Aquatic Toxicology 89 (2008) 172–179

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Endocrine effects of contaminated sediments on the freshwater snail *Potamopyrgus antipodarum in vivo* and in the cell bioassays *in vitro*

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ARTICLE INFO

Article history:
Received 27 February 2008
Received in revised form 30 May 2008
Accepted 20 June 2008

Keywords:
Sediment toxicity
AhR-mediated toxicity
ER-mediated toxicity
AR-mediated toxicity
Reproduction toxicity
Mudsnail biotest

ABSTRACT

Lake Pilnok located in the black coal-mining region Ostrava-Karvina, Czech Republic, contains sediments highly contaminated with powdered waste coal. Moreover, population of the endangered species of narrow-clawed crayfish *Pontastacus leptodactylus* with high proportion of intersex individuals (18%) was observed at this site. These findings motivated our work that aimed to evaluate contamination, endocrine disruptive potency using *in vitro* assays and *in vivo* effects of contaminated sediments on reproduction of sediment-dwelling invertebrates. Chemical analyses revealed low concentrations of persistent chlorinated compounds and heavy metals but concentrations of polycyclic aromatic hydrocarbons (PAH) were high (sum of 16 PAHs 10 μg/g dw). Organic extracts from sediments caused significant *in vitro* AhR-mediated activity in the bioassay with H4IIE-luc cells, estrogenicity in MVLN cells and anti-androgenicity in recombinant yeast assay, and these effects could be attributed to non-persistent compounds derived from the waste coal. We have also observed significant *in vivo* effects of the sediments in laboratory experiments with the Prosobranchian euryhaline mud snail *Potamopyrgus antipodarum*. Sediments from Lake Pilnok as well as organic extracts of the sediments (externally added to the control sediment) significantly affected fecundity during 8 weeks of exposure. The effects were stimulations of fecundity at lower concentrations at the beginning of the experiment followed by inhibitions of fecundity and general toxicity. Our study indicates presence of chemicals that affected endocrine balance in invertebrates, and emphasizes the need for integrated approaches combining *in vitro* and *in vivo* bioassays with identification of chemicals to elucidate ecotoxicogical impacts of contaminated sediment samples.

1. Introduction

Despite ongoing efforts of the European Union (EU) to control and ensure adequate surface water quality including its functioning as a habitat for wildlife, numerous freshwater ecosystems (especially in Eastern Europe), remain highly polluted. In particular, sediments are sinks/sources of contaminants such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), or organochlorine pesticides (OCPs) (Wirth et al., 1998; Hilscherová et al., 2001, 2002). Governments worldwide (including the European Commission via the European Water Framework Directive and others; FDEP, 2003) intend to set concentration limits or sediment quality criteria (SQC) for priority contaminants. These criteria, however, cover mostly "old" (traditional) persistent chemicals, whereas "modern" substances like hormones, pharmaceuticals, or personal care products (or various derivatives) are rarely included. Since these substances are known to occur in very low concentrations in the environment, they cannot easily be detected by routine analytical methods. Nevertheless, a number of studies have described the potential effects of these substances on aquatic organisms (Hallare et al., 2005; Verslycke et al., 2007).

The present study investigates reproduction-related endocrine disruptive effects of sediments from the contaminated Lake Pilnok, which is situated in the black coal-mining region of Ostrava-Karvina in the Czech Republic. Lake Pilnok is an artificial pond, which

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doi:10.1016/j.aquatox.2008.06.013

originated as flooded ground depression that has been used as a dumping site for powdered waste coal since the middle of the 20th century. In spite of intensive black coal-mining activities, basic parameters of water quality (oxygen content and transparency) supplied by underground springs remained stable, and the narrow-clawed crayfish *Pontastacus* (syn. *Astacus*) *leptodactylus* (Decapoda, Crustacea) Eschscholtz, 1823 lives in many reservoirs spread over the Ostrava-Karvina region. However, an abnormal population of this endangered species has been observed only in the Lake Pilnok with about 18% female-like individuals that possessed both female and male sexual characteristics (Ďuriš et al., unpublished results). Similar observations (abnormalities in external sexual characteristics or histologically determined ootestis) were previously described in crustaceans such as gammarids (Dunn et al., 1994; Ladewig et al., 2002) or decapods (Rudolph, 1999; Kozák et al., 2007) with proportion of intersex individuals about 10%. This abnormality was termed intersex in crustaceans and it was related to partial hermafroditism and plasticity of phenotypical sex determination or other factors such as parasitic infestation or environmental contamination (Medley and Rouse, 1993; Rudolph, 1999; Ford, 2008). The coincidence of the intersex and the waste coal powder suggested the presence of unknown compounds that might be causing endocrine disruption in this crayfish species.

The assessment of toxicity of sediments generally requires application of at least a single suitable biological test (biotest) along with chemical analyses. For instance, the combined TRIAD approach (chemical analyses of known compounds, whole-sediment toxicity testing and evaluation of benthic biodiversity) has been discussed and used (Chapman and Hollert, 2006; Sørensen et al., 2007). Thus, testing of *in vivo* effects plays a key role in sediment toxicity evaluation and several model organisms have been used to assess various groups of contaminants (Jobling et al., 2003; De Lange et al., 2005).

Some *in vivo* toxicity models have been shown to be particularly suitable for studying reproductive and developmental effects (Duft et al., 2007; Kusk and Wollenberger, 2007; Verslycke et al., 2007). Prosobranchian snails are sensitive organisms for the detection of (xeno-)hormones (Jobling et al., 2003), and bioassays with the euryhaline mud snail (*P. antipodarum* Gray, 1843) have been successfully used to study sediment toxicity (Duft et al., 2003; Oetken et al., 2005). The major advantages of this species are the continuous fertility of parthenogenic females, few maintenance requirements and relatively great sensitivity to compounds that may affect reproduction (Oetken et al., 2005).

The objectives of the study were: (1) to determine whether sediments of Lake Pilnok contain chemicals with endocrine disruptive potential and (2) to evaluate if the sediments can affect model invertebrate *P. antipodarum in vivo*. The approach used in this study combined chemical analyses (heavy metals and major organic contaminants), *in vitro* bioassays with cell lines (to study arylhydrocarbon (AhR), estrogen (ER) and androgen (AR) receptor-mediated effects) as well as *in vivo* experiments with *P. antipodarum* to assess mortality and reproduction in this sediment-dwelling animal. The comparison of effects observed in exposures to natural sediment versus control sediment spiked with its organic extract enabled evaluation of the importance of the extracted organic pollutants and their availability for the studied endpoints.

2. Methods

2.1. Experimental design

Samples of sediments were collected from a "contaminated" (Lake Pilnok) and "reference/control" site (Steinlach creek near Talheim, situated in a protected nature reserve, state of Baden-Württemberg, Germany). Extracts of sediments were analysed for (1) concentrations of chemical pollutants (metals, PAHs, PCBs, OCPs) and (2) the presence of compounds interfering with AhR, ER and AR using *in vitro* bioassays. Furthermore, *in vivo* effects of sediments on *P. antipodarum* snails were studied in two experimental settings: (1) whole-sediment toxicity assays with control sediment, contaminated Lake Pilnok sediment and two mixtures of both sediments, comprising 50% and 75% Lake Pilnok sediment, respectively and (2) toxicity assays using control sediment which was spiked with different volumes of organic extract from Lake Pilnok sediment (three doses equivalent to 50%, 75% and 100% of original Lake Pilnok sediment). All *in vivo* experiments were performed with 120 individuals for every exposure group (divided into two replicates of 60 animals each).

2.2. Sediment sampling and preparation of sediment organic extracts

Sediments of Lake Pilnok and Steinlach creek were collected from three places at each location, mixed, transported into the lab and prepared for use in studies. Sediments were stored frozen at −20 °C until further processing for analyses and experiments. A mass of 1.5 kg (fresh weight) of sediment from Lake Pilnok was extracted for 12 h with dichloromethane in a Soxhlet apparatus. Thawed sediment was ground with anhydrous sodium sulphate until it reached a paste-like consistency; the lump was placed in Soxhlet cartridges and extracted. The extract containing extractable organic fraction was concentrated by rotary evaporation and divided into two portions. The solvent of the first portion was changed to acetone. Acetone extract was used for *in vivo* experiments (fast evaporation after dosing). The second portion of the extract was transferred to dimethylsulfoxide, the carrier used during *in vitro* experiments with cells.

2.3. Analyses of organic contaminants

A portion of the organic extract was used for chemical analyses of 16 PAHs, 7 indicator PCBs and OCPs (hexachlorocyclobenzene, 4 HCH stereoisomers, 2 congeners of each DDE, DDD and DDT). Activated copper was used to remove sulphur from the extract prior to analyses. Fractionation was achieved on silica gel columns; a sulphuric acid modified silica gel column was used for PCB/OCP samples. Samples were analysed using GC–ECD (HP 5890) equipped with a Quadrex fused silica column 5% Phe for PCBs and OCPs. The 16 US EPA polycyclic aromatic hydrocarbons were determined in all samples using a GC–MS instrument (HP 6890–HP 5973) equipped with a J&W Scientific fused silica column DB-5MS. Samples were quantified using Pesticide Mix 13 (Dr. Ehrenstorfer, Augsburg, Germany) and PAH Mix 27 (LCG Promochem, Teddington, UK) standard mixtures. To assure quality of analyses, laboratory blanks and certified reference material BCR-536 were analysed in parallel, and surrogate recovery standards were used (D10-phenanthrene and D12-perylene for PAH analyses; *para*-terphenyl and PCB 121 for PCB/OCP analyses). Recoveries were 55% and 68% for PAHs analyses in control and Lake Pilnok sediment: 68% and 94% for PCBs in control and Lake Pilnok sediment. Blanks run in parallel always contained less than 1% of the concentrations determined in the studied samples.

2.4. Analyses of heavy metals

Concentrations of heavy metals (vanadium: V, chromium: Cr, cobalt: Co, nickel: Ni, copper: Cu, zinc: Zn, arsenic: As, cadmium: Cd, lead: Pb and mercury: Hg) in sediment samples were analysed according to ISO 11466, method adapted to analytical instrumen-

tation. Dry sediment (1 g dw) was leached with 2.3 ml HNO_3 and 7 ml HCl overnight followed by heating under reflux for 2 h, and after cooling the mixture was diluted for analyses using inductively coupled plasma-mass spectrometry (ICP-MS Agilent 7500ce, Agilent Technologies, Japan). Elements (isotopes) suffering from polyatomic interferences were measured in He collision mode using Octopole Reaction System. Ions of Ge, In and Bi were used as internal standards, methodology was verified by analyses of soil certified reference materials (ANA 7001–7004). Total content of mercury was determined by thermooxidation method using AMA-254 analyzer (Altec, Czech Republic).

2.5. In vitro assays

The potential of the sediment extracts to induce AhR-mediated (dioxin-like) effects were determined with the H4IIE.luc bioassay. The ER-mediated activity of the sediment extracts was evaluated using a bioassay with the MVLN cell line. Methodological details for both luciferase reporter gene-based assays have been described previously (Hilscherová et al., 2001, 2002). In brief, cells were seeded in 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) and exposed to dilutions of sediment extracts for 24 h in three replicates. The activity of AhR- or ER-induced luciferase was quantified using Promega Steady Glo Kit (Promega, Mannheim, Germany). After the initial range-finding experiments, full concentration–response curves for induction of AhR- and ER-mediated responses were generated. Besides the effects of crude extract to induce AhR-mediated effects in the H4IIE.luc assay, responses were also determined for extracts that had been treated with sulphuric acid to remove labile compounds such as PAHs. The effects of sediment extracts were related to the luciferase induction by the reference compounds: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for AhR-mediated effects, 17β-estradiol (E2) for ER-mediated effects using methods described previously (Villeneuve et al., 2000).

The potency of sediment extracts to modulate AR-mediated responses was examined by a yeast reporter assay comprising a recombinant yeast cell line (Leskinen et al., 2005). Yeast seeded in 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) were exposed to dilutions of sediment extracts in three replicates for 3 h. The effects of sediment extracts were assessed in comparison with the reference compound testosterone as AR-mediated luciferase activity. The anti-androgenicity (competitive activity) of sediment extract was measured as the decrease of AR-mediated luciferase activity in exposures using extracts supplemented with 10^{-8} M testosterone.

2.6. In vivo assays

The sediment biotest using parthenogenic females of the prosobranchian snail *P. antipodarum* was originally developed by Duft et al. (2003) and our studies followed these guidelines. The exposures were performed in 1.5 L glass aquaria using the thawed wet sediment to cover the ground (equivalents of 60 g dry weight, (dw), per aquarium). The aqueous medium (800 mL per aquarium) was a mixture of stream water from Steinlach creek and tap water at a ratio of 1:1. To establish equilibrium between sediment and water phase, sediment/water systems were set up 7 days prior to commencing experiments. A volume of 500 mL water was renewed weekly. The exposure (8 week) was performed under constant conditions at a temperature of $15.6 \pm 0.14\,^{\circ}C$ and a light:dark cycle of 14:10 h.

The exposure was performed with (1) natural sediments in quantitatively different mixtures and (2) control sediment spiked with different volumes of the organic extract from the Lake Pilnok sediment. The experiments with the natural sediments contained exposure of snails to Lake Pilnok sediment ("100% Lake Pilnok" = 60 g dw Lake Pilnok sediment), and to its mixtures with control sediment ("75% Lake Pilnok" = 45 g dw Lake Pilnok sediment + 15 g dw control Steinlach sediment; "50% Lake Pilnok" = 30 g dw Lake Pilnok sediment + 30 g dw control sediment). 60 g (dw) of Steinlach sediment served as control. The experiments with the organic extract used 60 g (dw) of Steinlach sediment to which different volumes of the extract prepared from the Lake Pilnok sediment was added. The sediment extract was dosed as 3 mL acetone solution in concentrations that corresponded to "50% Lake Pilnok" (total extract of 30 g dw Lake Pilnok sediment), "75% Lake Pilnok" (total extract of 45 g dw Lake Pilnok sediment), or "100% Lake Pilnok" (total extract of 60 g dw Lake Pilnok sediment). The control sediment spiked with 3 mL of acetone was used as the solvent control. After sediment spiking, the solvent was let to evaporate for 3 days in the dark before the aqueous medium was added to exposure systems.

All variants (control + 3 variants with whole sediment, solvent control + 3 variants with sediment extract) were performed in duplicate aquaria containing 60 *P. antipodarum* (parthenogenic females) per aquarium. Twenty animals were sampled from each aquarium at the end of the second week (after 14 days of exposure) and the 5th week (40 days exposure); all other surviving animals were examined at the end of the experiment (8 week exposure). Females were dissected and the embryos held in the brood pouch of each individual were counted under a stereomicroscope. Embryos were classified as either "early embryos" (without developed shell) or "further developed embryos" (after formation of a shell).

2.7. Data analysis

EC_{50} values (derived from H4IIE.luc, MVLN bioassays) were estimated using least-squares regression of the log-linear part of the full concentration–response curves. The assumptions of parallelism and equal efficacy of the unknown and standard curves were assessed by use of the method of comparing estimates of the EC20 and EC80 according to Villeneuve et al. (2000). TCDD equivalents (TEQ_{bio}) were calculated using the effect-equivalency approach by comparing the EC_{50} value of the TCDD standard calibration with the concentration of tested sample inducing the same bioassay response as the EC_{50} of TCDD (Hilscherová et al., 2000). Similarly, the estrogenicity was quantified as 17β-estradiol equivalents (E2-equivalents EEQs) by comparing the EC_{50} value of the E2 standard calibration with the concentration of tested sample inducing the same bioassay response as the EC_{50} of E2. Dioxin-equivalents (derived from the chemical analyses; TEQ_{chem}) were calculated from individual PAHs concentration and their relative potencies (REPs) calculated from H4IIE.luc bioassay according to Machala et al. (2001). Anti-androgenic effects of sediment extracts on the testosterone-induced luciferase were evaluated by analysis of variance (ANOVA) followed by Dunnet's test. *In vivo* experiments with *P. antipodarum* (all treatments) were performed in two duplicate aquaria. Differences between control aquaria and exposure variants were evaluated with the non-parametric Mann–Whitney *U* test. The threshold for significance of all statistical assays was set to $p < 0.05$.

3. Results

3.1. Chemical concentrations

Concentrations of both organic and inorganic chemicals were low in sediment from Steinlach creek while they were much higher

Table 1
Contamination of sediments by polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and metals

	Control (ng/g dw)	Lake Pilnok (ng/g dw)	FDEP-A/PEC[a] (ng/g dw)	REPs[b]
PAHs—sum of 16 PAHs	422	10,122	23,000	
Naphthalene	11	1071	560	n.a.
Acenaphthylene	5	40	130	n.a.
Acenaphthene	2	195	89	n.a.
Fluorene	6	1281	540	n.a.
Phenanthrene	57	3782	1200	n.a.
Anthracene	17	68	850	n.a.
Fluoranthene	101	356	2200	2.27×10^{-8}
Pyrene	72	594	1500	1.78×10^{-6}
Benzo[*a*]anthracene	37	434	1100	7.04×10^{-6}
Chrysene	37	900	1300	1.01×10^{-4}
Benzo[*b*]fluoranthene	23	541	n.a.	3.35×10^{-5}
Benzo[*k*]fluoranthene	12	58	n.a.	1.64×10^{-3}
Benzo[*a*]pyrene	20	278	1500	9.01×10^{-5}
Indeno[1,2,3-*cd*]pyrene	11	106	n.a.	2.96×10^{-4}
Dibenzo[*a*,*h*]anthracene	2	60	140	1.17×10^{-3}
Benzo[*g*,*h*,*i*]perylene	10	358	n.a.	6.19×10^{-6}
Sum of 7 PCBs[c]	0.86	4.30		
Sum of 8 OCPs[d]	0.52	2.94		
	(ng TCDD/g dw)	(ng TCDD/g dw)		
TEQ_{chem}[e]	0.032	0.338		
TEQ_{bio}[f]	2.4	70		
Metals	Control (μg/g dw)	Lake Pilnok (μg/g dw)	FDEP-TEC (μg/g dw)	
V	9.47	25.03	n.a.	
Cr	5.78	22.28	110	
Co	1.26	7.61	n.a.	
Ni	6.14	20.14	49	
Cu	2.94	27.76	150	
Zn	21.53	38.90	460	
As	1.38	3.37	33	
Cd	0.25	0.14	5	
Pb	3.46	45.85	130	
Hg	<0.01	0.06	1.1	

n.a.: data not available.

[a] Guideline values recommended by the Florida Department of Environmental Protection (FDEP, 2003) for the protection of sediment-dwelling organisms (anticipated/probable effect concentrations, A/PEC, for PAHs; threshold effects concentrations, TEC, for metals).

[b] Relative potencies (REPs) to induce AhR-mediated effects *in vitro* by PAHs (Machala et al., 2001).

[c] The sum of PCBs—seven indicator compounds: PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138, PCB 180.

[d] The sum of OCPs—hexachlorocyclobenzene, four hexachlorocyclohexane stereoisomers (α-HCH, β-HCH, γ-HCH, δ-HCH), *p*,*p*′- and *o*,*p*′-congeners of DDE, DDD and DDT.

[e] Calculated toxic equivalents (TEQ_{chem}).

[f] Toxic equivalents derived from the H4IIE.luc bioassay (TEQ_{bio}).

in sediment from Lake Pilnok (Table 1). The sum of analysed PAHs in Steinlach sediment was 422 ng ∑PAH/g dw, which is equivalent to 0.032 ng TEQ_{chem}/g dw. The concentration of ∑PAH in the Lake Pilnok was 1.0×10^4 ng/g dw, TEQ_{chem} = 0.34 ng TEQ_{chem}/g dw (Table 1). Concentrations of PCBs and OCPs in Lake Pilnok were about fivefold higher than in the control sediment. Also the concentrations of heavy metals were significantly higher in the Lake Pilnok sediment (e.g. 10-fold higher copper, lead, cobalt and molybdenum in comparison to the control).

3.2. *In vitro assays*

Concentrations of TEQ_{bio} were relatively low in Steinlach sediment (2.4 ng TEQ_{bio}/g dw) while concentrations of TEQ_{bio} were higher in sediments from Lake Pilnok (approximately 70 ng TEQ_{bio}/g dw sediment; Fig. 1A, Table 1). TEQ_{bio} were not detected in extracts treated with sulphuric acid to remove labile compounds, such as PAHs. This indicates that there was little contribution of persistent compounds such as PCBs and/or polychlorinated dibenzo-*p*-dioxins or dibenzofurans (PCDD/DF) to the observed dioxin-like activities.

While no significant (anti-)estrogenicity or (anti-)androgenicity were observed in sediments from Steinlach (Fig. 1B and C), there was significant activity in sediments from Lake Pilnok. Lake Pilnok sediment has elicited estrogenic potency approximately 4.5 ng EEQ/g dw (Fig. 1B). These extracts also displayed significant anti-androgenic effects (significant and dose-dependent inhibition of testosterone-induced AR-activation; Fig. 1C).

3.3. *In vivo test with P. antipodarum*

Mortality of *P. antipodarum* during the 8-week exposure was low. Six out of 60 individuals died in one of the control aquaria (control sediments and solvent controls), while no mortality was observed in the second replicate. Lake Pilnok sediment caused mortalities in the range of 1–13% of exposed animals (i.e. maximum 7 dead individuals out of 60). Steinlach sediment spiked with the extract from Lake Pilnok caused 0–18% mortality (i.e. maximum 11 dead individuals out of 60). The differences in mortalities between contaminated and control sediments were not statistically significant.

Fecundity of snails varied among treatments. The number of "early embryos" (with undeveloped, shell) was significantly increased after 2-week exposure to 50% Lake Pilnok sediment (Mann–Whitney *U* test, $p < 0.05$; Fig. 2A) followed by an apparent inhibition of reproduction at all concentrations at the end

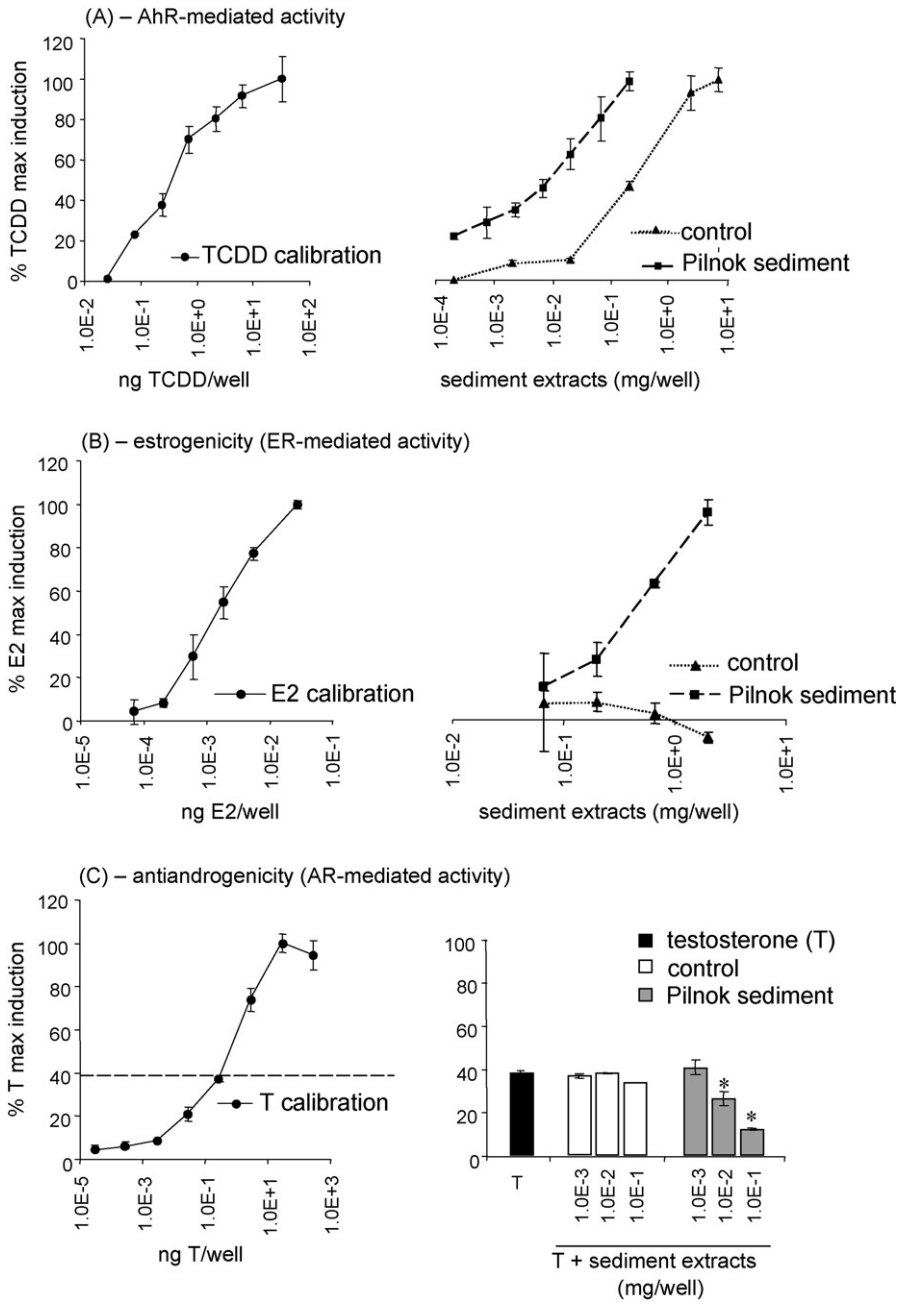

Fig. 1. Concentration–response curves of reference chemicals and sediment extracts. (A) Arylhydrocarbon receptor (AhR-) dependent luciferase activity in the H4IIE.luc cell bioassay; (B) estrogen receptor (ER-) dependent luciferase activity in the MVLN cell bioassay and (C) androgen receptor (AR-) dependent luciferase in recombinant yeast bioassay. Reference compounds were 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); 17β-estradiol (E2); testosterone (T). Graphs display means and standard deviation from three replicates. *Statistically significant anti-androgenicity of the Lake Pilnok sediment extract (suppression of the effect induced by 10^{-8} M testosterone; ANOVA followed by Dunnet's post-test; $p < 0.05$).

of exposure (8 week) ($p < 0.05$; Fig. 2A). More "further developed" (later stage with shell) embryos were observed at 5 week than 2 week of exposures to 50% and 75% Lake Pilnok, but prolonged (8 week) exposures suppressed numbers of embryos as well (Fig. 2B). Steinlach sediment spiked with organic extract from the Lake Pilnok sediment caused apparent reductions in the production of early embryos, and the effects were present both in the beginning of exposure (2 week) and at the end (8 week; 50%, 75% and 100% Lake Pilnok; $p < 0.05$; Fig. 3); numbers of "further developed" embryos were not affected (Fig. 3B).

4. Discussion

In our study, chemical and ecotoxicological investigations were combined to investigate contaminated sediments from the Lake Pilnok. Chemical analyses revealed high concentrations of PAHs. Concentrations of indicator PCBs and selected OCPs were about five times higher than in a control but contamination by these persistent organochlorine compounds seems to be generally lower than other polluted sites in the Czech Republic (Eljarrat et al., 2001; Hilscherová et al., 2001). Also concentrations of heavy metals (with the exception of cadmium) were higher in Lake Pilnok than

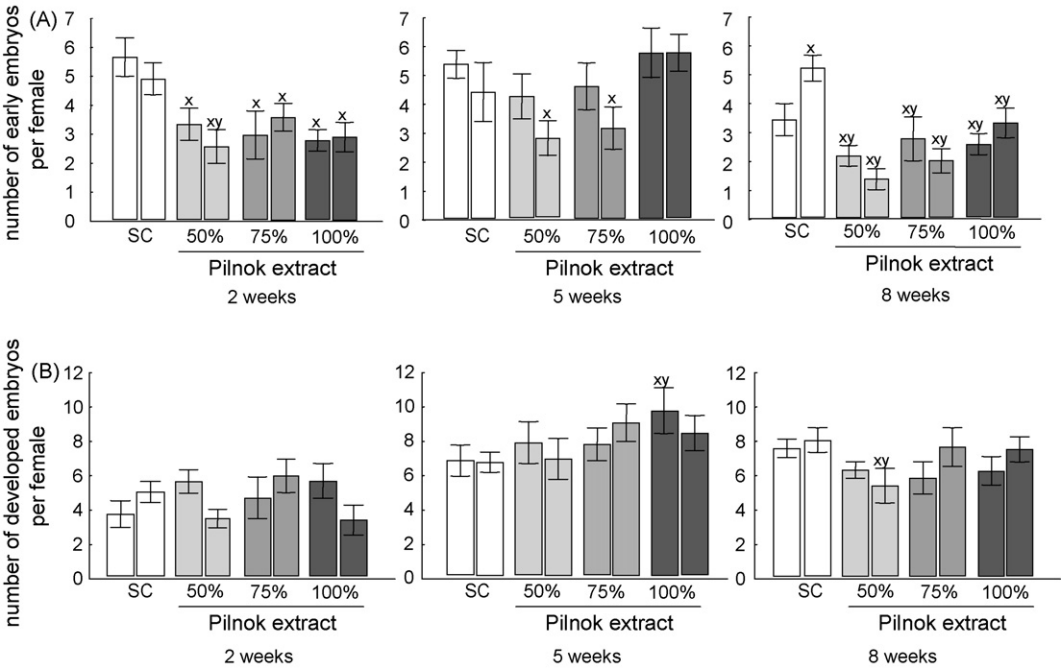

Fig. 2. Effects of Steinlach sediment spiked with the organic extract prepared from Lake Pilnok sediment. Number of (A) early embryos (i.e. embryos without developed shell) and (B) further developed embryos (with shell) in the brood pouch of female *Potamopyrgus antipodarum*; data given as numbers per individual. SC: solvent control (control Steinlach sediment with added and evaporated acetone); '50% Lake Pilnok', '75% Lake Pilnok' and '100% Lake Pilnok': three doses of the organic extract prepared from Lake Pilnok sediment, see Section 2). All variants were performed in duplicates—each column represents results from individual aquarium (average number of embryos per female; 20 females investigated; error bars: standard error of mean). Statistically significant differences from controls are marked with letters (*x*, *y*: significant difference from the first (*x*) and the second (*y*) control aquarium; Mann–Whitney *U* test; $p < 0.05$).

in Steinlach Creek but in comparison to other polluted sites they indicate minimally to moderately impaired sediments (Wiesner et al., 2001; Negri et al., 2006). The concentrations of metals were less than the threshold effect concentrations (TEC) proposed by FDEP (2003) indicating negligible effects on sediment organisms.

Concentrations of $\sum$PAH in Lake Pilnok were approximately 20-fold higher than in reference sediment from Steinlach Creek, and

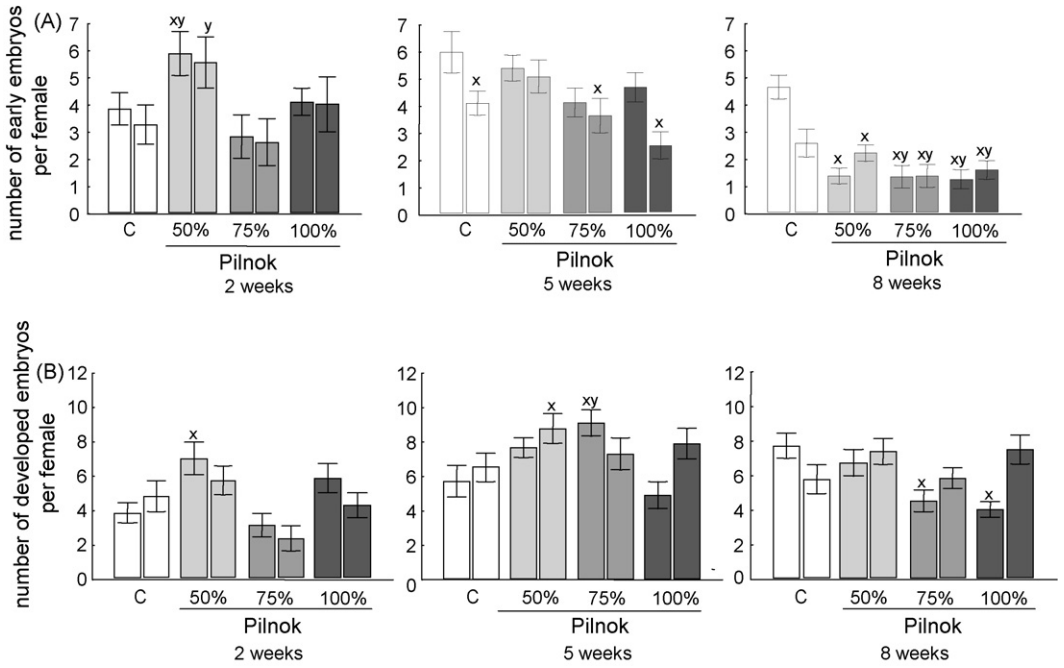

Fig. 3. Effects of the sediment exposures on the number of (A) early embryos (i.e. embryos without developed shell) and (B) further developed embryos (with shell) in the brood pouch of female *P. antipodarum*; data given as numbers per individual. (C) control sediment, '100% Lake Pilnok': untreated contaminated sediment; '50% Lake Pilnok' and '75% Lake Pilnok': appropriate mixtures of Lake Pilnok and control sediments. All variants were performed in duplicates—each column represents results from individual aquarium (average number of embryos per female; 20 females investigated; error bars: standard error of mean). Statistically significant differences from controls are marked with letters (*x*, *y*: significant difference from the first (*x*) and the second (*y*) control aquarium; Mann–Whitney *U* test; $p < 0.05$).

they were comparable to other contaminated sediments from the Czech Republic (Hilscherová et al., 2001) or worldwide (Giacalone et al., 2004). The PAHs in the two studied sediments, were, based upon the ratios of phenanthrene/anthracene and fluoranthene/pyrene, derived from different sources (Sanders et al., 2002). In sediments from Steinlach Creek, the ratios Phe/Ant = 3.4 and Flu/Pyr = 1.4 suggest a pyrogenic origin which may be preferentially derived from the traffic and farming activities in the vicinity, while the ratios Phe/Ant = 55.6 and Flu/Pyr = 0.6 confirmed the petrogenic source of PAHs in Lake Pilnok sediment. These conclusions are consistent with the historical use of Lake Pilnok for the storage of black coal waste.

Concentrations of PAHs measured in Lake Pilnok sediment (with the exception of anthracene and fluoranthene) were higher than the TEC used by FDEP (2003), and the concentrations of specific compounds (naphthalene, acenaphthene, fluorene and phenanthrene) were higher than probable effect limits (PEC) of the FDEP (2003). These findings represent one line of evidence that contamination of Lake Pilnok sediments with PAH may potentially cause adverse effects *in situ*.

The concentration of 70 ng TEQ_{bio}/g dw in extracts of Lake Pilnok sediment was relatively high, compared with sediments with similar concentrations of PAHs (Hilscherová et al., 2001) where concentrations ranged from 1.9 to 23 TEQ_{bio}/g dw. Furthermore, the concentration of TEQ_{bio} measured in the H4IIE.luc bioassay was approximately 200-fold higher than the concentration of TEQ_{chem} calculated from the concentrations of individual PAHs and their respective REP values. This result suggests that Lake Pilnok sediment contains more AhR-active compounds than can be accounted for by PAHs determined by instrumental analyses. However, it is unlikely that PCDD/Fs were responsible for this activity because treatment of the extract with sulphuric acid (which would remove only labile compounds and not PCDD/F), completely removed AhR-mediated activity. One explanation is the possible presence of AhR-active compounds originating from the deposited powdered waste coal that were not quantified in the PAHs studied here. A substantial part of coal is comprised of solid matter called maceral (ASTM, 1979), which is rich in organic compounds such as aliphatic hydrocarbons, cycloalkanes and also polyaromatics including miscellaneous substituted compounds (Schacht et al., 1999). Other studies (Orem et al., 1999; Frouz et al., 2005) have demonstrated that solid fossil organic substrates contain a bio-accessible fraction of organic compounds such as polyphenols. All these organic compounds (which are not analysed in routine chemical screenings) could contribute to unusually high AhR-mediated activity observed in our study, and they should be further explored as they may potentially harm living organisms *in vivo*.

Extracts from Lake Pilnok sediment were also estrogenic and anti-androgenic *in vitro*. PAHs could also play some role in these effects since previous studies have shown potential of some compounds (such as benzo[*a*]anthracene and dibenzo[*a*,*h*]anthracene) to activate ER and also act as anti-androgens (Vinggaard et al., 2000; Villeneuve et al., 2002). Furthermore, there is also evidence that anti-androgenic effects could be, at least in part, caused indirectly by high activation of AhR as shown for PAHs (Kizu et al., 2003). Similar to AhR-mediated effects, estrogenicity (EEQ) in Lake Pilnok extract was higher than previously reported for sediments with comparable concentrations of PAHs (Hilscherová et al., 2002). These results, taken together indicate the presence of organic compounds with estrogenic and anti-androgenic potential (derived most probably from the coal) that may display remarkable effects on aquatic organisms, possibly by means of affecting hormonally regulated processes.

The results of the *in vivo* studies of *P. antipodarum* were variable, even among individuals within the same exposure aquarium, including controls. However, some general trends were observed. For example, the number of "early embryos" was a more sensitive indicator of developmental toxicity than was the number of "further developed" (shelled embryos). This is similar to the results of previous studies (Duft et al., 2003). The early development of embryos in the brood pouch seems to reflect actual effects of those toxicants that immediately affect the general health condition and the reproductive status of the females.

The temporal profiles of effects exerted by the whole-sediment exposures (initial stimulations in the number of embryos followed by the decrease at higher doses or longer exposures) seem to correspond to the general character of the dose-response curve in the model species. Such effects have been observed in aquatic organisms exposed to 17β-estradiol, and complex estrogenic mixtures such as wastewater effluents (Jobling et al., 2003). The initial increase in fecundity was later suppressed by possible re-allocation of energy for processes involved in detoxifying xenobiotics or synthesizing functional or structural proteins damaged by toxicity. General inhibitory effects on fecundity were much stronger pronounced in animals exposed to sediments with external organic extracts. This may be most probably explained by a considerably higher fraction of readily bioavailable toxicants (rapidly released from the extract after spiking) in comparison with exposures to the natural 'undisturbed' contaminated sediment. Our *in vivo* experiments with *P. antipodarum* thus seem to suggest the presence of organic compounds that may stimulate fecundity, but such effects may be later masked by the toxicity of the complex contaminant mixture in the sediment.

5. Conclusions

Overall, our study provides evidence that organic sediment contaminants from the Lake Pilnok may affect reproductive ability of invertebrates. The results demonstrate that non-persistent organic compounds which originated from powdered waste coal but are not analysed by routine chemical analyses exert AhR-mediated activity, estrogenicity and anti-androgenicity *in vitro*, and that they also affect *in vivo* reproduction of a model invertebrate *P. antipodarum.* Our study emphasizes the need of integrated approaches including *in vitro* and *in vivo* toxicological studies along with detailed chemical analyses for the evaluation of complex contaminated environmental samples.

Acknowledgements

The research was supported by the Grant Agency of the Czech Republic (GAČR 525/05/P160) and the European Union (FP6 project ECODIS, No. 518043-1). A scholarship of E.M. was provided by Deutsche Bundesstiftung Umwelt (DBU). The authors are greatly indebted to Jörg Oehlmann and Claudia Schmitt (Aquatic Ecotoxicology Department, University Frankfurt/Main, Germany) who have provided a laboratory culture of the snails and introduced us to the sediment biotest. We also acknowledge the initial stimulation of this research by Zdeněk Ďuriš, University of Ostrava, Czech Republic.

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