate of the range is more appropriate than a single point estimate due to the nonparallelism of the dose-response curves (the lower the range, the more parallel the curves are) [23]. The values based on the response equivalent to EC50 were used for statistical comparison among cell lines and samples.

Caution must be exercised when calculating the TEQs for complex environmental mixtures. The effective concentration (EC) value used as a reference must elicit a response within the linear portion of the log-transformed sample dose-response curve; otherwise, the calculation would lead to significant under- or overestimation. The responses of RLT2.0 cells to sample extracts 5B and 7B were 66 to 120% of TCDD_{max}. Thus, the EC50 for TCDD could not be used for TCDD-EQ calculations. In these cases, the calculation was based on EC90 values, which were within the linear portion of the sample dose-response curves. However, due to the fact that these curves did not reach the same maximum, application of a different basis for calculations may have resulted in overestimation when compared with the other samples.

The dioxin-like activity expressed as ng TEQ/g sediment (TCDD-EQ) was cell-line specific (Table 2). However, while the absolute TCDD-EQ varied among cell lines, there was good correlation among TCDD-EQs of the whole extracts estimated by different cell lines (Table 3). The rank order of potency agreed well among different cell lines, with samples 5BF, 7BF, and 4AF exhibiting the greatest activity and sample 8AF the least. The results from the H4IIE-wt and H4IIE-luc were highly correlated ($R^2 > 0.85$, $p < 0.01$); the TCDD-EQs derived from

Table 3. Coefficients of determination (R^2) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQs) calculated on median effective concentration EC50 values evaluated by the different cell lines and toxic equivalents (TEQs) calculated from the results of chemical analysis (ANALYT)^a

	Cell line			
	H4IIE-WT	H4IIE-LUC	PHLC-1	RLT2.0
H4IIE-LUC	0.88			
PHLC-1	0.88	0.90		
RLT2.0	0.75	0.93	0.75	
ANALYT.	0.88	0.88	0.96	0.79

^a All correlations are significant at $p < 0.05$ ($n = 8$).

the recombinant cells were consistently greater by as much as 2.5-fold. Greater relative potencies of some polyhalogenated aromatic hydrocarbons in the H4IIE-luc cells compared with the wild-type cells have been previously reported [16].

Concentrations of TCDD-EQ determined by fish cell lines were generally greater than those determined by mammalian cells (Mann–Whitney test, $p < 0.05$). As suggested by studies developing species-specific TEFs for organochlorine compounds [20,25], some PCDD and PCDF congeners can have greater relative potency in fish (specifically rainbow trout) cell lines, whereas other compounds, such as mono- and di-*ortho*-substituted PCBs, elicit greater relative potencies in mammalian cells. Relative potencies for hexachloro-dibenzo-*p*-dioxin and dibenzofuran congeners in RLT2.0 cells were 4- to 45-fold greater relative to mammalian cell lines [20]. Other classes of chemicals may also have contributed to the differences. Differences in ligand-binding affinity and ligand specificity among species and tissues have been previously reported [29]. The reasons for cell-line-specific differences in induction include different structure, quantity, and physicochemical properties of the AhR, transcription factors, and other associated proteins [11,25]. Another potentially important factor could be metabolism of the compounds in the mixture. Studies with single compounds have documented faster metabolism of some organochlorines in rat cells compared with fish cells [30].

Concentrations of residues in sediments

Relatively great concentrations of PAHs were measured in all sediments (Table 4). The sum concentrations of 16 PAHs

Table 4. Levels of polycyclic aromatic hydrocarbons (PAHs) [$\mu\text{g/g}$ dry wt], polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) [pg/g dry wt], and non-*ortho*-substituted polychlorinated biphenyls (PCBs) [pg/g dry wt] in sediments; for dioxins/furans, sum concentrations and dominant compound are listed; total PAHs = sum of the 16 PAHs listed in the methods

Sample	Total PAHs	OCDD	Total PCDDs	23478-PeCDF ^a	Total PCDFs	Non- <i>ortho</i> -substituted PCBs				Total
						#81	#77	#126	#169	
3B	3.5	0.88	0.88	0.25	0.25	0.45	4.7	9.6	0.5	15
4B	16.5	0.75	0.95	0.2	0.2	1.1	13	9.2	1.15	25
5B	12.4	0.62	0.7	0.12	0.12	1	11	3.6	<0.02	16
6B	11.9	1.2	1.5	0.18	0.38	1.6	21	29	0.95	52
7B	39.9	0.7	0.7	0.16	0.56	0.96	11	3.6	<0.02	15
8B	20.4	0.8	1.1	0.2	0.2	1.9	31	12	0.93	46
9B	10.5	0.95	0.95	0.58	0.58	4.4	56	24	<0.02	85
3A	61.7	0.62	0.76	0.12	0.2	<0.02	8.9	3.8	<0.02	13
4A	20	1.6	1.8	0.24	0.24	0.72	8.6	3	0.58	13
5A	16.7	0.52	0.56	0.1	0.14	1.2	13	3.7	<0.02	18
8A	11.3	0.62	0.7	0.1	0.1	0.46	4	2.7	1.6	9
9A	8.4	0.54	0.58	0.08	1.16	2.1	27.9	4.5	<0.02	34
10A	8.8	0.52	0.52	0.12	0.16	<0.02	6.3	3.1	0.02	10

^a 23478-PeCDF = 2,3,4,7,8-Pentachlorodibenzofuran.

ranged from 1,132 to 40,000 ng/g dry weight. Pyrene, fluoranthene, and benzo[*b*]fluoranthene occurred at the greatest concentrations. No risk limits for concentrations of PAHs in sediments have been promulgated in the Czech Republic. However, in comparison with maximal permissible concentrations (MPCs, concentrations above which the risk of adverse effects is considered unacceptable) used as risk limits in The Netherlands [31], at least two of the PAHs in each sample, except for sample 8AF, exceeded the limits. In some samples collected before the floods (7BF, 5BF, 4BF), concentrations of most PAHs were greater than the MPCs for sediments.

Concentrations of organochlorine compounds in sediments were relatively low. This is the first study to report concentrations of PCDD/DFs and coplanar PCBs in the study area. Concentrations of the dominant congeners along with the sums for PCDD/DFs and coplanar PCBs are given (Table 4). Concentrations of both PCDDs and PCDFs were generally near the limit of detection of 0.02 pg/g dry weight in F1, with the total concentration less than 2.2 pg/g dry weight. However, PCDD/DFs that eluted in F2 were not analyzed in this study. The concentration of PCDD/Fs in this fraction were generally low (<50% of the concentration in F1). Coplanar PCB concentrations were less than 90 pg/g dry weight, with major contributions from congeners 77 and 126. The sum of other PCBs ranged from 14 to 114 ng/g dry weight. There was no significant difference in concentrations of PCDD/Fs or coplanar PCBs among locations.

Concentrations of TCDD-EQs in sediments

Because the H4IIE-luc bioassay was found to have the greatest sensitivity, least variability, and greater tolerance to cytotoxic effects, this cell line was chosen for detailed studies of all the samples taken before (BF) and after floods (AF). Extracts equivalent to as little as 0.1 mg dry weight sediment were sufficient to cause significant induction in this assay. Complete dose–response curves were obtained for all samples with maximal efficacies between 80 and 150% of the maximal induction observed for TCDD. The TCDD-EQs based on the EC50 response equivalent were used for comparison with analytical results.

To estimate the relative contribution of each analyte identified in the sediment extract to the whole sediment TCDD-EQs, the toxic equivalency quotients for each analyte were

Table 5. Contribution of the identified analytes to the total concentrations of toxic equivalents (TEQs) of whole sediment extracts^a

Sample	TEQs (pg TCDD-equivalent/g dry wt sediment)					
	PAHs	PCDDs	PCDFs	non-ortho PCBs	mono-ortho and di-orthoPCBs	Sum of TEQs
3B	2,803	0.00009	0.12	0.96	0.94	2,805
4B	8,812	0.02	0.1	0.94	1.1	8,814
5B	22,294	0.008	0.06	0.36	0.25	22,295
6B	6,565	0.003	0.1	2.9	0.24	6,570
7B	21,808	0.023	0	0.98	0.7	21,810
8B	12,627	0.23	0.1	1.2	1.2	12,630
9B	5,469	0.0001	0.29	2.4	1.5	5,473
3A	3,752	0.068	0.06	0.38	0.48	3,753
4A	15,072	0.003	0.12	0.31	0.6	15,073
5A	9,039	0.04	0.05	0.38	0.35	9,040
8A	781	0.0009	0.05	0.28	0.19	782
9A	6,382	0.04005	0.051	0.45	0.5	6,383
10A	5,911	0.00005	0.064	0.32	0.37	5,912

^a TEQ = toxic equivalents; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH = polycyclic aromatic hydrocarbons; PCDD = polychlorinated dibenzo-*p*-dioxin; PCDF = polychlorinated dibenzofuran; PCB = polychlorinated biphenyl.

calculated by multiplying the analyte's concentration by its toxic equivalence factor or specific relative potency. Contributions of PCDD/DFs, PCBs, and PAHs to the total TEQs are presented (Table 5). The TEFs used for dioxins, furans, and PCBs were WHO I-TEFs for humans/mammals, which are consensus values based on many different endpoints [13]. They represent overestimates of the actual potency because they are meant to be protective. They were used because this is the most complete source of TEFs available and is widely accepted. None of the organochlorine compounds, including PCBs, PCDDs, and PCDFs, contributed significantly to the total TEQs. Despite the great TEF values of organochlorines relative to RPs determined in the respective bioassays, their contribution was less than 1% of the total TEQs in all sediments. There are no consensus TEF values for PAHs. In this study, we have applied relative potencies determined from *in vitro* bioassays with H4IIE-wt cells [24]. Because TEFs or RPs can vary among cell types, calculated TEQs were interpreted in a semiquantitative manner. The greatest contributions to PAH-TEQs were by benzo[*b*]fluoranthene and benzo[*k*]fluoranthene. Polycyclic aromatic hydrocarbons contributed the greatest proportion of the TEQs due to their relative great concentrations, representing over 99% of the total TEQs. Significant induction of TCDD-like activities by PAHs has been documented in studies testing effects of extracts of air particulate material [32], fly ash samples [33], or semipermeable membrane devices exposed to stream water [34]. Possible concern for comparison of the bioassay and analytical results could be losses of some compounds, namely PAHs, due to metabolization in longer exposure studies. However, recent time-series studies of induction of the cell lines with PAH indicate that there is no loss of potency of PAHs with time. This is probably because the interactions with the AhR, which initiates the responses of the cells, occur very rapidly at the beginning of the incubation (Villeneuve et al., unpublished data).

The TCDD-EQs derived by H4IIE-luc bioassay were significantly correlated with TEQs ($R^2 > 0.8$, $p < 0.01$; see Fig. 3). The differences between TCDD-EQs and TEQs were mostly insignificant, considering the variability in bioassay results, the uncertainty of TEFs, and analytical errors within the assays. Most of the compounds responsible for the AhR-mediated activity have been accounted for. Only in some samples (3B,

8AF) did unknown compounds contribute significantly to the total TEQ. Cases where concentrations of TCDD-EQ were significantly less than those of TEQ suggest possible less-than-additive (antagonistic) interactions among compounds in the mixture. Previous studies have reported that some compounds, especially di-*ortho*-PCBs, can act as antagonists or partial ag-

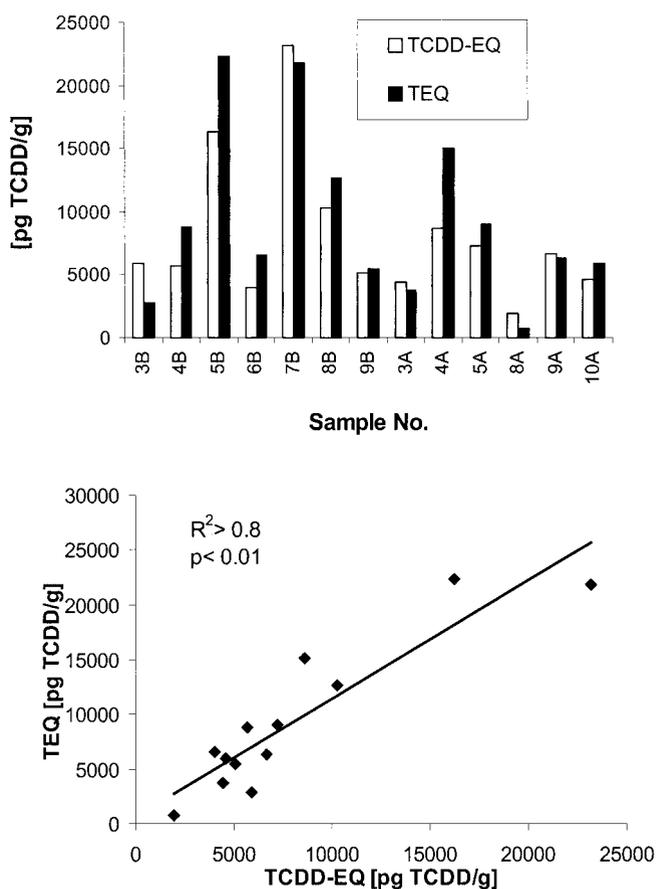


Fig. 3. Relationship between toxic equivalents determined from H4IIE-luc bioassays (2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents [TCDD-EQ] based on response equivalent to 50% [EC₅₀] of the maximal response produced by the standard [TCDD_{max}]) and those calculated from analytical toxic equivalents (TEQ).

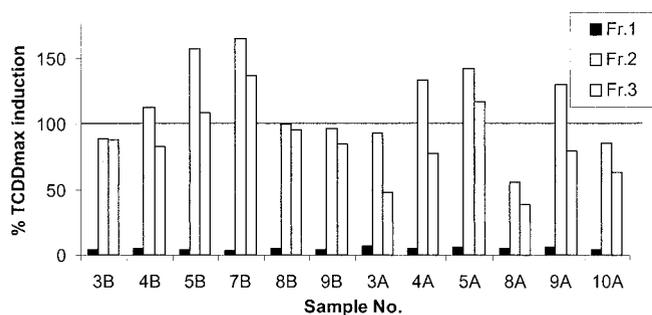


Fig. 4. Luciferase induction in the H4IIE-luc cell bioassay elicited by sediment fractions separated from the whole extract (nondiluted, 1:1). Response magnitude is expressed as percentage of maximal induction caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard (TCDD_{max}).

onists for the AhR, thus reducing the total potency of the mixture [12,13,16].

Fractionation

Compounds in the sediment extracts were separated, based on their polarity, into three different fractions by use of florisil column chromatography (Fig. 2). Two concentrations of each fraction (1:1 and 1:3 dilutions) were then tested with H4IIE-luc cells (Fig. 4). Little activity was observed in the first fraction (F1), with significant responses elicited in 3AF-F1 and most of the BF-F1 samples. The greatest induction was only about 1.5-fold greater than that of the solvent control. The nonpolar compounds eluted in the first fraction include PCBs and some of the PCDD/DFs, compounds with relatively great AhR-mediated activity [7,22,35]. Since the concentrations of these compounds were small, their small contribution to the total TEQs was expected (Tables 4 and 5).

The greatest induction of luciferase activity was caused by compounds in the second fraction (F2) for all samples with magnitudes of induction ranging between 8- and 26-fold above that of the solvent control. Most of the BF-F2 samples and also some AF-F2 samples (5, 4, 9) elicited responses greater than TCDD_{max}. This fraction contained PAHs, their derivatives, organochlorine pesticides, and a portion of the PCDD/DFs. These results confirm the conclusion of the mass-balance calculation, which suggested that PAHs were the major source of TEQs. Also in studies measuring EROD activity induced by Swedish sediment extracts in livers of cultured or *in ovo* injected chicken embryos, the greatest induction was caused by the sediment fraction containing PAHs [36,37]. However, since there may be additional compounds in F2 that could be AhR-active, caution must be exercised in assigning the causality to PAHs as the major contributors to toxicity.

The third fraction (F3) also caused significant induction (5- to 23-fold greater than the solvent control), but the maximal induction was less than that caused by F2. For some samples (5AF, 5BF, 7BF), the magnitude of induction was greater than TCDD_{max}. The compounds responsible for dioxin-like activity in F3 are unknown. They may include polar compounds that are relatively weak AhR agonists, which may be of either natural or synthetic origin. Recently, some studies have suggested additional types of AhR ligands and inducers with a wide structural variety, some of which could have been present in our sediment samples at concentrations sufficient to cause the observed induction [38]. Also, studies with marine sediments have documented the presence of unknown AhR-active

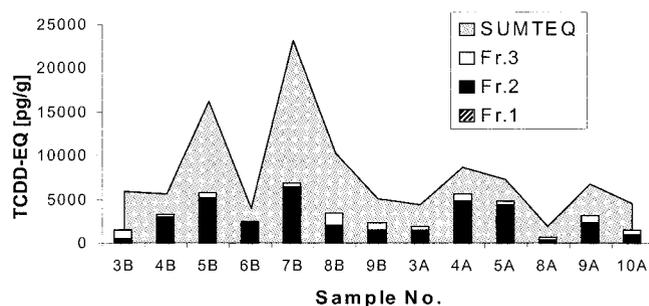


Fig. 5. Comparison of toxic equivalents determined as the sum of the toxic equivalent estimates from the three individual fractions and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) of the whole extract (SUMTEQ).

compounds in the most polar fractions of sediment extracts [39].

Our results demonstrate that the individual fractions can elicit induction as great as the total extract and greater than maximal induction caused by TCDD. Presented results suggest that the interactions within complex mixtures can lead to induction greater than the maximal induction caused by TCDD, standard reference AhR agonist. Maximal responses greater than those caused by TCDD have been reported for complex mixtures [23,39] as well as for single compounds [32]. Some PAHs tested in recombinant mouse cells, benzo[*a*]pyrene in particular, induced a response significantly greater than that caused by TCDD even though their AhR-inducing potency was less than that for TCDD [32]. Benzo[*a*]pyrene was present in all the sediment samples studied.

Semiquantitative estimates of toxic equivalents of individual fractions were determined based on the magnitude of induction calculated from the log-transformed TCDD calibration (Fig. 5). The sum of the toxic equivalents from the three fractions were significantly correlated with TCDD-EQs from the total extract ($R^2 = 0.72$, $p < 0.05$). But sums of the TCDD-EQs from the fractions were significantly less than the TCDD-EQs estimated for total extract. This may suggest synergistic interactions among compounds in different fractions. However, the estimates for the fractions were based on single induction values. The shape of the sample dose-response curve was unknown and the magnitude of induction was directly projected to the standard dose-response curve. Furthermore, the florisil column could have adsorbed some of the compounds that could elicit dioxin-like activity.

Effect of floods on dioxin-like activity in sediments

Comparison of the results for sediment samples collected both in the fall 1996 and of 1997 provided information on the potential effects of great floods that occurred in the studied rivers in the summer of 1997. The results of the comparison of dioxin-like activity between the two sampling periods indicate that there was little change (sites 3, 4, 9) or the TCDD-EQs were significantly less after floods (sites 5, 8). Concentrations of PCDD/DFs were small and did not change significantly. Concentrations of coplanar PCBs were less after the floods. There was no clear trend for concentrations of PAHs, but the apparent decrease that occurred at sites 5 and 8 confirmed the results of the bioassays. Thus, the influence of floods on contaminant concentration in sediments was site specific. Decreases in concentrations at sites 5 and 8 suggest resuspension, transport, and redistribution of contaminated sedi-

ments during floods. Slight increases in concentrations at sites 4 and 9 may be due to increased runoff from the denudation area of the rivers during floods.

CONCLUSIONS

In vitro bioassays proved their applicability for assessment of the dioxin-like activity of complex environmental mixtures. All analyzed sediment samples elicited significant dioxin-like activity in in vitro bioassays. The results correlated well among the cell lines, with greater toxic equivalents for fish than mammalian cell lines. The H4IIE-luc cells were the least variable and most sensitive cell system. Great caution must be taken when calculating the toxic potencies from nonideal dose-response curves obtained from complex mixtures. The mass-balance calculations based on chemical analyses suggested that PAHs can account for a considerable portion of the dioxin-like activity. These results were confirmed by a fractionation approach, where little activity was observed in the first fraction, which contained relatively small concentrations of organochlorine pollutants, and the greatest activity was observed in the second fraction containing PAHs. Detection of significant dioxin-like activity in the third fraction suggests the presence of unidentified polar AhR-active compounds in the sediments. The effect of floods on dioxin-like activity in the sediment is site-specific, with no obvious trend.

Caution must be applied when assessing the risk posed by TCDD-EQs in sediments. The approach presented here is meant to be a screening tool to allow for prioritizing of contaminated sediments for subsequent study including instrumental analyses. For instance, since the fractionation demonstrated that PCDD/DFs and PCBs contributed little to the TEQ, they would not be considered contaminants of concern and there would be no requirement for subsequent analyses of these compounds by use of resource-intensive high-resolution mass spectrometry.

While PAHs can be toxic to benthic invertebrates, they would not be expected to be biomagnified by vertebrates like PCDD/DFs and PCBs are. The results of this study indicate that the use of the H4IIE-luc bioassay in combination with fractionation was effective and accurate and allowed most of the conclusions to be made that would have been made based on extensive instrumental analyses. In this study, the results of the bioassays were verified by instrumental analyses. Therefore, it can be concluded that the approach could be a cost-effective alternative to the more resource-intensive and time-consuming instrumental analyses in initial screening of river sediments.

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