ELSEVIER

Aquatic Toxicology 44 (1998) 47–67

AQUATIC TOXICOLOGY

Comparison of two bioassays, a fish liver cell line (PLHC-1) and a midge (*Chironomus riparius*), in monitoring freshwater sediments

Sirpa E. Huuskonen [a,b,*], Tiina E. Ristola [c], Arvo Tuvikene [d,e], Mark E. Hahn [b], Jussi V.K. Kukkonen [c], Pirjo Lindström-Seppä [a]

[a] *University of Kuopio, Department of Physiology, P.O.B. 1627, Kuopio, FIN-70211, Finland*
[b] *Woods Hole Oceanographic Institution, Biology Department, Woods Hole, MA 02543, USA*
[c] *University of Joensuu, Department of Biology, P.O.B. 111, Joensuu, FIN-80101, Finland*
[d] *Institute of Zoology and Botany, Limnological Station, Rannu, EE-2454, Estonia*
[e] *University of Tartu, Institute of Zoology and Hydrobiology, Tartu, EE-2400, Estonia*

Received 15 June 1997; received in revised form 2 April 1998; accepted 15 April 1998

Abstract

Two bioassays, a fish hepatoma cell line (PLHC-1) and a midge (*Chironomus riparius*), were used to monitor surficial sediments from Lake Võrtsjärv and River Narva, Estonia. L. Võrtsjärv is polluted with mainly polycyclic aromatic hydrocarbons (PAHs) but R. Narva possesses complex contamination (PAHs, heavy metals, sulphates, chlorides). The PLHC-1 cells were exposed to the extracted lipid soluble compounds (PAH fraction) of the sediments, after which the cytotoxicity (total protein content) and cytochrome P4501A (CYP1A) inducibility (7-ethoxyresorufin *O*-deethylase activity (EROD) and CYP1A protein content) were measured. The midges were grown in whole sediments after which the midge growth (larval growth and survival as endpoints) or the emergence (larval survival and adult emergence) were tested. Contents of selected PAHs and heavy metals in the sediments were also evaluated. In the PLHC-1 screening experiments, most of the sediments from R. Narva were more toxic and caused higher EROD activities at lower doses than the sediments from L. Võrtsjärv. The most polluted sediment in R. Narva (total PAH content 744 ng g^{-1} dry weight sediment) gave 15 mg dry sediment ml^{-1} as the ED_{50} for induction of EROD activity in the cells exposed for 3 days. In the midge growth test, larvae seemed to grow better in the sediments from L. Võrtsjärv than from R. Narva and the mortality was somewhat higher in two areas in R. Narva than in other study areas. Adult emergence did not show such clear trends between these two watersheds, though emergence was accelerated in some sediments. A sediment from a point source of pollution (accidental release of asphalt) from R.

* Corresponding author. Tel.: +358 17 163095; fax: +358 17 163112; e-mail: sirpa.huuskonen@uku.fi

PII S0166-445X(98)00070-8

Narva was also studied and found to be the most toxic for the PLHC-1 cells and the most potent inducer of CYP1A (ED_{50}s 0.59 and 0.56 mg dry sediment ml^{-1}). None of the midge larvae survived in this sediment. In a time-course study, the highest EROD activity in the PLHC-1 cells was reached at lower doses of PAH fraction after 24-h exposure than after 48 or 72 h, suggesting metabolism of PAHs in the cultures. Further, CYP1A induction was still seen as elevated amounts of CYP1A protein in cases where catalytic EROD activity was decreased at higher doses of PAH fraction. Overall, the PLHC-1 bioassays were shown to be sensitive methods for detecting PAH pollution. The midge bioassays reflected better the bioavailability and the in situ effects of the complex mixture of compounds in the sediments.

Keywords: CYP1A; Heavy metals; Midge; PAHs; PLHC-1; Sediment; Toxicity

1. Introduction

Lake and river sediments are an ultimate sink for many persistent and accumulating chemicals. Bioavailability and bioaccumulation of these sediment-associated pollutants are affected by various abiotic and biotic factors (Schindler et al., 1995; Landrum et al., 1996). Some factors, like photoactivation caused by UV light, may transform the chemicals to even more toxic compounds (Ankley et al., 1994). Thus, because of the apparent or potential hazard of the chemicals in the sediments for the aquatic biota, approaches to biomonitor the sediments are essential.

Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants in aquatic ecosystems arising from a variety of anthropogenic activities (Neff, 1985). All PAHs are solids, and are sparingly soluble in water. PAHs are less persistent in aerobic surficial sediments than in deeper, anoxic sediments but the former may have enhanced toxicities and be more accessible to biota (Cerniglia, 1984; Ankley et al., 1994). Some of the PAHs (like benzo(*a*)pyrene, benz(*a*)anthracene and chrysene) are documented to be metabolized through cytochrome P4501A (CYP1A) and also act to induce this protein (Stegeman and Hahn, 1994; Van der Weiden et al., 1994). This biotransformation process can also convert PAH to more harmful compounds, including potential carcinogens (Stegeman and Lech, 1991). CYP1A induction in fish has been used as a biomarker of waters or sediments polluted with PAHs (Payne and Penrose, 1975; Van Veld et al., 1990; Celander et al., 1994; Engwall et al., 1994; George et al., 1995; Munkittrick et al., 1995; Tuvikene et al., 1996).

Some established fish hepatoma cell lines have an inducible CYP1A system (Fryer et al., 1981; Hightower and Renfro, 1988; Lee et al., 1993). One of these, derived from a hepatocellular carcinoma of *Poeciliopsis lucida* (PLHC-1), has been used in detecting the cytotoxicity of various compounds as well as in studies of CYP1A induction and inhibition (Babich et al., 1991; Hahn et al., 1993; Ryan and Hightower, 1994; Brüschweiler et al., 1995, 1996; Celander et al., 1996; Hahn et al., 1996).

Another group of potent pollutants in aquatic sediments are heavy metals. Heavy metals precipitate to the sediments as insoluble complexes with organic particulates or inorganic anions, such as carbonates. In water, however, metals may remain in solution as free ions or as soluble complexes of organic and inorganic anions (Hodson, 1988). The availability of the metals to aquatic organisms is affected by salinity, pH, and temperature, as well as types and quantities of dissolved organics and particulates (Pritchard, 1993; Schindler et al., 1995). Further, metal bioavailability, bioaccumulation and sediment toxicity can vary spatially and temporally (Krantzberg, 1994; Schindler et al., 1995).

Since larval stages of the *Chironomidae* are intimately associated with the surficial sediments, they have been widely used in bioassays in detecting the toxicity of sediment contamination (Krantzberg, 1994; Besser et al., 1995; Ristola et al., 1996b). These studies have revealed that both organic and inorganic contaminants may have effects on larvae.

Various in vitro tests have been considered as alternatives for live animal studies. The range of the in vitro tests and methods makes it possible to choose a suitable assay according to the aim of the study. For example, the PLHC-1 cell line may serve as a laboratory bioassay for detecting the fish-specific effects of lipid soluble compounds in the sediments. Further, comparative studies between various in vitro assays as well as between in vitro and in vivo studies are recommended in order to facilitate aquatic risk assessment. Invertebrate larvae living in the sediments serve as an in vivo assay to study the effects of the whole sediment, thus reflecting the bioavailability and in situ exposure of various pollutants.

The objective of the present study was to compare two different types of bioassays, a fish hepatoma cell line (PLHC-1) and a midge, *Chironomus riparius*, in the monitoring of aquatic sediments. The PLHC-1 cell line serves as a specific, sensitive and relatively novel approach for assessing the effects of sediment extracts. Midge bioassays have been studied more in biomonitoring purposes; they were selected to give a more holistic view of the hazard of contaminants in the sediments. Surficial sediments were collected from two Estonian inland waters characterized by different sources and degrees of chemical contamination. Lake Võrtsjärv is contaminated with mainly PAHs, River Narva with considerable amounts of various pollutants. In addition, reference sediments were collected from Lake Höytiäinen, Finland. The PLHC-1 cells were exposed to dichloromethane extracts of the sediments, after which cytotoxicity and CYP1A inducibility were assayed. Toxicity of the whole sediment was detected with a midge growth test and with an emergence test. To characterize the extent of pollution in the sediments, contents of selected PAHs (ranging from 3- to 6-ring) and heavy metals (Cd, cadmium; Cu, copper; Pb, lead; Hg, mercury), were analysed.

2. Materials and methods

2.1. Chemicals used in biochemical analyses

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), used as a positive control, was a generous gift from Dr Sirpa Kärenlampi (University of Kuopio, Finland). 2,4-Dinitrophenol (DNP), Eagle's minimum essential medium (MEM), Hepes buffer and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO), 7-ethoxyresorufin from Boehringer Mannheim (Germany) and trypsin EDTA from NordCell (Sweden). Part of the reagents and chemicals were obtained as described previously by Hahn et al. (Hahn et al., 1993, 1996). All other chemicals were of analytical grade.

2.2. Study sites

Sediments were collected from selected areas in Lake Võrtsjärv (South Estonia) and River Narva (North-East Estonia) (Fig. 1). The pollution in L. Võrtsjärv consists mainly of PAHs arising from the surrounding watershed (agricultural sources) and oil spills of fishing vessels. Part of the pollution is brought by two rivers which run into the northern part of the lake (River Tänassilma) and into the southern part (River Väike Emajõgi). In L. Võrtsjärv, sediments were collected from a northern (near Jõesuu) and a southern (Salu) part of the lake as well as from a proposed reference area in the middle of the lake (Vehendi).

R. Narva, running from L. Peipsi into the Gulf of Finland, receives its pollution mainly from drainage water from oil shale ash plateaus of Baltic and Estonian thermal power plants (TPPs) and partly from two oil shale mines (Sirgala and Narva). The main pollutants are sulphates, chlorides, heavy metals and PAHs. Further, municipal waste waters from the town of Narva are released into the river as mechanically and biologically, though still inefficiently, purified effluents. The waters of North-East Estonia receive also considerable amounts of airborne pollutants, mainly from the TPPs (Ots, 1992). The proposed reference area (in River Mustajõgi, a tributary of R. Narva) was selected 25 km upstream from the town of Narva. The site named Baltic TPP is situated 5 km upstream from the town of Narva and receives pollution mainly from the Baltic TPP but also from the Estonian TPP. Another contaminated site, Riigiküla 1, is located 5 km down-

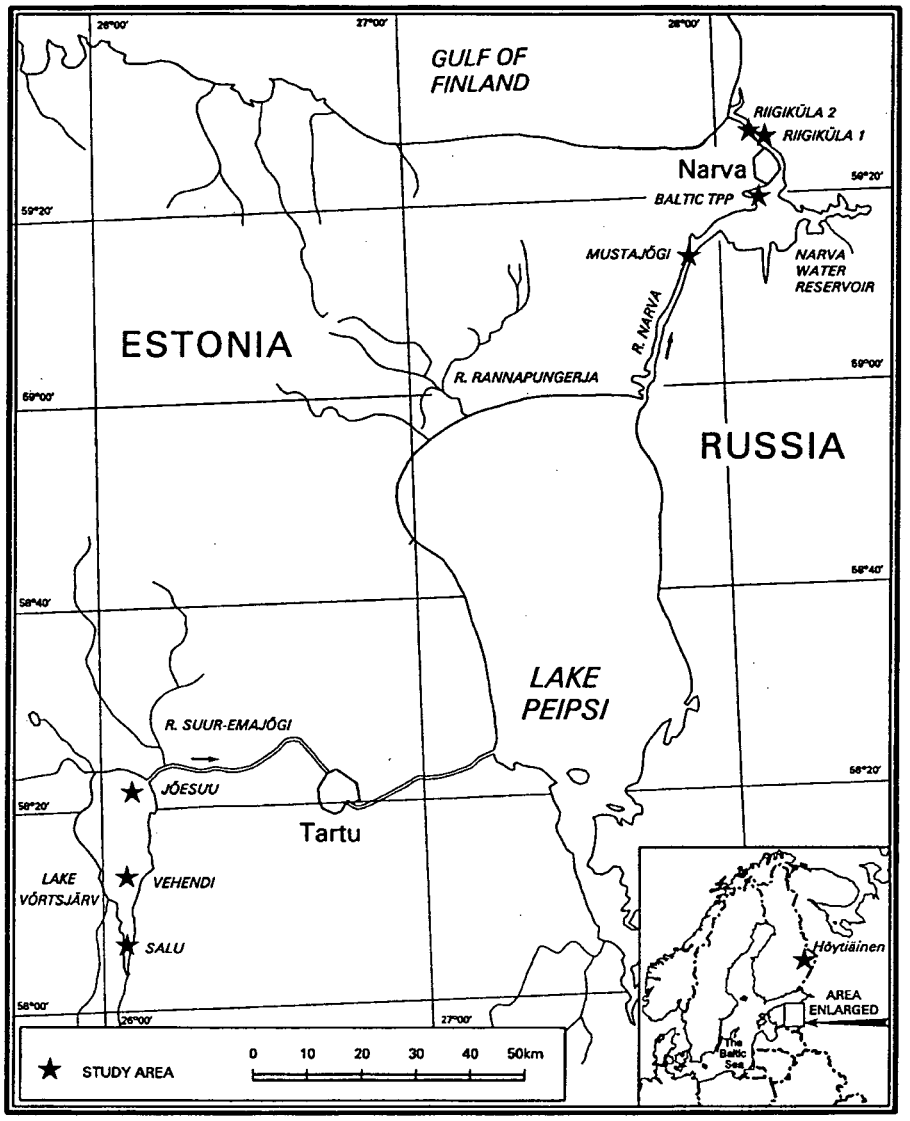

Fig. 1. Study areas in Lake Võrtsjärv and River Narva, Estonia. In L. Võrtsjärv, Jõesuu and Salu represent contaminated sites and Vehendi a reference site. In R. Narva, Baltic TPP and Riigiküla 1 represent contaminated sites and Mustajõgi a proposed reference site. An additional sample from Riigiküla was collected from a place where an asphalt spill had occurred (Riigiküla 2). For comparison, reference sediments were collected from L. Höytiäinen, Finland (Höytiäinen 1 and Höytiäinen 2, shown in the small overview map).

stream from the town of Narva and receives its pollution from the Baltic and Estonian TPPs and also as municipal sewage from the town of Narva. At Riigiküla, the other sediments (Riigiküla 2) were collected downstream from Riigiküla 1 at a place where we observed a point source of pollution. This pollution was caused by an accidental release of asphalt into the river water (Tiiu Sizova, personal communication).

Additional reference sediments for midge assays were collected from two relatively unpolluted sites (Höytiäinen 1 and Höytiäinen 2) in Lake Höytiäinen in Eastern Finland.

2.3. Sediment samples

The upper layer (5 cm) of sediment was collected with a bottom grabsampler (Ekman modifi-

cation) and divided into polyethylene boxes (for the PLHC-1 cells and chemistry) and into glass jars washed with acetone (for the midge bioassays). The sediments were stored at 4°C for the bioassays and at −20°C for the chemical analyses.

For the PLHC-1 assays, the sediments were first dried at 65°C overnight. To obtain lipid soluble compounds (PAH fraction), the samples were packed into glass fiber thimbles and extracted in a Soxhlet extractor with 300 ml of dichloromethane for 16 h. Dichloromethane was evaporated under vacuum into dryness and the dry extract was stored at −20°C. Before use, the sample was taken up in 1 ml of acetone.

For the midge bioassays, the sediment subsamples were first homogenized by stirring and then sieved to remove large particles and indigenous animals (mesh size 1 or 2 mm). In addition to bioassays, samples were analyzed for percentage dry matter (105°C until constant weight), loss of ignition (550°C for 2 h) and organic carbon content (Carlo Erba Elemental Analyzer). Fine particle fraction (< 63 μm) was determined by wet sieving.

2.4. PAH analyses

PAHs were extracted from dry sediments by *n*-hexane utilizing two steps of ultra-sound extraction and evaporated to a volume of 0.3 ml. The evaporated extracts were separated by thin-layer chromatography using aluminium oxide and a 4:1 mixture of hexane-benzene as the eluent. Dichloromethane (used in preparing the extracts for the cell assays) and *n*-hexane (used in preparing chemical samples) should extract nearly the same composition of lipid soluble compounds. Thus, though the solvents were different, PAH data should be valid in expressing the chemical background of the sediment extracts for the cell experiments.

Measuring of PAH content (of phenanthrene, anthracene, fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*e*)pyrene + benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene and benzo(*ghi*)perylene) was carried out by means of high performance liquid chromatography (HPLC; model 1311, Minsk, Belorussia). For chromatographic determination of the PAHs, the residue was dissolved in 0.2 ml of ethanol or acetone. The mixture of acetonitrile-water 93:7 (by volume) with flow rate of 8 μl min^{-1} was used as an eluting solvent. Fluorometric detection at two excitation wavelengths (254 and 296 nm) was used for identification and quantification. 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% (more details in Trapido and Palm, 1991). The detection limit was 2 ng per sample for each PAH.

A method based on the Schpol'skii effect, a specific fluorescence emission spectra in frozen hexane at 77 K (Khesina et al., 1983), was also applied for quantitive determination of benzo(*a*)-pyrene.

2.5. Heavy metal analyses

Samples for Cd, Cu, Pb or Hg analyses were first dried and then powdered in a mortar. For Hg analyses, 2.5 ml HNO_3 (conc.) and 10 ml H_2SO_4 (conc.) were added to 1 g of sample and the sample was allowed to stand for 24 h. After that the sample was heated at 70°C for 2 h, cooled and diluted when necessary. A 5% solution of $KMnO_4$ was added up to a point where violet color was stable. Further, 3% solution of hydroxylamine was added until the violet color disappeared. Dilution was made with deionized water. For Cd, Cu and Pb analyses, 1 g of sample was heated at 120°C under pressure (200 kPa) for 30 min with 20 ml of HNO_3 (conc.). Samples were diluted with deionized water.

Metal contents of Cd, Cu and Pb in sediments were measured with Varian SpectrAA 250 Plus atomic absorbtion spectrophotometer, applying the method of direct flame atomization (Finnish Standards Association, 1980a,b). Hg was measured by a cold vapour method using the same atomic absorption spectrophotometer equipped with a vapor generation accessory VGA-77. $SnCl_2$ was used as reducing agent.

Detection limits in metal analyses were 0.01 mg kg^{-1} dry weight sediment for Cd, 1 mg kg^{-1} for Cu, 0.1 mg kg^{-1} for Pb and 0.005 mg kg^{-1} for Hg. Recovery of standards was at least 80%.

2.6. PLHC-1 cell line

The PLHC-1 cell line, derived from a hepatocellular carcinoma of the topminnow *Poeciliopsis lucida* (Hightower and Renfro, 1988), was maintained in a humidified, 5% CO_2 atmosphere at 30°C in MEM, containing Earle's salts, non-essential amino acids, L-glutamine and 10% calf serum. Cells were grown in a 94/16-mm dish in 10 ml of medium, which was changed every 3–4 days. When confluent, the cells were usually subcultured at a split ratio of 1:3. For some experiments, cells were grown in 75-cm^2 flasks at 30°C in MEM and subcultured as described previously by Hahn et al. (1993).

2.7. PLHC-1 biotests: screening of cytotoxicity and EROD activity

Survival of the cells was assayed by measuring the total protein content. The cells were incubated on a 24-well plate. To calibrate the experiments, a stock solution of 20 mg DNP in 1 ml of DMSO was used. Medium (500 μl) was added to each well, except the last one in a row. Then 1 ml of medium containing DNP or the sediment extract was added to the last well, from which a continuous 1:1 dilution series was made. Then 2.5×10^5 cells (counted with a Coulter Counter®) were seeded in 500 μl of medium into every well. After 3 days of exposure, the medium was removed, and the cells were washed twice with 1 ml of buffered phosphate saline (PBS; 171 mM NaCl, 3.35 mM KCl, 1.84 mM KH_2PO_4, 10.1 mM $Na_2HPO_4 \times 2$ H_2O, pH 7.2). The cells were then dissolved in the wells with 0.05 M NaOH. Protein was assayed by the method of Bradford (1976). The total protein content in wells containing pure medium exceeded 100 μg ml^{-1} in every plate.

In the CYP1A induction test, 2×10^6 cells were first seeded into cell culture dishes in 10 ml of medium. The next day the medium was replaced by fresh medium containing different amounts of the studied samples diluted into acetone, and the diluent was used as a control. To compare the results of different exposures, 10 μl of 2 μM TCDD in DMSO (2 nM in test system) was added as a positive control, and 10 μl of DMSO as a negative control. The amount of DMSO or acetone in the medium did not exceed 0.5% of the total volume (50 μl per 10 ml).

After 3-day incubation, the cells were rinsed twice with 5 ml of ice-cold PBS. Then 1 ml of PBS was added, and the cells were scraped off the dish, pelleted with centrifugation and stored at −80°C. For assay of EROD activity, the cell pellets were thawed (+4°C) and sonicated (3×4 s, 10 μm from peak to peak), in 250 μl of ice-cold homogenizing buffer (50 mM Tris, 0.15 M KCl, pH 7.4). The *O*-deethylation of 7-ethoxyresorufin (EROD) was measured in a kinetic reaction with RF-5001PC spectrofluorometer (Shimadzu) at excitation wavelength of 530 nm and emission wavelength of 585 nm (Burke and Mayer, 1974). Cell sonicate (50–100 μl), Tris buffer (50 mM Tris, 25 mM $MgCl_2$, pH 7.5), 10 μl of 2 mM dicumarol and 7-ethoxyresorufin (1 μM in the final volume) were placed in a cuvette (total volume 2 ml). The reaction was initiated with 25 μl of 10 mM NADPH and measured at 30°C. The measurement was calibrated with 20 μl of 1 μM resorufin.

2.8. PLHC-1 biotests: time-course study and detection of CYP1A

A time-course study and detection of CYP1A were conducted with modifications of methods described by Hahn et al. (1996).

In the time-course study, 4×10^5 cells were first seeded in 0.5 ml of MEM into 48-well plates. On the next day the medium was replaced with fresh medium. The cells were dosed with different concentrations of the selected PAH fractions (diluted into DMSO + acetone) by adding 2.5 μl of each solution into the well. The diluent and 2 nM TCDD (final concentration) were used as controls. After 24-, 48- or 72-h exposure at 30°C, the medium was aspirated and the cells were washed with 0.5 ml of PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Then

200 μl of 2 nM 7-ethoxyresorufin in 50 mM sodium phosphate buffer (pH 8.0) was added in each well and the resorufin fluorescence was followed in a kinetic reaction with a multiwell plate reader (Cytofluor 2300 fluorescence plate reader, Millipore; 530 and 590 nm excitation and emission filters, respectively). Total protein content was measured after adding 100 μl of 1.08 mM fluorescamine (FA) in acetonitrile and incubating for 10 min at room temperature (Kennedy et al., 1995).

After the time-course study, 24 h was selected for the exposure time for detecting CYP1A. EROD activity and total protein content were measured simultaneously as endpoints. The *O*-deethylation of 7-ethoxyresorufin was initiated with 200 μl of 2 μM 7-ethoxyresorufin and the reaction was run for 10 min. The reaction was stopped with 100 μl of FA solution and the plate was allowed to sit for 10 min prior the measurement (Kennedy et al., 1995). For Western blot samples, the plates were treated with similar dosing as for plates used for EROD assay. After the exposure, the medium was aspirated, the cells were washed with ice-cold PBS and stored at −80°C. Whole cell lysates were prepared by solubilizing the cells on ice in 100 μl of sample treatment buffer (0.25 M Tris–HCl, pH 6.8, 40% (v/v) glycerol, 4% (w/v) sodium lauryl sulfate, 0.008% bromphenol blue, and 5% (v/v) 2-mercaptoethanol) (Hahn et al., 1996). After 10 min on ice, the plates were shaken, and the lysates were pipetted into Eppendorf tubes. Lysates in sample treatment buffer were placed in a thermocycler (Perkin Elmer) and heated to 95°C for 4 min. Samples and CYP1A standards (purified CYP1A1 from scup, *Stenotomus chrysops*) were analyzed by denaturing gel electrophoresis on 6–15% acrylamide gradient gels. Proteins were electrophoretically transferred onto Nytran nylon membranes (Schleicher and Schuell) and incubated with monoclonal antibody 1-12-3 (anti-scup CYP1A1; Park et al., 1986) at 10 μg ml^{-1}, then with goat anti-mouse IgG linked to alkaline phosphatase (Schleicher and Schuell; 1/5000 dilution). Color was developed by enhanced chemiluminescence as directed for the Schleicher and Schuell Rad-Free Chemiluminescence Detection Kit, using Kodak X-AR film. Fluorographs were digitized with a Kodak DCS200 digital camera and Adobe Photoshop, and band intensities were quantified by videoimaging densitometry using NIH Image software. Values for CYP1A equivalents were determined from the integrated optical density of the MAb 1-12-3 cross-reactive proteins relative to that of scup CYP1A1 standards.

2.9. Midge bioassays

The toxicity of whole sediments was assessed with two bioassays of a midge (*Chironomus riparius*): (1) a growth test (10 d), using larval growth and survival as endpoints, and (2) an emergence test (65 d), using larval survival and adult emergence as endpoints. Both tests were started with first instar larvae, which originated from at least three egg masses produced by laboratory cultured adults (original stock received from the Netherlands, maintained in our laboratory since 1990).

For both midge bioassays, 15 replicate test beakers with 10 g of wet sediment and 30 ml of artificial fresh water were prepared. The artificial water was prepared by adding the following inorganic salts to deionized water: $MgSO_4 \cdot 7\ H_2O$ (24.65 mg l^{-1}), $CaCl_2 \cdot H_2O$ (58.80 mg l^{-1}), KCl (1.15 mg l^{-1}) and $NaHCO_3$ (12.95 mg l^{-1}) (Ca + Mg hardness 0.5 mmol l^{-1}; Finnish Standards Association, 1984). In addition, sodium phosphate ($Na_2HPO_4 \cdot 2\ H_2O$ and $NaH_2PO_4 \cdot H_2O$, 1 mM) buffer solution was added and pH adjusted to 6.5. Test beakers were aerated overnight, after which one larva was transferred below the water level into each jar. Larvae were fed with an aqueous suspension of finely ground TetraMin® (TetraWerke) fish food. Daily food dose was 0.12 mg TetraMin® per larva.

Experiments were conducted at constant temperature (20 ± 2°C) and under a light regime of 16 h light and 8 h dark. In the growth test, overlying water in each test beaker was aerated constantly. In the emergence test, however, the beakers were aerated only until the beginning of emergence to avoid disturbance of emerging adults. After this aeration was continued at short intervals three times a week. Water lost due to evaporation was replaced with Millipore water. Temperature and

Table 1
Sediment characteristics in Lake Võrtsjärv (sites Salu and Jõesuu) and River Narva (sites Mustajõgi, Baltic TPP, Riigiküla 1 and Riigiküla 2), Estonia, and in Lake Höytiäinen (Höytiäinen 1 and Höytiäinen 2), Finland

	Salu	Jõesuu	Mustajõgi	Baltic TPP	Riigiküla 1	Riigiküla 2	Höytiäinen 1	Höytiäinen 2
Dry matter, %	9.7 ± 0.01	35.9 ± 0.05	50.2 ± 0.22	55.5 ± 0.17	52.2 ± 0.05	45.6 ± 0.07	23.6 ± 0.02	79.5 ± 0.63
Org. C, %	14.1 ± 0.04	4.4 ± 0.48	2.1 ± 0.11	3.8 ± 0.60	4.3 ± 0.62	23.1 ± 0.33	3.7 ± 0.02	0.3 ± 0.02
C:N	10.6 ± 0.06	12.5 ± 1.6	8.1 ± 2.0	19.5 ± 0.58	16.2 ± 0.19	63.6 ± 3.0	11.5 ± 0.10	8.1 ± 2.2
<63-μm particles, %	32.3 ± 4.7	33.9 ± 1.6	15.8 ± 2.8	19.8 ± 0.30	27.5 ± 1.2	60.7 ± 2.5	85.5 ± 1.2	6.5 ± 1.0

Mean ± S.D., $n = 3$.

dissolved oxygen in overlying water were monitored every 5 days in three randomly selected beakers of each set of replicates. Dissolved oxygen content of the water was mostly 60–85% of saturation throughout the tests, but on one occasion O_2 level of 52% was measured in a Riigiküla 2 sample.

At the end of the growth test (10 d), larvae were separated from the sediment by sieving (mesh size 63 μm). The surviving larvae were counted and their growth and development stage was determined by measuring body and head capsule length under a stereomicroscope. Total wet weight (ww) and dry weight (dw, at 60°C until constant weight) of each larvae was determined. The emergence test was continued as long as there were emerging adults. A net trap above each vessel retained the adults, which were counted and sexed daily.

2.10. Data analyses

Fluorescence data obtained from the Cytofluor plate reader were imported into SigmaPlot (Jandel Scientific) for analysis and curve fitting. Data obtained from EROD assays and densitometry of immunoblots were normalized to total cellular protein and analyzed by non-linear regression using the curve-fitting subroutine of SigmaPlot. EROD activity data obtained from exposures on 94/16-mm dishes and measured with Shimadzu spectrofluorometer were first calculated in SPSS for MS Windows (release 6.0) and the mean values (pmol min^{-1} mg^{-1} prot.) were used for curve fitting in SigmaPlot. Both sets of EROD data were fitted to a modified Gaussian function (for biphasic relationships) or to a logistic function (for sigmoid relationships) (Kennedy et al., 1993). In the case of data from exposures made on dishes, EC_{50} values were calculated for only those results for which EROD activity reached a plateau.

The results of the midge growth test were analysed using the Kruskall–Wallis non-parametric test and a non-parametric multiple comparison test by Dunn (1964) (Zar, 1996, p. 227). Survival data was analyzed using Fisher's exact test. Emergence curves of the surviving midges in different sediments were compared by Kaplan–Meier survival time analysis followed by Breslow statistics. Differences among sediments were considered statistically significant, when $P < 0.05$.

Principal component analysis (PCA; analysed with SYSTAT for the Machintosh) was used for classification of the sediment according to the results obtained in chemical analyses and bioassays. The general goal of multivariate modeling techniques (which include PCA) is to simplify the information in a complex data set. In PCA this is done by first creating a few PCs from many original variables and then plotting these PCs instead of plotting the individual variables. For the PLHC-1 cells, estimated NOEC (no observable effect concentration) values for EROD activity (7.1 mg ml^{-1} at Salu, 15.7 mg ml^{-1} at Jõesuu, 8.2 mg ml^{-1} at Mustajõgi, 79.7 mg ml^{-1} at Baltic TPP, 3.9 mg ml^{-1} at Riigiküla 1 and 0.11 mg ml^{-1} at Riigiküla 2) were used for PCA. Variables were autoscaled to unit variance before modelling.

Table 2
Content of selected polycyclic aromatic hydrocarbons (ng g^{-1} dw) in the sediments from Lake Võrtsjärv (sites Vehendi, Salu and Jõesuu), River Narva (sites Mustajõgi, Baltic TPP, Riigiküla 1 and Riigiküla 2) and Lake Höytiäinen (Höytiäinen 1)

Compound	Vehendi	Salu	Jõesuu	Mustajõgi	Baltic TPP	Riigiküla 1	Riigiküla 2	Höytiäinen 1[a]
3-Ring								
Phenanthrene	15.3	53.0	0.5	9.5	15.1	29.2	38 700	10
Anthracene	0.4	ND	ND	1.1	0.2	5.1	ND	0.9
4-Ring								
Fluoranthene	9.1	23.9	2.1	9.5	2.0	97.0	29 600	13
Pyrene	31.5	150.0	4.9	6.0	19.0	460.0	174 500	9.5
Benz(*a*)anthracene	3.9	14.8	2.8	3.1	1.9	38.8	24 400	ND
Chrysene	5.0	38.1	4.9	9.9	4.8	34.4	8360	ND
5-Ring								
Benzo(*e*)pyrene +								ND
Benzo(*b*)fluoranthene	4.7	40.4	5.2	11.5	8.2	18.0	1690	54
Benzo(*k*)fluoranthene	1.6	9.5	ND	ND	ND	11.3	372	ND
Benzo(*a*)pyrene	1.5	16.7	1.1	1.4	7.3	20.3	373	7.5
6-Ring								
Benzo(*ghi*)perylene	7.4	49.1	ND	ND	10.3	30.2	405	23
Total	80	396	22	52	69	744	278 400	118

ND, not detected.
[a] Data from Ristola et al. (1996a).

Table 3
Content of selected heavy metals (Cd, Cu, Pb, Hg) in the sediments from Lake Võrtsjärv (sites Vehendi, Salu and Jõesuu), River Narva (sites Mustajõgi, Baltic TPP, Riigiküla 1 and Riigiküla 2) and Lake Höytiäinen (Höytiäinen 1 and Höytiäinen 2)

Compound	Vehendi	Salu	Jõesuu	Mustajõgi	Baltic TPP	Riigiküla1	Riigiküla 2	Höytiäinen 1	Höytiäinen 2
Cd (mg kg^{-1})[a]	0.070	0.57	0.040	690	920	410	410	0.88[b]	ND
Cu (mg kg^{-1})	0.36	2.5	1.3	8.2	2200	3100	3400	–	–
Pb (mg kg^{-1})	4.6	22	6.1	5.9	12	28	24	–	–
Hg (μg kg^{-1})	130	66	7.0	50	24	40	93	103	24

ND, not detected; –, not analysed.
[a] Metal content (mg or μg) in dry weight sediment (kg).
[b] Lake Höytiäinen: measurement of Cd with Graphite Furnace Atomic Adsorption Spectrometry (Hitachi Z-9000), and of Hg with Gold Film Mercury Analyzer (model 511, Jerome Instrument Corporation).

3. Results

3.1. Sediment characteristics

The lowest dry matter content of the sediment was at Salu (9.7%) and the highest at Höytiäinen 2 (79.5%; Table 1). The other areas showed values ranging from 23.6 to 55.5%. Organic carbon content was higher at Salu (14.1%) and at Riigiküla 2 (23.1%) than at the other study sites (range from 0.3 to 4.4%; Table 1). C:N atomic ratio was especially high at Riigiküla 2 (63.6) when compared to the other sites (range from 8.1 to 19.5; Table 1). The percentage of fine particles in the sediment was high at Riigiküla 2 (60.7% <63 μm particle size) and at Höytiäinen 1 (85.5%; Table 1). The opposite was seen at Höytiäinen 2 (6.5%). At the other locations, the particle size of under 63 μm covered from 15.8 to 33.9% of all particles.

3.2. PAHs and heavy metals

In L. Võrtsjärv, Salu had a moderate total PAH content (396 ng g^{-1} dry sediment; Table 2). Vehendi and Jõesuu did not show such PAH pollution (80 and 22 ng g^{-1}, respectively).

In R. Narva, the total PAH content in the sediment of the reference Mustajõgi was less than at the contaminated areas (Table 2). The calculated total PAH content at this area was 52 ng g^{-1} dry sediment. At Baltic TPP, the total PAH content was a little higher (69 ng g^{-1}), and Riigiküla 1 was clearly polluted with PAHs (total 744 ng g^{-1}). However, the total PAH content in Riigiküla 2 sediment (278400 ng g^{-1}) was almost three orders of magnitude higher than in Riigiküla 1.

The contents of selected heavy metals are shown in Table 3. In L. Võrtsjärv, the content of Cd ranged from 0.040 to 0.570 mg kg^{-1} dry sediment and the content of Cu from 0.36 to 2.5 mg kg^{-1}. With both heavy metals the contents in R. Narva were several times higher, ranging from 410 to 920 mg kg^{-1} for Cd and from 8.2 to 3400 mg kg^{-1} for Cu. The differences in the Pb and Hg contents between these two waterbodies were not so drastic. In L. Võrtsjärv the content of Pb ranged from 4.6 to 22.0 mg kg^{-1} and in R. Narva from 5.9 to 28.0 mg kg^{-1}. The respective Hg contents ranged from 0.007 to 0.130 mg kg^{-1} and from 0.024 to 0.093 mg kg^{-1}.

3.3. Screening of cytotoxicity and EROD activity in the PLHC-1 cells

Extracts of sediments collected from R. Narva were more toxic and caused higher CYP1A induction in the cells than those from L. Võrtsjärv (Figs. 2 and 3). In L. Võrtsjärv, Vehendi and Salu sediment extracts were not toxic at doses up to 75

and 71 mg ml^{-1}, respectively. Sediment extracts from the northern part of the lake (Jõesuu) reduced the total protein content of the cells by 77% at a dry sediment dose of 105 mg ml^{-1} (Fig. 2A). The Salu sediment extracts increased EROD activity in the cells gradually up to 14 pmol min^{-1} mg^{-1}, reaching this activity at 71 mg ml^{-1} (Fig. 3A). A sample from Jõesuu elevated EROD activity up to 8 pmol min^{-1} mg^{-1} at 103 mg ml^{-1} dose (Fig. 3A).

In R. Narva, sediment extracts from Riigiküla 1 reduced the total protein content by 76% at 78 mg dry sediment ml^{-1} and by 21% at 83 mg ml^{-1} (Fig. 2B). The sediments at Mustajõgi acted similarly to the sediments received from Riigiküla 1 (reduction of 92 and 15% at 84 and 80 mg ml^{-1}, respectively). The more toxic one of the Riigiküla 1 sediments (see above) increased EROD activity most, reaching 20 pmol min^{-1} mg^{-1} at 31 mg ml^{-1} dose (ED_{50} 15 mg ml^{-1}; Fig. 3B). Mustajõgi sediment increased EROD activity maximally up to 19 pmol min^{-1} mg^{-1} at 58 mg ml^{-1} dose (ED_{50} 33 mg ml^{-1}). However, sediments from the area nearest to TPPs

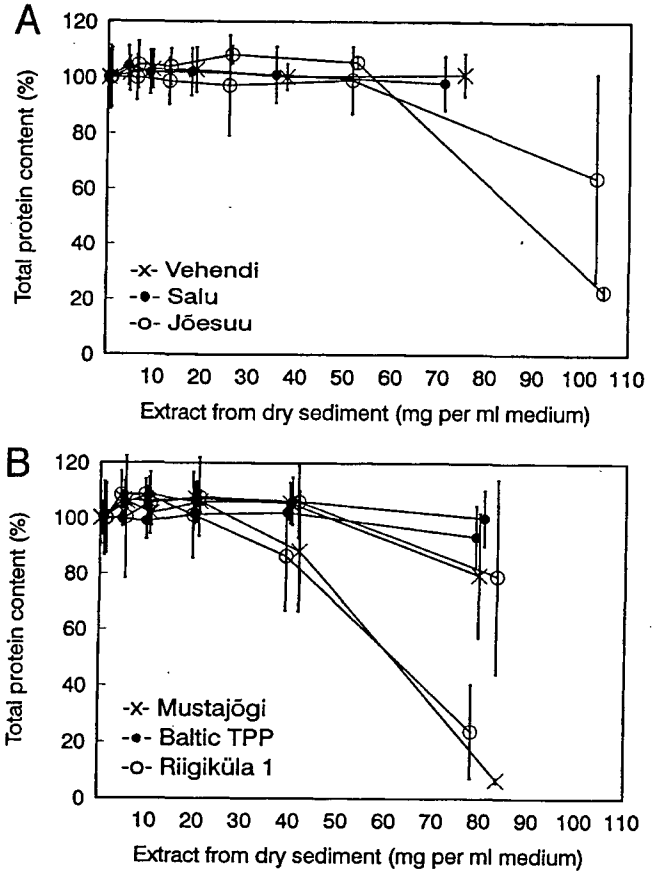

Fig. 2. Total protein content (%; 100% = control medium) of the PLHC-1 cells after 3-day exposure to the sediment extracts from (A) Lake Võrtsjärv and (B) River Narva. Mean ± S.D. from two to four experiments with different concentrations of the PAH fractions. Parallel samples from the same site are marked with the same symbol. DNP showed 50% reduction in total protein content at 33 ± 4 μg ml^{-1} (three cases). Different concentrations of the dichloromethane solvent did not cause cytotoxicity.

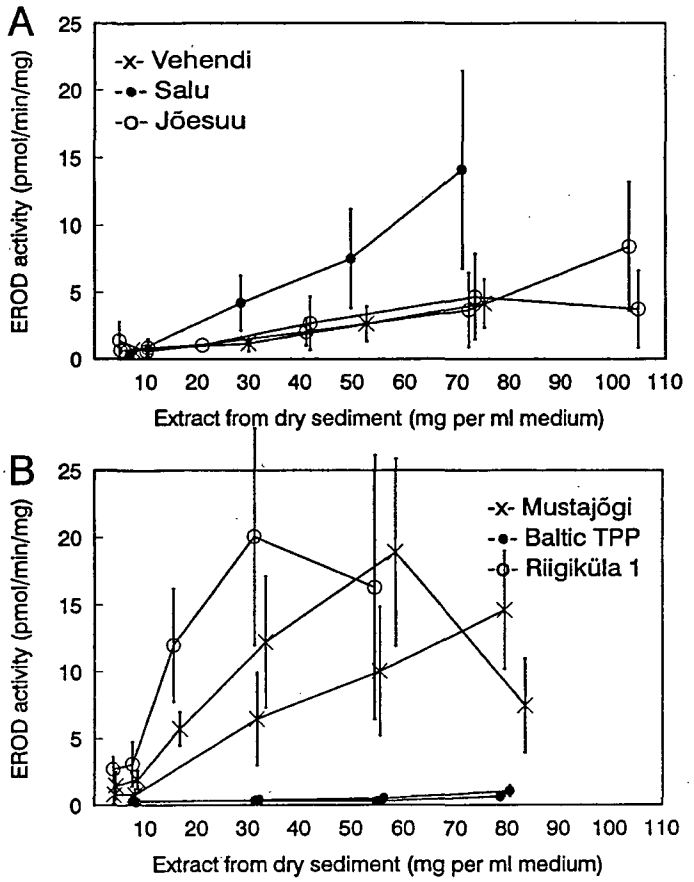

Fig. 3. 7-Ethoxyresorufin *O*-deethylase activity (EROD; pmol min^{-1} mg^{-1} protein) in the PLHC-1 cells after 3-day exposure to the sediment extracts from (A) Lake Võrtsjärv and (B) River Narva. Mean ± S.D. from two to four experiments with different concentrations of the PAH fractions. Parallel samples are marked with the same symbols. EROD activity for 2 nM TCDD was 199 ± 73 (six cases) and for DMSO 0.56 ± 0.39 (six cases). Different concentrations of the dichloromethane solvent did not induce EROD activity (not shown).

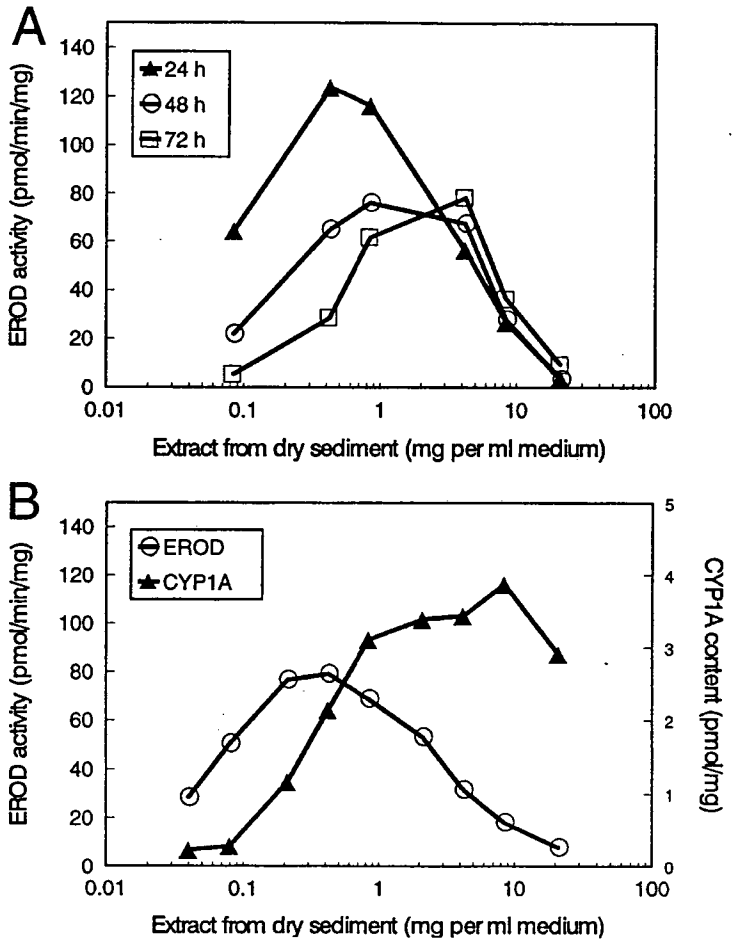

Fig. 4. (A) EROD activity (pmol min^{-1} mg prot.$^{-1}$) in the PLHC-1 cells after 24-, 48- and 72-h exposure to Riigiküla 2 sediment extracts and (B) EROD activity (pmol min^{-1} mg^{-1}) and CYP1A protein content (pmol mg^{-1}) in the PLHC-1 cells after 1-day exposure to the same sediment extracts. In the time-course study, EROD activities of 2 nM TCDD were 293, 211 and 214 pmol min^{-1} mg^{-1} after 24, 48 and 72 h, respectively. DMSO + acetone-treated cells showed EROD activities of 0.31, 0.21 and 0.12 pmol min^{-1} mg^{-1} after 24, 48 and 72 h, respectively. In the CYP1A induction detection, EROD activity of cells dosed with 2 nM TCDD was 200 pmol min^{-1} mg^{-1} and of DMSO + acetone – control 4.41 pmol min^{-1} mg^{-1}. TCDD treated cells induced CYP1A protein to 6.6 pmol mg^{-1}.

(Baltic TPP) were not toxic for the cells and did not elevate EROD activity.

As a comparison, a highly polluted site in R. Narva (Riigiküla 2) was examined (not shown). These sediment extracts reduced the total protein content by 97% at 67 mg ml^{-1} and by 80% at 42 mg ml^{-1}. Further, the EROD activity in the cells was highly induced, showing values as high as 51 pmol min^{-1} mg^{-1} at 1 mg ml^{-1} dose (ED_{50} values for parallel samples 0.59 and 0.56 mg ml^{-1}).

3.4. Time-course study and CYP1A in the PLHC-1 cells

A time-course study was conducted with the PAH fractions that were the most potent inducers of EROD activity in the PLHC-1 screening experiment (one sediment from Riigiküla 1, Fig. 3, and two samples from Riigiküla 2, not shown). For these samples, the apparent potency for induction of EROD activity was greatest after 24-h exposure, and declined thereafter. Thus, a maximum

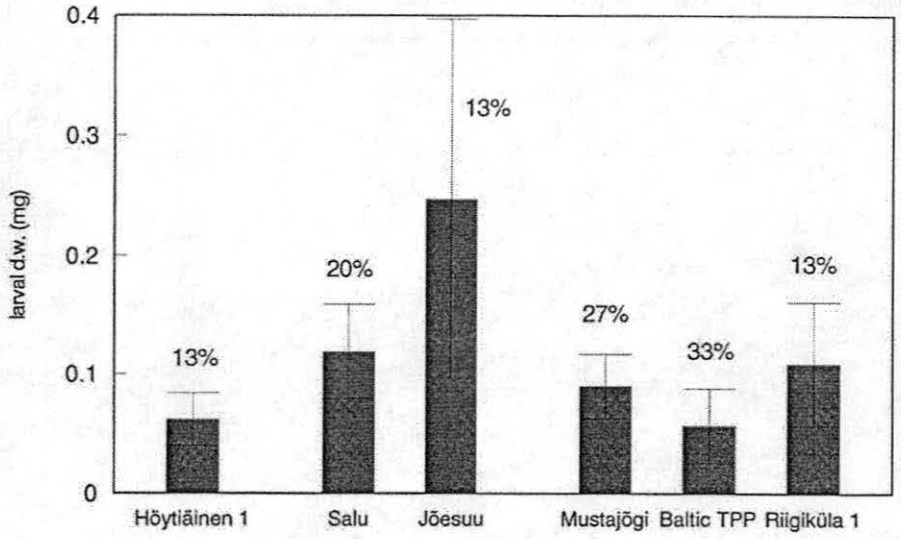

Fig. 5. Growth (measured as dry weight, mg per larva) of the midge *Chironomus riparius* after a 10-day exposure to the whole sediments from Lake Vorstjärv (Salu, Jõesuu) and River Narva (Mustajõgi, Baltic TPP, Riigiküla 1), Estonia, as well as from a reference site at Lake Höytiäinen, Finland. Mean ± S.D., number of larvae 10–13. Percent mortality of the larvae is expressed above the bars.

EROD activity occurred at lower doses of PAH fractions after 24 h than after 48 h (Fig. 4A). Further, the pattern continued showing maximum values at lower doses after 48 h than after 72 h.

Immunodetectable CYP1A protein was measured to determine whether the decrease in EROD activity at higher doses of some PAH fractions was due to inhibition of catalytic activity or due to the decline in CYP1A levels. CYP1A protein content was elevated at doses where catalytic activity was diminished after reaching the maximum induction (Fig. 4B). The CYP1A protein content continued to increase somewhat linearly after the decline of EROD activity (not shown) or reached a plateau (Fig. 4B). ED_{50} values for EROD activity were 2.6, 0.063 and 0.062 mg ml^{-1} for one Riigiküla 1 sample and two Riigiküla 2 samples, respectively. The respective ED_{50} values for CYP1A content were 9.3, 0.45 and 0.27 mg ml^{-1}.

3.5. Midge bioassays

In both midge bioassays, all larvae exposed to Riigiküla 2 sediments died. At the other sites, mortality did not differ significantly from the reference sediment though it was 27 and 33% in sediments Mustajõgi and Baltic TPP, respectively (Fig. 5).

In most of the test sediments, larvae grew equally well (Baltic TPP) or better than in the reference sediment during the 10-day bioassay (Fig. 5). Larval development was slowest in the reference and in the Baltic TPP sediment, where only 38 or 40% of the larvae had reached fourth instar, respectively. Larvae exposed to the Salu sediment developed significantly faster than those in the reference sediment ($P = 0.015$) and all the survivors were already fourth instars.

The fast larval development in the Salu sediment was reflected also in adult emergence rate, which was accelerated compared to the L. Höytiäinen reference sediment ($P = 0.040$; Fig. 6). Emergence was fast also in larvae exposed to Mustajõgi sediment, but the difference from the reference was only nearly significant ($P = 0.080$). In the Jõesuu sediment emergence was slightly delayed (Fig. 6) though this trend was not statistically significant ($P = 0.295$).

3.6. Principal component analysis

A PCA was used to classify sediments according to pollution level and toxicity. Riigiküla 2 was excluded from the analysis because its extreme contamination and toxicity could have masked

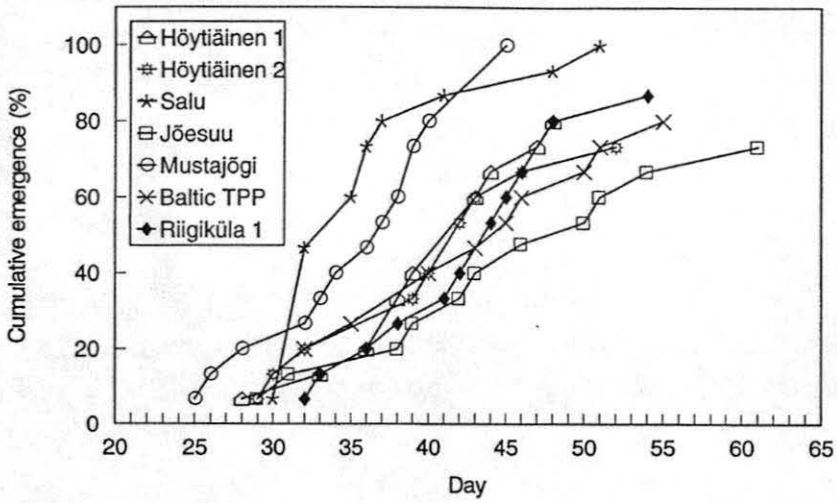

Fig. 6. Cumulative emergence (%) in the midge emergence test. Number of exposed larvae was 15 for each sediment. For further information see Fig. 5.

smaller differences among the other samples. PCA also requires that all the sites have values for all the parameters. We did not have cell results from Höytiäinen 1 and Höytiäinen 2 or midge results from Vehendi; therefore these sites were not analyzed in PCA.

A three-compartment PCA explained 89% of the total variance in the results. Cd and Hg concentrations, mortality in the growth test and NOEC for EROD activity in the cell assay got high positive and larval dry weight negative loadings on the first principal component (PC1, $r^2 = 36\%$). The second (PC2, $r^2 = 25\%$) was characterized by negative correlation with sediment Cu, Pb and total PAH concentration. Median emergence day and mortality in emergence test, proportion of third instar larvae in the end of the growth test as well as organic carbon content (the last one by negative loadings) characterized the third PC ($r^2 = 28\%$).

When the factor scores of PC1 and PC2 were plotted, high PAH, Cu and Pb levels isolated sediment Riigiküla 1 to the negative end of PC2 (Fig. 7A). The other sediment had scores which were near to zero on this axis. Sediment Jõesuu with low Cd and Hg concentration and high larval biomass in the growth test is located in the other side of PC1 from sediments Mustajõgi and Baltic TPP, which have opposite characteristics. PC3 plotted with PC1 gave negative scores on PC3 for sediments with high emergence rate and low mortality (Salu and Mustajõgi; Fig. 7B). The other sediments do not differ from each other on this axis.

4. Discussion

4.1. General aspects

Two inland waterbodies were monitored with a fish liver cell line (PLHC-1) and midge toxicity tests. These bioassays represented two approaches in detecting the effects of surficial sediments. The PLHC-1 cell line responded to the extracted lipid soluble compounds in the sediments. The midge assays revealed the effects of whole sediment and included bioaccumulation processes. Because the PLHC-1 bioassays were designed to monitor the effects of a certain type of compounds using sensitive and specific biomarkers, it is not surprising that the responses in the cell assays were stronger than in the midge assays. Though not as sensitive as the in vitro cell culture tests, the midge assays were able, however, to give more ecotoxicological knowledge of the effects of the pollutants.

Aquatic sediments typically contain concentrations of contaminants higher than those in overlying waters. Pore-water, being physically near the

sediments, has been shown to contain considerable amounts of pollutants. In our study, we did not measure the biological effects of the overlying or pore-water separately. Munkittrick et al. (1995) have studied the CYP1A inducing effects of the sediments and bottom waters on rainbow trout (*Oncorhynchus mykiss*) exposed in the laboratory. Fish responded to chemicals from the sediments, but not from bottom water. Harkey et al. (1994) compared the bioavailability of selected neutral hydrophobic contaminants in whole-sediment, elutriate and pore-water. In most cases the accumulation in freshwater benthic invertebrates, *Diporeia* spp., *Chironomus riparius* larvae and *Lumbriculus variegatus*, was less from aqueous extracts than from whole sediment. These studies provide evidence that sediment assessment experiments do not necessarily require the collection of overlying or pore-water as part of the test protocol.

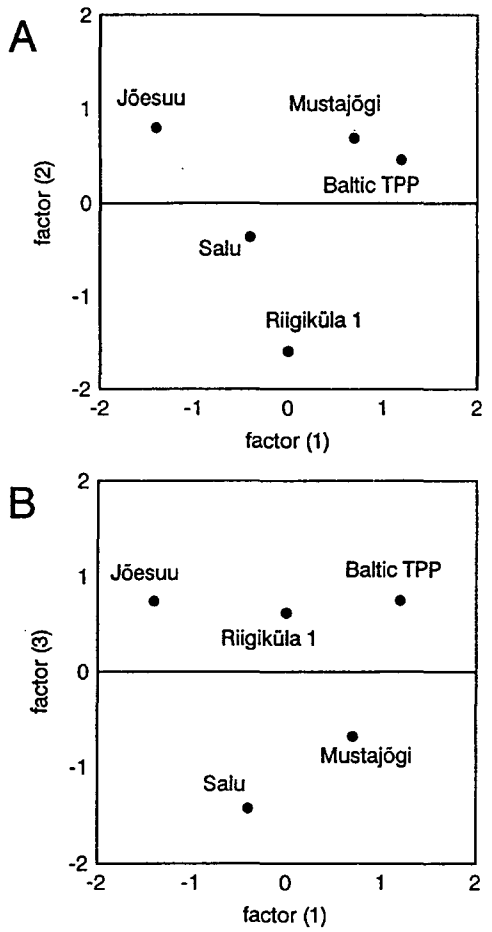

Fig. 7. Factor scores in principal component analysis (PCA); (A) PC1 versus PC2 as plotted, (B) PC1 versus PC3 as plotted. The extracted factors represent the underlying variables that explain all the covariability among chemical and toxicological variables.

Diffusion, bioturbation, and biodegradation of contaminants may be important in modifying their distribution in sediments. Complete burial may take many years (Schindler et al., 1995). We selected the upper layer of the sediment for our study material. The concentrations of contaminants in the top of sediments can be high; for example Wilcock et al. (1996) observed that the mass of selected PAHs was concentrated in the top 2 cm of interdial sandflats, where most losses also occurred. Further, downward movement of PAHs was slow. It can be concluded that surficial sediments are under continuous impact of the surroundings but are most relevant for effects on the biota.

4.2. PAH fraction and the PLHC-1-cells

The PAH fraction of some sediments was able to cause a manyfold increase of EROD activity in the PLHC-1 cells, when compared to control cells treated with the solvent. In previous in vitro studies with aquatic sediments, Mátlová et al. (1995) observed that the PAH fraction of river sediments caused CYP1A1 induction in mammalian Hepa-1 cell culture, though at higher concentrations EROD activity was decreased. This kind of suppression of catalytic enzyme activity was detected in some R. Narva samples in our study, as well. When CYP1A protein content was measured in these samples, induction of CYP1A was maintained even at the higher sediment doses. The phenomenon of inhibited catalytic activities in the PLHC-1 cells, which may mislead the interpretation of CYP1A induction results, has been previously described with both halogenated aromatic hydrocarbons (HAHs) and PAHs (Hahn et al., 1993, 1996; Brüschweiler et al., 1996; Celander et al., 1997). Our study indicates that the determination of CYP1A induction with PLHC-1 cells requires the measurement of CYP1A immunoprotein as part of the protocol when environmental samples with high or moderate PAH

pollution are monitored. However, as seen with the extremely polluted sediment from Riigiküla 2, CYP1A protein content also decreased when the doses were sufficiently high. Thus, both EROD activity and CYP1A content can have biphasic induction curves, as seen also in earlier studies with model compounds (Hahn et al., 1993, 1996; Lorenzen et al., 1997). Hahn et al. (1996) estimated relative potencies of the polychlorinated biphenyl (PCB) congeners as compared to TCDD. The apparent potencies calculated from EROD data were an order of magnitude greater than those calculated from CYP1A data. This trend was seen also in our samples when ED_{50} values of EROD activity and CYP1A protein content were compared.

In all the samples studied, CYP1A induction started at sublethal doses of PAH fraction. The same PAHs do not necessarily cause both CYP1A induction and, at higher doses, cytotoxicity. Still, the function of CYP1A at lower doses of the sediments may produce metabolites that can cause the death of the cells at higher doses. Evidence that the metabolism of PAHs really occurs in the PLHC-1 test system was obtained in the time-course study where the EROD-inducing potency decreased with exposure time. Celander et al. (1997) compared CYP1A induction by β-naphthoflavone (BNF, a PAH-like compound) in the PLHC-1 cells to that obtained with TCDD and observed noticeable differences between BNF and TCDD in the duration of induction, consistent with a greater rate of metabolism of BNF than of TCDD. Similar results were obtained by Pesonen et al. (1992) in trout hepatocytes. The presence of HAHs was not measured in our samples. If they were present in dichloromethane extracts, the effects of HAHs may have become more pronounced in the course of the experiment whereas the role of PAHs may have diminished.

Temporal studies with fish in vivo have also shown that experiment duration affects CYP1A induction. Zhang et al. (1990) injected rainbow trout with a low dose (50 $\mu g\ kg^{-1}$ body weight) or high dose (50 $mg\ kg^{-1}$) of BNF and followed the time-course effects of these treatments. More than 8 weeks was not long enough to allow CYP1A enzyme activities to return to basal levels after treatment with a high dose, whereas a recovery period of 8 days was long enough after treatment with a low dose. Van der Weiden et al. (1992) observed that 0.60- and 3.06-$\mu g\ kg^{-1}$ doses of TCDD still significantly induced EROD activity after 6 and 12 weeks after administration, whereas after treatment with a 0.30-$\mu g\ kg^{-1}$ dose EROD activity attained background levels after 12 weeks.

The total PAH contents in our sediments ranged from 22 to 744 ng PAH g^{-1} dry sediment at the actual study areas. At the asphalt release site of Riigiküla 2 the total PAH content reached 278400 ng g^{-1} dw sediment. Van Veld et al. (1990) collected fish over a sediment PAH concentration gradient that ranged from 9 to 96000 ng PAH g^{-1} dry sediment. In that study, liver CYP1A and associated EROD activity were detectable in all samples and were induced approximately eightfold at the most heavily contaminated site. Further, liver CYP1A correlated well with sediment PAH content. In a laboratory experiment of Munkittrick et al. (1995), CYP1A catalytic activities in rainbow trout paralleled more closely PAH levels in the sediments than the observed PCB concentrations. In our survey, at the sites more heavily loaded with PAHs, the potency of CYP1A induction in the PLHC-1 cells followed the order of the total PAH content (Salu < Riigiküla 1 < Riigiküla 2). However, the EROD induction did not follow the total content of PAHs in every case. For example, the effects of Mustajõgi and Baltic TPP sediment extracts were different though their total PAH contents were similar. Not all the PAHs are capable of causing CYP1A induction. Besides, different variations of synergistic or antagonistic effects may appear in a test system that contains a mixture of compounds. These are some of the factors that may complicate the interpretation of the results.

The percentage of 4-ring PAHs of the total PAH content was extremely high (85%) at the sites of Riigiküla. This was mostly due to high percentage of pyrene (over 60% of the total PAHs). When Van der Weiden et al. (1994) studied the temporal induction of CYP1A in the mirror carp (*Cyprinus carpio*), pyrene was not as strong an inducer as benzo(*a*)pyrene or chrysene.

In our study, however, pyrene was present in such amounts at Riigiküla (460 and 174500 ng g^{-1} dry sediment) that it could have been the main inducer of EROD activity. Fent and Baetscher (1998) listed the relative potencies of selected PAH compounds in a PLHC-1 test system. Five PAHs were the same as in our chemical analyses, namely benzo(*k*)fluoranthene, benzo(*a*)pyrene, chrysene, benz(*a*)anthracene and pyrene. When PAH-specific equivalent concentrations for our PAH chemical data were calculated according to Fent and Baetscher (1998), pyrene, indeed, had the highest equivalent concentration (EC) at Riigiküla 2 (not shown).

4.3. Variety of factors affecting the midges

There were no drastic changes in the growth or survival of the midge larvae, except in the presence of sediments from Riigiküla 2, which was heavily polluted especially with PAHs but also with Cd and Cu. Indeed, Cd and Cu concentrations in other R. Narva sediments were also sufficiently high to have been detrimental to benthic species (Persaud et al., 1992). In addition, PCA analysis showed an association between low larval biomass and high Cd and Hg levels at the Mustajõgi and Baltic TPP sites. Lack of more severe toxic responses could reflect the capacity of the midges to cope with the pollutants even when the environment is considerably loaded. While the PLHC-1 cells respond to the chemicals as such and have little possibility to avoid the loading in the test system, the midges might selectively use their physiological properties, e.g. in intake, metabolism and excretion of the pollutants, to minimize the hazard. Furthermore, the bioavailability and bioaccumulation properties of the compounds can affect the response seen in the midges. Additional experiments using chemical or biochemical indices of PAH and metal exposure will be needed to understand the low response of midges in this study.

Accelerated adult emergence in Salu and Mustajõgi sediments may reflect effects of chemical contamination. This type of toxic response has been observed also in other studies. For example, when stream invertebrates were exposed to atrazine, insects emerged earlier from the treatment mesocosms than from controls (Gruessner and Watzin, 1996). A possible explanation for this is that atrazine triggered a physical response in some insects resulting in earlier emergence. Also Lowell et al. (1995) have observed that exposure to biologically-treated, bleached-kraft pulp mill effluent stimulated growth and emergence of mayfly (*Baetis tricaudatus* Dodds). The authors suggested that the stimulatory effect involved more than just an increase in food availability and that the effluent may have stimulated mayfly growth directly via hormonal or other growth-stimulation effects.

Some general characteristics of the sediment could affect the midge bioassays, as well. The percentage of fine-grained particles was high at Riigiküla 2 and at Höytiäinen 1. Furthermore, organic carbon content was elevated at Salu and at Riigiküla 2. These findings fail to explain the severe toxicity caused by Riigiküla 2 sediment. The high organic carbon content at Salu, however, could be connected with the rapid development of larvae and emergence of adults through improved food supply. Suedel and Rodgers (1994) observed that *Chironomus tentans* tolerated a wide variety of particle-size regimes and organic matter content of sediments. The authors suggested that reduced survival due to physical characteristics rather than chemical contamination does not result until *C. tentans* are exposed to sediments with organic matter content $< 0.91\%$. In our study, fed midges grew well in the sediment from Höytiäinen 2 where organic carbon content was only 0.3%. Earlier bioassays with various unpolluted sediments and a similar feeding regime as here (Ristola et al., 1998) also suggest that sediment organic content would not cause the kinds of differences in larval growth, development rate or survival observed in this study. Biological factors, like the amount of food available and rearing density, can also be of great importance in the midge bioassays. To avoid the unnecessary interference of the biological factors in our test system, the midges were fed during the experiment and raised individually. Therefore, it is suggested that the observed responses in midge bioassays were caused by chemical contamination, not by sediment general characteristics.

4.4. Comparison of the PLHC-1 and midge biotests

In our surveys, surficial sediments showed stronger biological responses in the PLHC-1 cells than in the midges. This can be partly explained by the selection of parameters, e.g. CYP1A is very specific for one class of chemicals. In addition, other factors may affect the difference. First, the samples in the PLHC-1 bioassays were extracted, thus being concentrated and potentially more bioavailable to the cells. However, bioavailability in the midge test is not known. Second, the exposure times in our bioassays were different. PAHs are metabolized and excreted relatively rapidly in fish and fish cells (Collier and Varanasi, 1991; Celander et al., 1997). There is evidence also of rapid biotransformation of PAHs, such as benzo(*a*)pyrene, in *Chironomus riparius* (Leversee et al., 1982). Due to the relatively short-term exposures, the PLHC-1 bioassays likely reflect the biological effects of slightly metabolized PAHs as supported by results of the time-course study. In the midge assays, in contrast the PAHs may have been more transformed during the exposure. Our study does not, however, clarify which were the possible metabolites and whether the metabolites were less or more active than the parent PAHs.

When the results from the two bioassays were compared, the toxicity of the Riigiküla 2 sediment was easily observed with both bioassays. However, the correlations with the other samples were not so clear. Larval mortality was somewhat higher at Mustajõgi and at Baltic TPP than in the reference sediments. However, only the Mustajõgi sample was toxic for the PLHC-1 cells. In Jõesuu sediment, the adult emergence of the midge was slightly delayed. This sample was toxic for the PLHC-1 cells. Adults emerged early from Mustajõgi and Salu sediments. Both of these samples had a clear induction of EROD activity in the PLHC-1 cells. These examples show that certain trends between these test systems can be found, though the interpretation of the results may not be exact or easy. Therefore, PCA were run to statistically compare the biological and chemical data.

As seen in PCA, some changes in midge bioassays were connected with low or high metal concentrations. In the PLHC-1 bioassays, metals were not present in the extracts. Apparently PLHC-1 cells are affected by PAHs but the midges by other pollutants, as well. This could also explain why according to PCA the less potent sediments for the cells (high NOEC for EROD activity) would have been deleterious for the midges (mortality high, larval dry weight low). In the future it would be useful to study the effects of metal contamination on the PLHC-1 cells. This approach has been used in a study of Ryan and Hightower (1994) where heavy-metal ion toxicity in fish cells was detected using a combined stress protein and cytotoxicity assay. Gunther et al. (1997) compared the survival of amphipods in sediment bioassays to induction of EROD activity in speckled sanddabs (*Citharichthys stigmaeus*). The authors documented a highly significant correlation between the responses of these invertebrate and fish bioassays, suggesting that sediment contaminants were causing the effects in both assays.

The loadings of study sites in PCs, when several parameters were included into the PCA, seemed to reveal even slight biological changes in midge assays better than those in PLHC-1 assays. In this way, PCA served especially midge assays in showing the agents correlating with biological effects. The studied water areas have different histories of pollution, L. Võrtsjärv being contaminated mainly with PAHs and R. Narva also with other pollutants, like sulphates, chlorides and heavy metals. In this sense, the exposures with the PAH fraction on the PLHC-1 cells failed to express the overall pollution in R. Narva. In the midge bioassays with whole sediments, however, all the pollutants, depending on their bioavailability, can affect the larvae.

4.5. Validity of reference areas

In R. Narva, the sediments from the proposed reference area showed both cytotoxicity and EROD induction in the PLHC-1 cells. In L. Võrtsjärv, however, the middle site of the lake was appropriate to be used as a reference area when

the responses of the cells were measured. The total PAH content in Mustajõgi and Vehendi sediments was almost the same. It is possible that the Mustajõgi sediment extract contained, in addition to PAHs, also other lipid soluble compounds capable of affecting the cells.

Mortality in the midge growth test was slightly elevated in Mustajõgi sediment. Indeed, based on our experiments, this area seems not to be a valid reference area for further studies. Probably Mustajõgi is situated too close to the heavy pollution sources. The contaminants may have entered this water area, for example, via air. Höytiäinen 1 and Höytiäinen 2, however, provided suitable reference material in midge assays. The Vehendi area was, unfortunately, not studied in the midge assays.

5. Conclusion

The PLHC-1 cells responded sensitively to the PAH fraction of the surficial sediments. In this way, the cells proved their strength in categorizing different sites with respect to sediment PAH contamination. However, the effects seen in the PLHC-1 cells represented more likely the potential than the actual hazard of lipid soluble compounds in the sediments. While the PLHC-1 bioassays expressed the toxicological and physiological effects of only a certain type of pollutants, the PAH fraction, the midge bioassays expressed the toxicological effects of the whole sediment. Therefore, the midges were able to respond, e.g. to heavy metals, as well as PAHs, and thus may be a more relevant indicator of the severity of the harmful effects caused by pollution. Differences in the responses of the studied bioassays suggest that test batteries may help to clarify the effects of aquatic pollution from various perspectives.

Acknowledgements

We thank Marina Trapido for chemical analysis of PAHs and Ülis Sõukand for analysis of heavy metals. We are grateful to Lawrence E. Hightower for the PLHC-1 cells and to John J. Stegeman for the antibody MAb 1-12-3. We appreciate the cooperation of Kari Koponen, Ossi Ritola and Sue Bello, the helpful comments of Päivi Kopponen as well as the technical assistance provided by Riitta Venäläinen, Eeva-Liisa Palkispää and Pälvi Pöyhönen. This research was supported by the Academy of Finland (Research Council for Environmental Sciences and for Environment and Natural Resources, project 3036), the Finnish Ministry of Agriculture and Forestry (Veterinary and Food Department) and the Saastamoinen Foundation as well as by the Finnish Graduate School for Integrated Aquatic Hazard Assessment, the Estonian Science Foundation (grant number 2397) and Sven and Dagmar Salén Foundation. This research was also supported in part by the U.S.N.O.A.A. (grant NA46RG0470; W.H.O.I. Sea Grant project No. R/B-124). Contribution No. 9465 from the Woods Hole Oceanographic Institution.

References

Ankley, G.T., Collyard, S.A., Monson, P.D., Kosian, P.A., 1994. Influence of ultraviolet light on the toxicity of sediments contaminated with polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 13 (11), 1791–1796.

Babich, H., Rosenberg, D.W., Borenfreund, E., 1991. In vitro cytotoxicity studies with the fish hepatoma cell line, PLHC-1 (*Poeciliopsis lucida*). Ecotoxicol. Environ. Saf. 21, 327–336.

Besser, J.M., Kubitz, J.A., Ingersoll, C.G., Braselton, W.E., Giesy, J.P., 1995. Influences on copper bioaccumulation, growth, and survival of the midge, *Chironomus tentans*, in metal-contaminated sediments. J. Aquat. Ecosys. Health 4, 157–168.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brüschweiler, B.J., Würgler, F.E., Fent, K., 1995. Cytotoxicity in vitro of organotin compounds to fish hepatoma cells PLHC-1 (*Poeciliopsis lucida*). Aquat. Toxicol. 32, 143–160.

Brüschweiler, B.J., Würgler, F.E., Fent, K., 1996. An ELISA assay for cytochrome P4501A in fish liver cells. Environ. Toxicol. Chem. 15 (4), 592–596.

Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorometric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2, 583–588.

Celander, M., Näf, C., Broman, D., Förlin, L., 1994. Temporal aspects of induction of hepatic cytochrome P4501A and

conjugating enzymes in the viviparous blenny (*Zoarces viviparus*) treated with petroleum hydrocarbons. Aquat. Toxicol. 29, 183–196.

Celander, M., Hahn, M.E., Stegeman, J.J., 1996. Cytochromes P450 (CYP) in the *Poeciliopsis lucida* hepatocellular carcinoma cell line (PLHC-1): dose- and time-dependent glucocorticoid potentiation of CYP1A induction without induction of CYP3A. Arch. Biochem. Biophys. 329 (1), 113–122.

Celander, M., Bremer, J., Hahn, M.E., Stegeman, J.J., 1997. Glucocorticoid-xenobiotic interactions: dexamethasone-mediated potentiation of cytochrome P4501A induction by β-naphthoflavone in a fish hepatoma cell line (PLHC-1). Environ. Toxicol. Chem. 16 (5), 900–907.

Cerniglia, C.E., 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. 30, 31–71.

Collier, T.K., Varanasi, U., 1991. Hepatic activities of xenobiotics metabolizing enzymes and biliary levels of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. Arch. Environ. Contam. Toxicol. 20, 462–473.

Dunn, O.J., 1964. Multiple contrasts using rank sums. Technometrics 6, 241–252.

Engwall, M., Brunström, B., Brewer, A., Norrgren, L., 1994. Cytochrome P4501A induction by a coplanar PCB, a PAH mixture, and PCB-contaminated sediment extracts following microinjection of rainbow trout sac-fry. Aquat. Toxicol. 30, 311–324.

Fent, K., Baetscher, R., 1998. Assessing toxic potencies of polycyclic aromatic hydrocarbons in environmental samples using a fish hepatoma cell line PLHC-1 (manuscript in preparation).

Finnish Standards Association, 1980a. Metal content of water, sludge and sediment determined by atomic absorption spectroscopy, atomization in flame. General principles and guidelines (in Finnish). Standard SFS 3044. Finnish Standards Association, Helsinki.

Finnish Standards Association, 1980b. Metal content of water, sludge and sediment determined by atomic absorption spectroscopy, atomization in flame. Special guidelines for lead, iron, cadmium, cobalt, copper, nickel and zinc (in Finnish). Standard SFS 3047. Finnish Standards Association, Helsinki.

Finnish Standards Association, 1984. Water quality. Determination of the acute toxicity with water flea, *Daphnia magna* Straus (in Finnish). Standard SFS 5062. Finnish Standards Association, Helsinki.

Fryer, J.L., McCain, B.B., Leong, J.C., 1981. A cell line derived from rainbow trout (*Salmo gairdneri*) hepatoma. Fish Pathol. 15, 193–200.

George, S.G., Wright, J., Conroy, J., 1995. Temporal studies of the impact of the Braer oilspill on inshore feral fish from Shetland, Scotland. Arch. Environ. Contam. Toxicol. 29, 530–534.

Gruessner, B., Watzin, M.C., 1996. Response of aquatic communities from a Vermont stream to environmentally realistic atrazine exposure in laboratory microcosms. Environ. Toxicol. Chem. 15 (4), 410–419.

Gunther, A.J., Spies, R.B., Stegeman, J., Woodin, B., Carney, D., Oakden, J., Hain, L., 1997. EROD activity in fish as an independent measure of contaminant-induced mortality of invertebrates in sediment bioassays. Mar. Environ. Res. 44 (1), 41–49.

Hahn, M.E., Lamb, T.M., Schultz, M.E., Smolowitz, R.M., Stegeman, J.J., 1993. Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an *Ah* receptor-containing fish hepatoma cell line (PLHC-1). Aquat. Toxicol. 26, 185–208.

Hahn, M.E., Woodward, B.L., Stegeman, J.J., Kennedy, S.W., 1996. Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: response to TCDD, TCDF, and two planar PCBs. Environ. Toxicol. Chem. 15 (4), 582–591.

Harkey, G.A., Landrum, P.F., Klaine, S.J., 1994. Comparison of whole-sediment, elutriate and pore-water exposures for use in assessing sediment-associated organic contaminants in bioassays. Environ. Toxicol. Chem. 13 (8), 1315–1329.

Hightower, L.E., Renfro, J.L., 1988. Recent applications of fish cell culture to biomedical research. J. Exp. Zool. 248, 290–302.

Hodson, P.V., 1988. The effect of metal metabolism on uptake, disposition and toxicity in fish. Aquat. Toxicol. 11, 3–18.

Kennedy, S.W., Lorenzen, A., James, C.A., Collins, B.T., 1993. Ethoxyresorufin *O*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multi-well plate reader. Anal. Biochem. 211, 102–112.

Kennedy, S.W., Jones, S.P., Bastien, L.J., 1995. Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. Anal. Biochem. 226, 362–370.

Khesina, A.Y., Hitrovo, L.A., Gevorkyan, B.Z., 1983. On the quantitive determination of polycyclic aromatic hydrocarbons in environmental pollutants utilizing luminescence and fluorescence spectrometry (in Russian). J. Appl. Spectrom. 6, 928–934.

Krantzberg, G., 1994. Spatial and temporal variability in metal bioavailability and toxicity of sediment from Hamilton Harbour, Lake Ontario. Environ. Toxicol. Chem. 13 (10), 1685–1698.

Landrum, P.F., Harkey, G.A., Kukkonen, J., 1996. Evaluation of organic contaminant exposure in aquatic organisms: the significance of bioconcentration and bioaccumulation. In: Newman, M.C., Jagoe, C.H. (Eds.), Ecotoxicology: A Hierarchial Treatment. CRC Press, Boca Raton, FL, pp. 85–131.

Lee, L.E.J., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K.-B., Pasitschniak-Arts, M., Mosser, D.D., Bols, N.C., 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biol. Toxicol. 9 (3), 279–294.

Leversee, G.J., Giesy, J.P., Landrum, P.F., Gerould, S., Bowling, J.W., Fannin, T.E., Haddock, J.D., Bartell, S.M., 1982. Kinetics and biotransformation of benzo(*a*)pyrene in *Chironomus riparius*. Arch. Environ. Contam. Toxicol. 11, 25–31.

Lorenzen, A., Kennedy, S.W., Bastien, L.J., Hahn, M.E., 1997. Halogenated aromatic hydrocarbon-mediated porphyrin accumulation and induction of cytochrome P4501A in chicken embryo hepatocytes. Biochem. Pharmacol. 53, 373–384.

Lowell, R.B., Culp, J.M., Wrona, F.J., 1995. Stimulation of increased short-term growth and development of mayflies by pulp mill effluent. Environ. Toxicol. Chem. 14 (9), 1529–1541.

Mátlová, L., Machala, M., Nezveda, K., Granátová, M., Nevoránková, Z., 1995. Biochemical screening of highly toxic aromatic contaminants in river sediment and comparison of sensitivity of biological model systems. Chemosphere 30 (7), 1363–1371.

Munkittrick, K.R., Blunt, B.R., Leggett, M., Huestis, S., McCarthy, L.H., 1995. Development of a sediment bioassay to determine bioavailability of PAHs to fish. J. Aquat. Ecosys. Health 4, 169–181.

Neff, J.M., 1985. Polycyclic aromatic hydrocarbons. In: Rand, G.M., Petrocelli, S.R. (Eds.), Fundamentals of Aquatic Toxicology: Methods and Applications. Hemisphere Publishing, New York, pp. 416–454.

Ots, A., 1992. Formation of air-polluting compounds while burning oil shale. Oil Shale 9 (1), 63–75.

Park, S.S., Miller, H., Klotz, A.V., Kloepper-Sams, P.J., Stegeman, J.J., Gelboin, H.V., 1986. Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): crossreactivity with 3-methylcholanthrene induced rat cytochrome P-450. Arch. Biochem. Biophys. 249, 339–350.

Payne, J.F., Penrose, W.R., 1975. Induction of aryl hydrocarbon (benzo(*a*)pyrene) hydroxylase in fish by petroleum. Bull. Environ. Contam. Toxicol. 14, 112–116.

Persaud, D., Jaagumagi, R., Hayton, A., 1992. Guidelines for the protection and management of aquatic sediment quality in Ontario. Ontario Ministry of the Environment, Water Resources Branch, Toronto, 23 pp.

Pesonen, M., Goksøyr, A., Andersson, T., 1992. Expression of P4501A1 in a primary culture of rainbow trout hepatocytes exposed to β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Arch. Biochem. Biophys. 292, 228–233.

Pritchard, J.B., 1993. Aquatic toxicology: past, present, and prospects. Environ. Health Perspect. 100, 249–257.

Ristola, T., Pellinen, J., Van Hoof, P.L., Leppänen, M., Kukkonen, J., 1996a. Characterization of Lake Ladoga sediments. II. Toxic chemicals. Chemosphere 32 (6), 1179–1192.

Ristola, T., Pellinen, J., Leppänen, M., Kukkonen, J., 1996b. Characterization of Lake Ladoga sediments. I. Toxicity to *Chironomus riparius* and *Daphnia magna*. Chemosphere 32 (6), 1165–1178.

Ristola, T., Pellinen, J., Ruokolainen, M., Kostamo, A., Kukkonen, J.V.K., 1998. Effects of sediment type, feeding level and larval density on growth and development of a midge, *Chironomus riparius*. Environ. Toxicol. Chem. (submitted).

Ryan, J.A., Hightower, L.E., 1994. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environ. Toxicol. Chem. 13 (8), 1231–1240.

Schindler, D.W., Kidd, K.A., Muir, D.C.G., Lockhart, W.L., 1995. The effects of ecosystem characteristics on contaminant distribution in northern freshwater lakes. Sci. Total Environ. 160/161, 1–17.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives. CRC Press, Boca Raton, FL, pp. 87–206.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. Environ. Health Perspect. 90, 101–109.

Suedel, B.C., Rodgers, J.H. Jr., 1994. Responses of *Hyalella azteca* and *Chironomus tentans* to particle-size distribution and organic matter content of formulated and natural freshwater sediments. Environ. Toxicol. Chem. 13 (10), 1639–1648.

Trapido, M., Palm, T., 1991. A study of the contents and convertibility of polycyclic aromatic hydrocarbons and sulphur in lichen *Hypogymnia physodes* and its substrate. Proc. Estonian Acad. Sci. Ecol. 1, 72–76.

Tuvikene, A., Huuskonen, S., Roy, S., Lindström-Seppä, P., 1996. Biomonitoring of South Estonian waters by means of xenobiotic metabolism of rainbow trout (*Oncorhynchus mykiss*) liver. Comp. Biochem. Physiol. 114C (2), 171–177.

Van der Weiden, M.E.J., van der Kolk, J., Bleumink, R., Seinen, W., van der Berg, M., 1992. Concurrence of P450 1A1 induction and toxic effects after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 24, 123–142.

Van der Weiden, M.E.J., Hanegraaf, F.H.M., Eggens, M.L., Celander, M., Seinen, W., van den Berg, M., 1994. Temporal induction of cytochrome P4501A in the mirror carp (*Cyprinus carpio*) after administration of several polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 13 (5), 797–802.

Van Veld, P.A., Westbrook, D.J., Woodin, B.R., Hale, R.C., Smith, C.L., Huggett, R.J., Stegeman, J.J., 1990. Induced cytochrome P-450 in intestine and liver of spot (*Leiostomus xanthurus*) from a polycyclic aromatic hydrocarbon contaminated environment. Aquat. Toxicol. 17, 119–132.

Wilcock, R.J., Corban, G.A., Northcott, G.L., Wilkins, A.L., Langdon, A.G., 1996. Persistence of polycyclic aromatic compounds of different molecular size and water solubility in surficial sediment of an interdial sandflat. Environ. Toxicol. Chem. 15 (5), 670–676.

Zar, J.H., 1996. Biostatistical Analysis, 3rd ed. Prentice-Hall, Englewood Cliffs, NJ, 662 pp.

Zhang, Y.S., Andersson, T., Förlin, L., 1990. Induction of hepatic xenobiotic biotransformation enzymes in rainbow trout by β-naphthoflavone. Time-course studies. Comp. Biochem. Physiol. 95B (2), 247–253.