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Cytochrome P4501A Induction and Porphyrin Accumulation in PLHC-1 Fish Cells Exposed to Sediment and Oil Shale Extracts

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Abstract. The present study describes the use of a fish hepatoma cell line (PLHC-1) in monitoring the biological effects of sediments collected from recipient waters of the oil shale industry. Sampling sites were located in River Purtse and River Kohtla in northeast Estonia. The effects of pure oil shale on the PLHC-1 cells were also studied. The cells were exposed to *n*-hexane–extracted samples in 48-well plates for 24 h, and 7-ethoxyresorufin *O*-deethylase (EROD) activity, total protein, and porphyrin content were measured in the exposed cells. Polycyclic aromatic hydrocarbon (PAH) contents in the samples were measured by high-performance liquid chromatography (HPLC). All the sediment and oil shale samples induced CYP1A activity and led to porphyrin accumulation in the cells. The most potent inducers were the sediments collected near the oil shale processing plants (site Lüganuse in River Purtse and Kohtla in River Kohtla), as well as those at the most downstream site in River Purtse (Purtse). These samples possessed high total PAH contents, ranging from 4,270 to nearly 150,000 μg/kg dry sediment. The presence of other lipophilic organic contaminants in the samples was not determined in this study. Both EROD activity and porphyrin content exhibited biphasic induction curves, and the ED_{50}[1] values for EROD activity were lower than the ED_{50}s for porphyrin content. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induction equivalents (TCDD-EQs) calculated from EROD induction potencies correlated well with total PAHs ($r^2 = 0.827$ and $p = 0.003$ for log-transformed data) and also with individual PAHs. TCDD-EQs for porphyrin content did not correlate significantly with total PAHs (log-log $r^2 = 0.785$, $p = 0.116$). The biological potency and PAH contamination of the samples showed the same rank order, except at Lüganuse, where sediment extracts induced CYP1A and porphyrins more than could have been expected based on PAH contents. Bioassay-derived induction EQs (normalized to dibenz(*a*,*h*)anthracene) were 20- to 3,200-fold greater than EQs calculated from the concentrations of five PAHs, suggesting important contributions from other compounds or nonadditive effects. The PLHC-1 cells proved to be a sensitive bioanalytical tool for sediments contaminated with PAH-type pollutants in the oil shale processing area. We suggest further use of this bioassay in screening and monitoring waters with similar background of pollution as in northeast Estonia.

Huge oil shale reserves in northeast Estonia are under continuous mining, combustion, and thermal processing. The active consumption reserve of oil shale is nearly 4 billion tons, of which 872 million tons were mined up to 1995. This extensive production and utilization of oil shale release a number of various pollutants into air, water, and soil. The contamination includes pollutants such as phenols, sulfates, chlorides, sulfites, polycyclic aromatic hydrocarbons (PAHs), heavy metals, and high alkalinity (Liblik and Rätsep 1994; Punning *et al.* 1997).

Among watersheds at the oil-shale area of Estonia, River Purtse with its tributaries is polluted with water from coke-ash dumps of oil shale processing plants (RAS Kiviter and RAS Eesti Kiviõli) and with oil shale drainage water from underground mines Kohtla, Sompa, Tammiku, and Kiviõli, and open-cast pit Aidu. In fact, River Purtse is regarded as one of the most polluted rivers in Europe (Kahru *et al.* 1996). The extreme contamination of River Purtse impacts not only this river and its tributaries but also the Gulf of Finland. There is great concern about the aquatic problems caused by the oil shale industry in River Purtse, and attempts to solve them have been made (*e.g.*, reconstruction of waste water treatment plant in Kohtla-Järve). In spite of this, it may require several years to rehabilitate this river and its tributaries to a more natural and less contaminated condition. Thus, efforts to minimize the contaminant loading and to repair existing environmental damage are needed. Moreover, the abiotic and biotic effects of

[1] ED_{50} = effective dose for 50% of maximal effect, used here for unknown concentrations of compounds; EC_{50} = effective concentration for 50% of maximal effect, for exact chemical concentrations.

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contaminants in the ecosystems of northeast Estonian rivers should be thoroughly investigated and continuously monitored.

The toxicological and various physiological effects of various contaminants in effluents released from oil shale processing activities have been shown in studies with mammals (Nelson *et al.* 1978), bacteria (Kahru *et al.* 1996; Pruul *et al.* 1996; Heinaru *et al.* 1997), and aquatic species (Woodward *et al.* 1985; Tuvikene *et al.* 1999). In addition, the effects of lipophilic organic contaminants in sediments collected from the recipient waters of the oil shale thermal power plants have been detected with a fish hepatoma cell line, PLHC-1, *in vitro* (Huuskonen *et al.* 1998b). The use of the PLHC-1 cells in environmental monitoring is based on the knowledge that some PAHs and halogenated aromatic hydrocarbons (HAHs) are documented to cause Ah receptor–mediated induction of cytochrome P4501A (CYP1A) hemoprotein in this cell line (Hahn *et al.* 1993, 1996; Brüschweiler *et al.* 1996a; Celander *et al.* 1997; Fent and Bätscher 1999). As an indicator of toxicant-induced changes in biological systems, CYP1A induction is regarded as one of the most sensitive and specific biomarkers for hazardous PAHs and HAHs (Stegeman and Hahn 1994; Bucheli and Fent 1995). Because of this, CYP1A has been widely used to assess environmental contamination and health in aquatic environment (reviewed in Bucheli and Fent 1995).

It has also been observed that treatment with planar HAHs can lead to accumulation of porphyrins in the PLHC-1 cells (Hahn and Chandran 1996). HAHs cause a disruption in heme biosynthesis at least in part by stimulating the CYP1A-dependent oxidation of heme precursors (porphyrinogens) to porphyrins (Sinclair *et al.* 1984; Jacobs *et al.* 1989; Lambrecht *et al.* 1992). A relationship between CYP1A induction and porphyrin accumulation in PLHC-1 cells has been suggested; porphyrin accumulation can be either directly or indirectly regulated via the Ah receptor and/or the induced CYP1A (Hahn and Chandran 1996).

The benefits of utilizing PLHC-1 cells or other cells in *in vitro* toxicological research are numerous. The cell culture system allows better control of environmental conditions, eliminates interactive systemic effects, produces highly reproducible results, requires only small amounts of chemicals, and is relatively rapid and inexpensive in use (Baksi and Frazier 1990). Cell cultures could also provide information about the concentration range at which a chemical exerts adverse effects on aquatic organisms (Isomaa and Lilius 1995). This information is necessary in making predictions and establishing safety factors in risk assessment. In addition, cell culture bioassays can provide taxon-specific data (Hahn *et al.* 1993, 1996). For example, data obtained in PLHC-1 cells can have greater relevance to fish than estimates of dioxin equivalents measured in rat hepatoma cells or dioxin equivalents calculated using mammalian toxic equivalency factors (TEFs). Furthermore, PLHC-1 bioassays could be useful for studying the interactive effects of PAHs and/or HAHs in fish.

The objective of our study was to evaluate the use of the PLHC-1 cell line as a bioassay in monitoring the aquatic hazard of oil shale processing and mining. Our interest was in the effects of PAHs, contaminants that are often associated with the use and disposal of petroleum-containing materials or their derivatives. The PLHC-1 cells were exposed to the *n*-hexane extracts of surficial sediments from River Purtse and River Kohtla. Furthermore, a proposed initial source of the hazardous chemicals—oil shale as raw material—was studied. The hypothesis was that the PLHC-1 cells would sensitively respond to the lipophilic organic contaminants in the samples, showing both CYP1A induction and porphyrin accumulation. The results obtained in the PLHC-1 bioassay were compared to the contents of PAHs in the samples.

Materials and Methods

Chemicals and Reagents Used in Bioassay

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD; stock 10 mg/ml in toluene, >98% purity) was obtained from Ultra Scientific (Hope, RI). PAH standards were purchased from Aldrich (Milwaukee, WI). Eagle's Minimum Essential Medium (MEM), calf serum, trypsin, resorufin, bovine serum albumin, and fluorescamine (FA) were purchased from Sigma (St. Louis, MO). 5-Aminolevulinic acid hydrochloride (ALA) was obtained from Aldrich, and uroporphyrin I standards from Porphyrin Products (Logan, UT). All the other reagents were of analytical grade.

Study Areas

Surficial sediments (two separate samples from each study site) were collected from River Purtse and River Kohtla in North-East Estonia (Figure 1). River Purtse and its tributaries River Kohtla and River Erra receive pollutants as releases (quantity of influxes 2.5–2.9 million m^3 per year) from chemical factories (RAS Eesti Kiviõli in the town of Kiviõli in River Erra; RAS Kiviter in the town of Kohtla-Järve in River Kohtla), which process oil shale into shale oil (Liblik and Rätsep 1994). Approximately 70 million tons of solid waste products of semicoking of oil shale are accumulated in big conical ash dumps. Due to the imperfect technology of retorting (semicoking) the solid waste contains harmful components. Ecologically careless handling of the coke-ash dumps makes it possible that toxic compounds flow into the river as runoffs: The main pollution in water is caused by phenols, oil products, sulphides, suspended solids, and high alkalinity. Furthermore, oil shale mines (Kohtla, Sompa, Tammiku, Kiviõli, and Aidu) in River Purtse and its tributaries release sulfates, chlorides, carbonates, oil products, and heavy metals with oil shale drainage water (influx 61–65 million m^3 per year).

As an example of contaminant amounts in waste waters, in 1989–1992 about 840 tons of phenolic compounds, including 210 tons of volatile ones, 35–40 tons of oil products, and 250–300 tons of sulfides have flown into River Purtse annually (Liblik and Rätsep 1994). As air pollution, the stationary pollution sources of the Kohtla-Järve and Kiviõli oil shale processing plants emit annually about 8–11 thousand tons of organic substances, including over 6,000 tons of hydrocarbons, about 1,800 tons of benzene, 900 tons of toluene, and other toxic compounds (phenol, formaldehyde, styrene, ethylbenzene, etc.) (Liblik and Rätsep 1994).

In River Purtse, study sites Lüganuse and Purtse are situated 5 and 10 km, respectively, downstream from RAS Eesti Kiviõli (Figure 1). RAS Eesti Kiviõli releases its effluents first to River Erra and then to River Purtse just before Lüganuse. In addition to waste waters from RAS Eesti Kiviõli, Purtse receives effluents from RAS Kiviter through River Kohtla. In River Kohtla, a study site Kohtla is situated near RAS Kiviter and Püssi 10 km downstream from Kiviter (Figure 1). Reference sediments were not included in the first sampling. However, Ereda sediments were collected as for references 3 km upstream from RAS Kiviter in a separate sampling.

The sediment sampling sites had accumulation bottom with low water current. Water depth was about 0.5 m at the sites. Especially

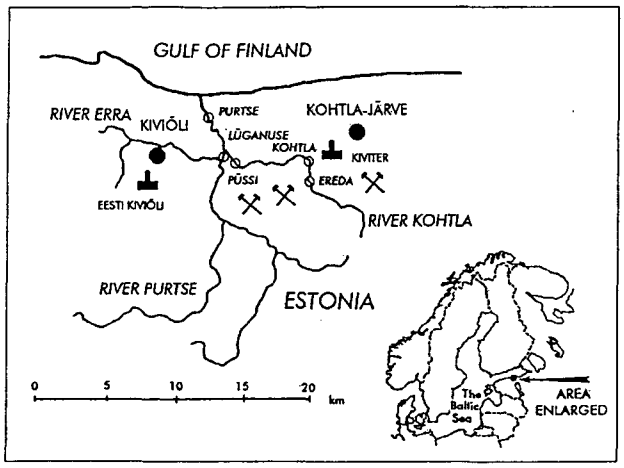

Fig. 1. Study areas of River Purtse (Lüganuse, Purtse) and its tributary River Kohtla (Kohtla, Püssi, Ereda) in northeast Estonia. Ereda is the proposed reference area

Kohtla sediments but also sediments from Lüganuse and Purtse had bad odor. The contaminants affect also higher life in River Purtse and its tributaries. Macroinvertebrate species vanish from some polluted areas during the period of high water level in spring and early summer. Macroinvertebrates appear again in late summer when there is no rain and drainage water from the ash heeps: the number of species increases in the order Kohtla, Püssi, Lüganuse, Purtse and Ereda (H. Timm, unpublished data).

As comparison for the sediments, two samples of pure oil shale were collected from the oil shale mine of Narva.

Sample Collection, Treatment, and Chemical Analyses

The upper layer (5 cm) of sediment was collected with a bottom grabsampler (Ekman modification) and divided into polyethylene boxes. The sediments were stored at 4°C prior to the extractions. Hard piece samples of oil shale were collected into soft plastic bags.

Various sediments and oil shale were first dried at 65°C for 24 h to constant weight, powdered, and then extracted with *n*-hexane to extract a number of lipid-soluble PAHs and HAHs. Dry sample (about 6–9 g) was transferred into glass fiber thimbles that had been preextracted with *n*-hexane. The sample was extracted in a Soxhlet extractor with 200 ml of *n*-hexane for 16 h. The extract was then concentrated to dryness, dissolved in 10 ml of *n*-hexane, and divided into two precleaned glass vials (5 ml in each, one for analyses in PLHC-1 cells and one for PAH analyses). The samples were concentrated to dryness and made up to 1 ml with *n*-hexane. As comparison, a sample containing the extraction solvent itself, *n*-hexane, was treated as the sediments and oil shale.

PAH content (phenanthrene [Phe], anthracene [Ant], fluoranthene [Flu], pyrene [Pyr], benz(*a*)anthracene [BaA], chrysene [Chr], benzo(*e*)pyrene + benzo(*b*)fluoranthene [BeP + BbF], benzo(*k*)fluoranthene [BkF], benzo(*a*)pyrene [BaP], and benzo(*g,h,i*)perylene [BghiP]) was measured by high-performance liquid chromatography (HPLC; model 1311, Minsk, Belorussia). For chromatographic determination of the PAHs, the sample in *n*-hexane was first dried, and the residue was dissolved in 0.2 ml of ethanol or acetone. A mixture of acetonitrile-water 93:7 (by volume) with a flow rate of 8 µl/min was used as an eluting solvent. The run did not last longer than 35 min. Fluorometric detection at two excitation wavelengths (254 nm and 296 nm) was used for identification and quantification. Both external and internal standards were used for HPLC quantitation. The emission range was 330–600 nm. The chromatographic column (0.5 × 300 mm) was filled with Silosorb C18 (Chemopol, Czechoslovakia). The coefficient of variation for the HPLC method was 1.5% (see Trapido and Palm 1991). The detection limit was 2 ng per sample for each PAH.

Prior to the cell exposures, the extracts were concentrated to dryness and dissolved in 0.5 or 1 ml of dimethylsulfoxide (DMSO). No cleanup step for the samples was undergone. Dilutions were prepared in DMSO, and the cells were exposed to the extracts as described below.

Cell Culturing and Treatment

The PLHC-1 fish cell line was derived from a hepatocellular carcinoma of a topminnow (*Poeciliopsis lucida*) (Hightower and Renfro 1988). The PLHC-1 cells were grown as monolayers in 75-cm^2 flasks at 30°C in MEM (containing Earle's salts, nonessential amino acids, L-glutamine, and 10% calf serum). The cells were subcultured as previously described by Hahn *et al.* (1993). The cells used in the exposures of this study were from passages 41 and 42.

The PLHC-1 cells were exposed to the extracts in 48-well plates (the method basically described in Kennedy *et al.* 1995; protocols for PLHC-1 cells in Hahn *et al.* 1996, and Hahn and Chandran 1996). On the first day, 4×10^5 cells in 0.5 ml of medium were placed into the wells. After 24 h at 30°C, the medium was aspirated and replaced with 0.5 ml of fresh medium. The sample extracts in DMSO were added (volume 2.5 µl) to the medium. The plate was incubated at 30°C for 24 h, after which the medium was removed, the wells were washed with 0.5 ml of buffered phosphate saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and 100 µl of sodium phosphate buffer (0.05 M, pH 8.0) was added. The *O*-deethylation of 7-ethoxyresorufin (EROD) was initiated by the addition (100 µl/well) of 7-ethoxyresorufin (final concentration 2 µM) in sodium phosphate buffer, and the reaction was run for 10 min at room temperature (RT). (Resorufin production increases linearly with time under these conditions [Hahn *et al.* 1996]). The reaction was stopped with 150 µl of cold FA solution (150 µg/ml in acetonitrile), and the plate was allowed to sit for 15 min at RT prior to the measurement of fluorescence with a plate reader (Cytofluor 2300 fluorescence plate reader, Millipore, Bedford, MA; wavelengths for excitation and emission filters were 530 nm and 590 nm for EROD activity, and 409 nm and 460 nm for protein content). When accumulation of porphyrins in the cells was measured, 100 µl of 3 mM ALA in 0.9% NaCl was first added to the medium 5 h before EROD and protein analyses. Following the EROD and protein assays, 400 µl of 3 N HCl was added to the wells, the plate was left for 30 min at RT, and the fluorescence of porphyrins was read on the cytofluor (excitation and emission filters 409 nm and 645 nm, respectively). Resorufin, bovine serum albumin, and uroporphyrin I standards were included on each plate (first two columns; see Kennedy *et al.* 1995).

Ereda sediments, which were collected in a separate sampling, and one sample from both Kohtla and Püssi were exposed in one set of exposure. This additional exposure was conducted three times in the facilities of another laboratory as the other cell experiments. Cells used in these exposures had been passed 65–70 times. After the exposures, EROD activity and protein content were analyzed with a Fluoroscan Ascent plate reader (Labsystems, Helsinki, Finland, excitation and emission filters 538 nm and 590 nm for EROD activity and 390 nm and 460 nm for protein content).

TCDD in 2 nM concentration was used as a positive control on each plate. In addition, concentration-response curves for TCDD were detected to obtain EC_{50}s for EROD activity and porphyrin content.

Data Processing

After analyses, fluorescence data was imported into SigmaPlot (Jandel Scientific, San Rafael, CA) for analysis and curve fitting. Data obtained from EROD and porphyrin assays were normalized to total cellular protein and analyzed by nonlinear regression using the curve-fitting subroutine of SigmaPlot. Data were fitted to a modified Gaussian function (Equation 1, for biphasic relationships) or to a logistic function (Equation 2, for sigmoid relationships) as described previously by Kennedy *et al.* (1993).

The Gaussian function was

$$y(d) = Y_b + (Y_m - Y_b)exp\,[-C[\ln(d) - \ln(d_m)]^2] \quad \text{(Eq. 1)}$$

where $C = \ln(2)/[\ln(EC_{50}) - \ln(d_m)]^2$, $y(d)$ is EROD activity or porphyrin content at inducer concentration d, Y_b is basal EROD activity or porphyrin content, Y_m is maximal EROD activity or porphyrin content, d_m is the inducer concentration producing maximal EROD activity or porphyrin content, and EC_{50} is the inducer concentration producing 50% of maximal EROD activity or porphyrin content.

The logistic function was

$$y(d) = Y_b + (Y_m - Y_b)\,[1 + \exp[-g(\ln(d) - \ln(EC_{50}))]]^{-1} \quad \text{(Eq. 2)}$$

where $y(d)$, Y_b, Y_m, and EC_{50} are as for Equation 1, and g is a slope parameter.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induction equivalents (TCDD-EQs) for EROD activity and porphyrin content were calculated according to the formula:

PLHC-1 TCDD-EQ (pg/g)
= [TCDD EC_{50} (pg/ml)]/[extract ED_{50} (mg/ml)] × 1,000 (mg/g)

Bioassay-derived dibenz(*a*,*h*)anthracene induction equivalents (PLHC-1 DBahA-EQs) for EROD activity were calculated respectively, by replacing TCDD EC_{50} with DBahA EC_{50} (Fent and Bätscher 1999; see also Fent *et al.* 1998).

Pearson's bivaric log-log correlation analyses between TCDD-EQ values from biological assays and PAH results and between PLHC-1 bioassay-derived DBahA-EQs and calculated analytical DBahA-EQs were run with SPSS for MS Windows (Release 6.1.3).

Results

PAH Compounds

The order of the PAH contamination in the sediments and oil shale, based on total PAHs, was Kohtla > Purtse > Lüganuse > Püssi > Ereda > oil shale (Table 1). PAH concentrations were especially high in the Kohtla and Purtse sediments (149,100 µg/kg and 74,650 µg/kg in Kohtla and 28,560 µg/kg and 26,310 µg/kg in Purtse). The major contributor to total PAHs was Flu in Kohtla sediments (41% of total PAHs) and BghiP in Purtse sediments (33%). The second most common individual PAH in Kohtla and Purtse sediments was Pyr (25 and 30%, respectively). Pyr was the major PAH in other samples (Lüganuse 55%, Ereda ~ 65%, Püssi and oil shale 30–35%).

Biochemical Analyses

The experiments for measuring EROD activity in the exposed cells were run in two sets (experiments 1 and 2 in tables and figures). In the first experiment, even the lowest doses of most sediment extracts caused EROD induction, and the highest doses of some samples strongly inhibited EROD activity (Table 2). Therefore, the first experiment was used to determine the doses for the second experiment and to establish the doses leading to maximal EROD activities (54 to 82 pmol/min/mg, Table 2). These maximal EROD values were reached at relatively similar doses of Lüganuse, Purtse, and Kohtla sediments (0.081–0.432 mg dry sediment per ml medium); at doses 2.25 and 7.48 mg/ml of Püssi sediments; and at doses 24.9 and 47.3 mg/ml of oil shale.

The second EROD experiment gave similar maximum values as the first one, the peak of EROD activity in the cells ranged from 48 to 86 pmol/min/mg (Table 2, Figure 2A). These peak values were achieved at sediment doses ranging from 0.159 to 0.244 mg/ml at Lüganuse, Purtse, and Kohtla and at doses of 2.25 and 3.74 mg/ml at Püssi. Oil shale increased EROD activity to a maximum of 74 pmol/min/mg at the 24.9 mg/ml dose and 65 pmol/min/mg at 24.7 mg/ml.

Peak porphyrin contents in the cells for the sediment and oil shale extracts ranged from 263 to 445 pmol/mg (Figure 2B). These values were achieved at sediment doses of 0.244, 19.1, 0.162, and 4.49 mg/ml in Lüganuse, Purtse, Kohtla, and Püssi samples, respectively. Oil shale induced porphyrins most at the 49.8 mg/ml dose. As with the EROD results, some of the porphyrin curves were biphasic.

All the presented EROD and porphyrin data were achieved at doses where total protein was not substantially affected: Total protein content in the cells was not decreased more than 24% in the first experiment and more than 27% in the second at any extract doses tested, when compared to the cells treated with pure medium (not shown). The highest sample doses did not reduce total protein content more than 21% or 20% in these two experiments. A comparison of protein yield at different cell densities and culture times has been described by Hahn *et al.* (1996).

Based on the ED_{50}s for both EROD activity and porphyrin content in the second experiment, the rank order potency of the samples was Kohtla (most potent) > Lüganuse > Purtse > Püssi > oil shale (least potent) (Table 3). The ED_{50}s for EROD activity of Lüganuse, Purtse, and Kohtla sediments (0.0186–0.0585 mg/ml) were about one order of magnitude less than the ED_{50}s of Püssi sediments (0.487 and 0.843 mg/ml) and about two orders of magnitude less than the ED_{50}s of oil shale (6.70 and 8.91 mg/ml; Table 3). For each sample, the ED_{50} for increased porphyrin content was greater than the ED_{50} for EROD induction (Table 3). For the positive control, TCDD, the EC_{50} for EROD induction was 0.186 nM and that for porphyrin accumulation was 0.662 nM (data from experiments run with cell passages 40 and 42, not shown).

To compare Ereda reference samples to other River Kohtla samples, an additional experiment was run three times. In these experiments, maximal EROD activities in the cells were reached after treatment with 28.4 and 15.2 mg/ml of Ereda sediment extracts (Figure 3). Kohtla and Püssi sediment extracts acted similarly as in the previous experiments (maximal EROD activities at 0.162 and 4.49 mg/ml, respectively;

Table 1. Content of PAH compounds (μg/kg dry weight) in the sediments and oil shale

Site	Phe	Ant	Flu	Pyr	BaA	Chr	BeP + BbF	BkF	BaP	BghiP	Total
River Purtse											
Lüganuse A[a]	510	42	815	3,220	205	524	226	11	126	188	5,867
Lüganuse B[a]	317	28	631	2,320	185	392	165	5	95	132	4,270
Purtse A	3,210	105	846	8,430	525	444	2,130	567	2,900	9,400	28,557
Purtse B	2,805	93	773	7,820	831	732	2,020	365	1,940	8,930	26,309
River Kohtla											
Kohtla A	12,200	5,700	61,200	37,185	2,160	10,400	3,410	833	2,750	13,300	149,138
Kohtla B	5,680	2,710	30,500	19,000	1,260	6,140	1,318	375	1,350	6,320	74,653
Püssi A	100	16	248	1,010	222	628	516	15	416	159	3,330
Püssi B	216	12	282	1,140	160	433	423	7	380	191	3,244
Ereda[b] A	110	3	76	877	32	65	17	2	25	87	1,294
Ereda B	202	3	110	943	44	80	16	2	24	80	1,504
Oil shale A	173	12	209	302	88	62	31	9	21	58	965
Oil shale B	190	14	272	367	126	80	35	10	24	58	1,176

[a] A and B separate samples from the same study site

[b] Ereda sediments collected in a separate sampling than the other samples

Phe = phenanthrene, Ant = anthracene, Flu = fluoranthene, Pyr = pyrene, BaA = benz(*a*)anthracene, Chr = chrysene, BeP + BbF = benzo(*e*)pyrene + benzo(*b*)fluoranthene, BkF = benzo(*k*)fluoranthene, BaP = benzo(*a*)pyrene, BghiP = benzo(*g,h,i*)perylene

Figure 3). The ED_{50}s for EROD induction appeared in order Kohtla < Püssi < Ereda. Thus, Ereda reference samples were less potent to induce EROD activity (ED_{50}s 16.0 and 12.1 mg/ml) than other River Kohtla samples (ED_{50} for Kohtla 0.0460 mg/ml and for Püssi 1.33 mg/ml). In these experiments, TCDD produced an EC_{50} for EROD induction at 0.182 nM concentration (not shown). At the doses studied these sediment extracts did not cause a substantial decrease in total protein (not shown).

Correlation Between Biological and Chemical Data

Based on the ED_{50}s of the sample extracts and EC_{50} of TCDD, we calculated PLHC-1 TCDD-EQs for both EROD activity and porphyrin content (Table 3). A Pearson's log-log correlation between total PAHs and TCDD-EQs for EROD activity produced an r^2 of 0.847 ($p = 0.001$, $n = 12$) with Ereda reference samples included (Figure 4) and an r^2 of 0.827 ($p = 0.003$, $n = 10$) without Ereda references (not shown). In contrast, a statistically significant correlation was not found between total PAHs and TCDD-EQs for porphyrin content (log-log $r^2 = 0.785$, $p = 0.116$, and $n = 5$) (not shown). Among individual PAHs, the best log-log correlation was seen with Pyr in relation to TCDD-EQs for both EROD activity ($r^2 = 0.897$, $p < 0.001$, and $n = 10$ without Ereda data) and porphyrin content ($r^2 = 0.861$, $p = 0.061$, $n = 5$) (not shown).

To compare the theoretical and bioassay-derived potencies of the samples, dibenz(*a,h*)anthracene equivalent concentrations (analytical DBahA-EQs; Fent and Bätscher 1999; see also Fent *et al.* 1998) were calculated for BkF, BaP, Chr, BaA, and Pyr (Table 4). As observed for PAH contents, Kohtla and Purtse sediments produced the highest EQs. Overall, BkF, BaP, and Chr made the greatest contribution to PAH-derived DBahA-EQs. The calculated PAH-specific chemical potency of the samples (analytical DBahA-EQs) was highly correlated with the PLHC-1 bioassay-derived biological potency (PLHC-1 DBahA-EQs) (Figure 5; log-log $r^2 = 0.852$, $p < 0.001$, $n = 12$), except at Lüganuse. However, the PLHC-1 DBahA-EQs were 20- to 3,200-fold greater than the calculated DBahA-EQs.

Discussion

We used induction of CYP1A and accumulation of porphyrins in PLHC-1 fish cells to study the biological effects of river sediments and oil shale that contained relatively high amounts of lipophilic organic contaminants. This *in vitro* approach proved to be successful with samples from the studied area in northeast Estonia, not only because of the clear biochemical activities elicited by the samples but also due to good correlations between biological and chemical data.

Biochemical Responses and Chemical Data

The most potent sediments for induction of CYP1A and porphyrins in the PLHC-1 cells were those collected at sites nearest to the oil shale processing plants (Kohtla and Lüganuse). In addition, the Purtse sediments, which received pollutants from several sources, were also very active. When the rank order of the samples based on biological potency and total PAHs were compared, Lüganuse sediments were more potent inducers of CYP1A and porphyrins than would have been expected from total PAHs. The total analytical DBahA-EQs of Lüganuse sediments were about one order of magnitude lower than those of Purtse sediments though bioassay-derived EQs for EROD activity in these samples were similar. CYP1A in PLHC-1 cells is known to be induced by both PAHs and planar HAHs (Hahn *et al.* 1993, 1996; Brüschweiler *et al.* 1996a; Fent and Bätscher 1999), but so far, only HAHs have been tested for porphyrin accumulation (Hahn and Chandran 1996). The biological effects of the Lüganuse samples cannot be assessed from only the PAHs measured; other compounds might also be contributing to the EROD induction at this site. Synergistic interactions of chemicals could also explain the higher cell responses in the Lüganuse samples. Furthermore, the compounds released from RAS Eesti Kiviõli (pollution source especially for Lüganuse) could differ in part from those released from RAS Kiviter (pollution source for Kohtla and Püssi).

Despite of the mismatch of Lüganuse sediments, total PAHs correlated well with EROD-inducing potency in the exposed

Table 2. 7-Ethoxyresorufin *O*-deethylase (EROD) activity (pmol/min/mg protein) in PLHC-1 cells exposed to sediment extracts from River Purtse (Lüganuse, Purtse and River Kohtla (Kohtla, Püssi) as well as to oil shale extracts

	Lüganuse				Purtse				Kohtla				Püssi				Oil Shale			
Dilution	4.87 g/ml[a]		4.32 g/ml		3.81 g/ml		3.85 g/ml		3.24 g/ml		3.17 g/ml		8.98 g/ml		7.48 g/ml		9.96 g/ml		9.86 g/ml	
Factor	1A[b]	2A	1B	2B	1A	2A	1B	2B	1A	2A	1B	2B	1A	2A	1B	2B	1A	2A	1B	2B
2,000,000	—	4 ± 1[c]	—	2 ± 0	—	2 ± 1	—	2 ± 1	—	5 ± 0	—	2 ± 0	—	—	—	—	—	—	—	—
1,000,000	—	5 ± 1	—	4 ± 1	—	5 ± 2	—	4 ± 0	—	8 ± 1	—	4 ± 0	—	—	—	—	—	—	—	—
200,000	—	20 ± 1	—	17 ± 1	—	17 ± 1	—	9 ± 1	—	28 ± 1	—	16 ± 0	—	—	—	—	—	—	—	—
100,000	41 ± 7	40 ± 3	35 ± 5	37 ± 1	19 ± 7	30 ± 3	15 ± 6	19 ± 2	37 ± 4	49 ± 2	24 ± 8	30 ± 0	—	9 ± 2	—	3 ± 0	—	—	—	—
40,000	53 ± 1	74 ± 7	53 ± 7	47 ± 4	52 ± 9	63 ± 4	43 ± 12	41 ± 3	**68 ± 2**	59 ± 3	50 ± 4	50 ± 4	—	24 ± 3	—	7 ± 1	—	4 ± 1	—	7 ± 1
20,000	**54 ± 5**[d]	**82 ± 3**	54 ± 5	**48 ± 2**	67 ± 4	**86 ± 3**	63 ± 5	**58 ± 2**	62 ± 7	**60 ± 5**	**57 ± 1**	**54 ± 3**	—	38 ± 8	—	11 ± 2	—	—	—	—
10,000	53 ± 1	56 ± 9	**54 ± 2**	41 ± 2	**70 ± 7**	83 ± 3	**75 ± 3**	52 ± 3	52 ± 7	48 ± 1	52 ± 4	43 ± 2	55 ± 7	65 ± 4	36 ± 1	23 ± 3	6 ± 1	8 ± 1	6 ± 1	7 ± 1
4,000	19 ± 4	—	23 ± 5	—	40 ± 6	—	50 ± 4	—	23 ± 3	—	45 ± 3	—	**67 ± 8**	**80 ± 3**	63 ± 1	43 ± 4	14 ± 1	16 ± 1	8 ± 1	14 ± 1
2,000	14 ± 2	15 ± 1	16 ± 2	9 ± 0	24 ± 1	34 ± 2	34 ± 4	24 ± 2	10 ± 0	11 ± 0	27 ± 2	14 ± 1	—	80 ± 3	—	**49 ± 2**	—	—	—	—
1,000	5 ± 0	6 ± 1	5 ± 2	5 ± 1	12 ± 2	14 ± 2	21 ± 3	14 ± 2	3 ± 1	5 ± 1	12 ± 3	10 ± 0	48 ± 4	59 ± 6	**82 ± 25**	45 ± 2	50 ± 1	52 ± 3	41 ± 2	39 ± 4
400	1 ± 1	—	2 ± 1	—	6 ± 0	—	6 ± 1	—	1 ± 1	—	5 ± 1	—	19 ± 2	32 ± 4	35 ± 3	30 ± 3	**64 ± 9**	**74 ± 4**	66 ± 2	**65 ± 4**
200	4 ± 0	2 ± 0	2 ± 1	1 ± 0	4 ± 0	4 ± 2	2 ± 0	3 ± 0	—	—	4 ± 3	1 ± 1	11 ± 3	14 ± 1	23 ± 3	17 ± 1	61 ± 7	72 ± 4	**71 ± 2**	64 ± 2
DMSO	1 ± 0	—	1 ± 0	—	2 ± 1	—	1 ± 1	—	0 ± 1	—	2 ± 1	—	—	1 ± 1	—	1 ± 1	—	—	—	—
2 nM TCDD	110 ± 15	114 ± 0	117 ± 4	107 ± 9	115 ± 1	105 ± 4	130 ± 9	98 ± 12	95 ± 5	107 ± 6	108 ± 3	116 ± 11	122 ± 9	85 ± 6	—	76 ± 2	135 ± 12	—	—	—

[a] Weight of stock dry sediment (g) extracted with *n*-hexane and dissolved in 1 ml of DMSO; stock weight/dilution factor = dose studied in test system (g/ml medium)
[b] 1 and 2 express separate experiments, A and B separate samples
[c] Mean from three wells ± SD
[d] Bold: maximal EROD activity

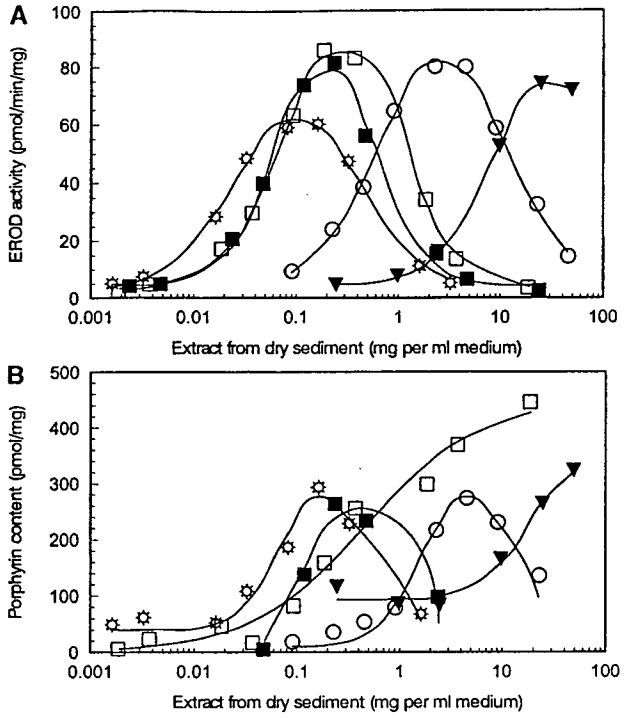

Fig. 2. A: EROD activity (pmol/min/mg), and B: porphyrin content (pmol/mg) in PLHC-1 cells after exposure to sediment and oil shale extracts. The sediments were collected from River Purtse at sites Lüganuse (■) and Purtse (□) as well as from River Kohtla at sites Kohtla (⌑) and Püssi (○). Oil shale (▼) was collected from the mine of Narva. Results of one sample from the second experiment (marked 2A in Table 2). Mean of three wells. EROD activity or porphyrin content of negative control (*n*-hexane, which was run through the extraction as such) did not exceed 13 pmol/min/mg or 88 pmol/mg, except at the highest dose (20 pmol/min/mg and 127 pmol/mg; not shown)

cells. Thus, our findings support the use of the PLHC-1 cells in environmental monitoring of PAHs in oil shale-polluted sediments. Similarly, Villeneuve *et al.* (1997) found that PLHC-1 cells were capable in monitoring the biological effects of lipophilic organic contaminants in urban storm water runoff where PAHs were the major pollutants. These authors measured EROD activity in the cells after exposure to complex mixture of lipophilic organic contaminants concentrated in semipermeable membrane devices.

A weaker correlation was seen between total PAHs and porphyrin content. Porphyrin accumulation is suggested to be mediated in part via Ah receptor, as with CYP1A induction (Hahn *et al.* 1988), but other factors are also important (Smith and Francis 1983; Sinclair *et al.* 1997). Porphyrin accumulation has been observed in studies with planar HAHs in PLHC-1 cells (Hahn and Chandran 1996). Lorenzen *et al.* (1997) further suggested that HAHs with a planar configuration mediated CYP1A induction and/or Ah receptor activation leading to porphyrin accumulation in an avian system *in vitro,* whereas nonplanar HAHs may cause porphyrin accumulation by a mechanism not involving induction of CYP1A. In our study all the downstream samples possessed levels of PAH contamination ranging from moderate to heavy, and, similarly, these samples also caused porphyrin accumulation in the cells. PAHs have shown to cause porphyrin accumulation in mammalian *in vivo* studies (Francis and Smith 1987). As an example from avian systems, planar HAHs were highly porphyrogenic in cultured chick embryo hepatocytes and stimulated uroporphyrin accumulation when added to 3-methylcholanthrene-treated cells, whereas nonhalogenated CYP1A inducers were only slightly porphyrogenic and blocked accumulation of uroporhyrin in planar HAH-treated chick cells (Sinclair *et al.* 1984, 1986; Rodman *et al.* 1989). To our knowledge, the ability of individual PAHs to cause porphyrin accumulation in the PLHC-1 cells has not yet been studied.

It is also possible that the samples contained planar HAHs. In principal, combustion of fossil fuel, in the presence of chlorine, can be a source of PCDDs and PCDFs (Fiedler *et al.* 1990). The ratio of TCDD-EQs for porphyrin content and TCDD-EQs for EROD activity was the highest in Lüganuse sediment. This suggests that the compounds in Lüganuse sediment were relatively more porphyrinogenic than those from the other sites. Unfortunately, planar HAHs were not determined in our study and, thus, whether these compounds were more important in causing biological effects at some study sites cannot be determined at this time. Some elevated levels of PCBs in biota, water, and sediment have been detected in the Gulf of Finland (Roots 1996).

Bioassay induction results often yield higher EQs than toxic equivalents calculated from chemical analyses (Engwall *et al.* 1996, 1997; Villeneuve *et al.* 1997; Willett *et al.* 1997). In our study the PLHC-1 DBahA-EQs for EROD activity were 20–3,200 times higher than the analytical DBahA-EQs for five PAHs. It is obvious that analytical EQs based on only five PAHs do not represent the effects of all the organic compounds in the samples. In any case, this further suggests that there were other lipophilic organic compounds in addition to the PAHs measured in the samples that were able to cause CYP1A induction and porphyrin accumulation in the cells. The complex mixture of lipophilic compounds may cause antagonistic and synergistic interactions. This may complicate the search for chemicals or chemical groups primarily responsible for biological effects.

Almost all of the samples examined in this study produced biphasic CYP1A induction curves. This has been observed in earlier studies with model compounds with PLHC-1 cells (Hahn *et al.* 1993, 1996; Brüschweiler *et al.* 1996a) and chicken embryo hepatocytes (Lorenzen *et al.* 1997). The occurrence of both induction and inhibition has been especially associated with CYP1A induction by PCBs (Gooch *et al.* 1989; Huuskonen *et al.* 1996). In the study of Gooch *et al.* (1989), a coplanar PCB, 3,3′,4,4′-tetrachlorobiphenyl, proved to be a potent competitive inhibitor of EROD activity in fish. Later, Hahn *et al.* (1993) observed an inhibition caused by the same PCB congener in PLHC-1 cells. Moreover, it has been shown that CYP1A inhibition in the PLHC-1 cells can occur with higher concentrations of PAH compounds, as well (Brüschweiler *et al.* 1996a; Celander *et al.* 1997; Fent and Bätscher 1999). Porphyrin accumulation also may be diminished at higher doses, as detected earlier with some samples in a study of Lorenzen *et al.* (1997). In that study, chicken embryo hepatocyte cultures were treated with individual HAHs as well as with a commercial mixture of PCBs (Aroclor 1254). The cause for that decline was not suggested.

In our study, all the downstream sediment extracts were more potent inducers of CYP1A and porphyrins than the oil shale

Table 3. ED_{50} (mg/ml) and TCDD-EQ (ng/g) for EROD activity and for porphyrin content, and DBahA-EQ (ng/g) for EROD activity in the PLHC-1 cells

Site	ED_{50} for EROD	TCDD-EQ for EROD[a]	DBahA-EQ for EROD[b]	ED_{50} for Porphyrin	TCDD-EQ for Porphyrin[c]
River Purtse					
Lüganuse A	0.0504	1.19×10^3	55.2×10^3	0.0800	2.66×10^3
Lüganuse B	0.0295	2.03×10^3	94.4×10^3	ND	ND
Purtse A	0.0574	1.04×10^3	48.5×10^3	0.479	445
Purtse B	0.0585	1.02×10^3	47.6×10^3	ND	ND
River Kohtla					
Kohtla A	0.0186	3.22×10^3	150×10^3	0.0581	3.67×10^3
Kohtla B	0.0275	2.18×10^3	101×10^3	ND	ND
Püssi A	0.487	123	5.72×10^3	1.41	151
Püssi B	0.843	71.0	3.30×10^3	ND	ND
Ereda A	16.0[d]	3.66[d]	174	ND	ND
Ereda B	12.1[d]	4.84[d]	230	ND	ND
Oil shale A	6.70	8.94	415	16.7	12.8
Oil shale B	8.91	6.72	312	ND	ND

[a] Calculated using the EC_{50} value for EROD induction by TCDD (0.186 nM [59.88 pg/ml])
[b] Calculated using the EC_{50} value for EROD induction by DBahA (1.0×10^{-8} M [2,783.6 pg/ml], after Fent and Bätscher (1999)
[c] Calculated using the EC_{50} value for porphyrin accumulation by TCDD (0.662 nM [213.14 pg/ml])
[d] Sediment data and TCDD EC_{50} value for EROD activity (0.182 nM) from the additional experiment
ND = not determined
For further explanations see Table 1 and for original EROD data Table 2 (experiment 2)

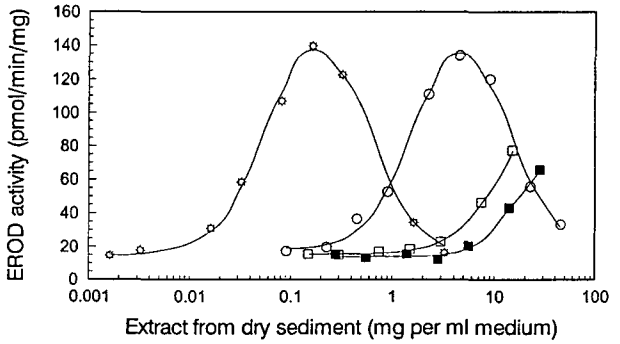

Fig. 3. EROD activity (pmol/min/mg) in PLHC-1 cells after exposure to sediment extracts. Samples Ereda A (■) and Ereda B (□), as well as Kohtla A (⧆) and Püssi B (○) were examined (see Table 1 for PAH contents). Mean from three experiments (all together nine wells). EROD activity after treatment with 2 nM TCDD was 214 ± 59 pmol/min/mg (19 replicates; not shown)

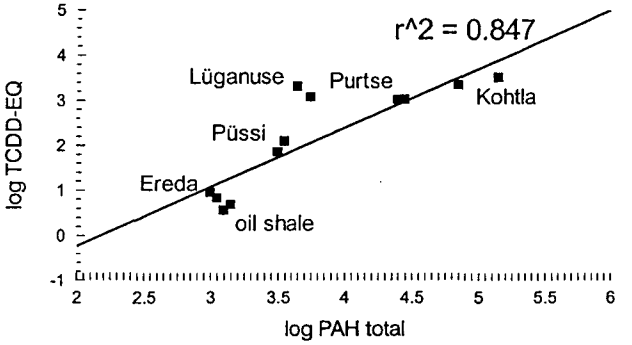

Fig. 4. A Pearson's log-log correlation between TCDD-EQs for EROD activity in PLHC-1 and total PAH content (chemical analysis). r^2 = 0.847 with Ereda reference samples and r^2 = 0.827 without references

extracts. However, oil shale was supposed to be the initial source of contamination. The sediments contained more PAHs than the oil shale but the major compounds were the same both in the oil shale and the sediments. PAHs released from the raw material in the processing and mining of oil shale may have been accumulated and selectively concentrated in the recipient river sediments during a long period. Indeed, the bottoms of River Purtse and River Kohtla are covered with mud with a high content of organic substances (thickness up to 0.3 m). Huge quantities of hydrocarbons and compounds typical of shale oil products have been found in this layer (reviewed in Liblik and Rätsep 1994). In addition, the most polluted sediments were collected at sites nearest to the oil shale–processing factories. It can be suggested that at least most if not all of the PAHs that were able to affect the PLHC-1 cells were released by the oil shale processing industry either from oil shale or combustion.

The sediments from the reference site, Ereda, showed moderate PAH contamination that was never the less much less than in the other sediments. Transportation of contaminants via air may have affected this area (Punning *et al.* 1997). Based on the ED_{50}s, Ereda samples were as potent inducers of CYP1A in the cells as the sediments from a Finnish reference lake (ED_{50}s 15 and 18 mg/ml; Huuskonen *et al.* 1998a). This suggests that *Ereda* samples were suitable as references. Difficulty in finding a valid reference site in northeast Estonia was noticed in a study of Huuskonen *et al.* (1998b).

When the present CYP1A results in the cells treated with sediment extracts from contaminated sites were compared to previous data (Huuskonen *et al.* 1998a, 1998b), EROD activity was found to be induced at lower doses in the present study. In River Narva, Estonia, a polluted sediment (744 µg total PAH/kg dry sediment) from an oil shale processing area gave an ED_{50} of 2.6 mg/ml for EROD activity in the PLHC-1 cells (Huuskonen *et al.* 1998b). Only a sediment polluted by an accidental release

Table 4. PAH-specific equivalent concentrations (analytical DBahA-EQs, ng/g) calculated according to relative potencies (RPs) by Fent and Bätscher (1999) where dibenz(*a*,*h*)anthracene was the most potent PAH compound (RP = 1.0, EC_{50} for EROD activity 1.0×10^{-8} M)

Site	Benzo(*k*)fluoranthene 0.334[a]	Benzo(*a*)pyrene 0.055	Chrysene 0.036	Benz(*a*)anthracene 0.006	Pyrene 0.003	Total
River Purtse						
Lüganuse A	3.7	6.9	18.9	1.2	9.7	40.4
Lüganuse B	1.7	5.2	14.1	1.1	7.0	29.1
Purtse A	189.4	159.5	16.0	3.2	25.3	393.4
Purtse B	121.9	106.7	26.4	5.0	23.5	283.5
River Kohtla						
Kohtla A	278.2	151.3	374.4	13.0	111.6	928.5
Kohtla B	125.3	74.3	221.0	7.6	57.0	485.2
Püssi A	5.0	22.9	22.6	1.3	3.0	54.8
Püssi B	2.3	20.9	15.6	1.0	3.4	43.2
Ereda A	0.7	1.4	2.3	0.2	2.6	7.2
Ereda B	0.7	1.3	2.9	0.3	2.8	8.0
Oil shale A	3.0	1.2	2.2	0.5	0.9	7.8
Oil shale B	3.3	1.3	2.9	0.8	1.1	9.4

[a] RPs of individual PAHs (calculated on pg/ml basis)
For PAH contents (µg/kg DW) and further explanations see Table 1

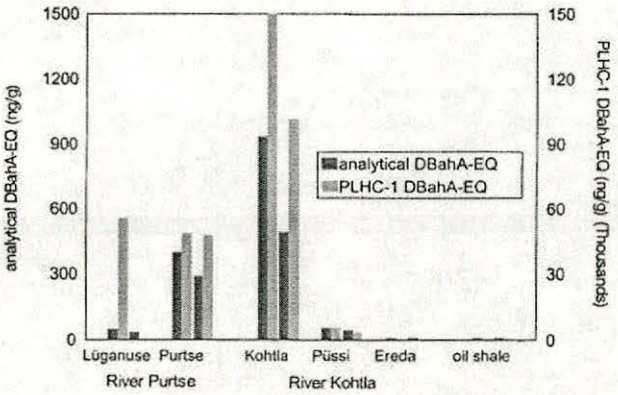

Fig. 5. Equivalent concentrations based on the PLHC-1 biological data (PLHC-1 DBahA-EQ for EROD activity; ng/g) and PAH-specific chemical data (analytical DBahA-EQ for five PAHs; ng/g) after Fent and Bätscher (1999)

of asphalt (278,400 µg total PAH/kg dry sediment) produced about the same ED_{50} values (0.062 and 0.063 mg/ml) as in the present study. Extracts of sediments from Lake Kernaala in Finland (ED_{50}s from 3.4 to 7.0 mg/ml at sites having a history of PCB pollution) were also less potent inducers of EROD activity as compared to the contaminated sediments of River Purtse and River Kohtla (Huuskonen *et al.* 1998a).

Validity and Utilization of the Results

There are numerous studies showing induction of CYP1A by lipophilic organic compounds in fish (*e.g.*, Gooch *et al.* 1989; van der Weiden *et al.* 1994b; Goksøyr *et al.* 1994; Stegeman and Hahn 1994; Bucheli and Fent 1995; Huuskonen *et al.* 1996; Tuvikene *et al.* 1996). Some studies emphasize specifically the CYP1A inducing effects of oil contamination (George *et al.* 1995; Spies *et al.* 1996). However, to our knowledge there is only a single report of hepatic porphyria in wild fish exposed to environmental HAHs (Koss *et al.* 1986). For example, van der Weiden *et al.* (1994a) did not observe altered porphyrin levels in fish after experimental treatment with HAHs. In our study, both CYP1A induction and porphyrin accumulation were enhanced in a PLHC-1 fish cell culture system. This suggests that porphyrin content measured *in vitro* could be used as a biomarker for lipophilic organic contaminants. However, it is not clear which chemicals (other than TCDD and planar PCBs [Hahn and Chandran 1996]) can cause porphyrin accumulation in the PLHC-1 cells.

In studies of Engwall *et al.* (1996, 1997), the bio-TEQ values were normalized to carbon contents of the sediment and settling particulate matter (SPM) samples instead of the dry weights. This was because lipophilic compounds are largely partitioned to the carbon fraction of sediment or SPM (Di Toro *et al.* 1991). However, it has also been shown that organic contaminants that possess similar solubilities in lipid associate differently with organic carbon (Harkey *et al.* 1994). We based our calculations on dry weight of sediment since the sediments had relatively similar properties.

The *n*-hexane extracts of the sediments and oil shale contained a mixture of chemicals in a concentrated form. Thus, these samples had a capacity to affect the PLHC-1 cells very effectively. It has been also observed that the effects of whole sediments on an aquatic organism, midge larvae (*Chironomus riparius*), were not as strong as the impact of sediment extracts on PLHC-1 cells (Huuskonen *et al.* 1998b). In the midge assays, the contaminants in whole sediments were not concentrated, and, in addition, other contaminants (both organic and inorganic) were present and able to affect the midges. It was concluded that the effects seen in the PLHC-1 cells represent the potential hazard of lipophilic organic contaminants in the samples. By widening the selection of assays to include a suite of endpoints in addition to CYP1A induction and porphyrin accumulation (see Babich *et al.* 1991; Ryan and Hightower 1994; Brüschweiler *et al.* 1995, 1996b), the PLHC-1 cells could serve as a tool to study other kinds of pollutants, as well. This approach would produce a more complete picture of the potential effects of contaminants in environmental samples.

A question has been raised whether sensitive single-species tests can be used to make predictions regarding complex ecosystems. To overcome this difficulty, more detailed comparisons between an *in vitro* PLHC-1 bioassay and various *in vivo* systems are needed to establish its utility in assessing contamination of ecosystems. The number of *in vitro* tests and/or endpoints can be also increased to develop test batteries. In our study, the PLHC-1 cells were used to detect the fish-specific effects of lipophilic organic contaminants in the sample extracts. The cells proved to be very promising in categorizing and monitoring of aquatic sediments in an oil shale processing and mining area of northeast Estonia, although not all the potentially toxic compounds were identified in the study. The PLHC-1 bioassays also showed that the lipophilic organic contaminants in River Purtse sediments were extremely biologically potent even at the site nearest to the Gulf of Finland. These results support the use of the PLHC-1 bioassays in suggesting ecotoxicological hazards.

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