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

E. Mazurová[a], K. Hilscherová[a,b], V. Jálová[a], H.-R. Köhler[c], R. Triebskorn[c,d], J.P. Giesy[e,f,g,h], L. Bláha[a,b,*]

[a] Masaryk University, RECETOX (Research Centre for Environmental Chemistry and Ecotoxicology), Kamenice 3, CZ-62500 Brno, Czech Republic
[b] Academy of Sciences of the Czech Republic, Institute of Botany, Kvetna 8, CZ-60365 Brno, Czech Republic
[c] Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany
[d] Steinbeis-Transfer Center for Ecotoxicology and Ecophysiology, Blumenstr. 13, D-72108 Rottenburg, Germany
[e] University of Saskatchewan, Department of Veterinary Biomedical Sciences and Toxicology Centre, 44 Campus Drive, Saskatoon, SK S7N 5B3, Canada
[f] Zoology Department, National Food Safety and Toxicology Center, and Center for Integrative Toxicology, Michigan State University, East Lansing 48824, USA
[g] Biology and Chemistry Department, City University of Hong Kong, Kowloon, Hong Kong, China
[h] School of the Environment, Nanjing University, Nanjing, China

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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

* Corresponding author at: Masaryk University, RECETOX (Research Centre for Environmental Chemistry and Ecotoxicology), Kamenice 3, CZ-62500, Brno, Czech Republic. Tel.: +420 549493194; fax: +420 549492840.
E-mail address: blaha@recetox.muni.cz (L. Bláha).

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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-