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Effect-directed analysis for estrogenic compounds in a fluvial sediment sample using transgenic *cyp19a1b*-GFP zebrafish embryos

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ABSTRACT

Xenoestrogens may persist in the environment by binding to sediments or suspended particulate matter serving as long-term reservoir and source of exposure, particularly for organisms living in or in contact with sediments. In this study, we present for the first time an effect-directed analysis (EDA) for identifying estrogenic compounds in a sediment sample using embryos of a transgenic reporter fish strain. In the tg(*cyp19a1b*-GFP) transgenic zebrafish strain, the expression of GFP (green fluorescent protein) in the brain is driven by an oestrogen responsive element in the promoter of the *cyp19a1b* (aromatase) gene. The selected sediment sample of the Czech river Bilina had already been analysed in a previous EDA using the yeast oestrogen screening assay and had revealed fractions containing estrogenic compounds. When normal phase HPLC (high performance liquid chromatography) fractionation was used for the separation of the sediment sample, the biotest with transgenic fish embryos revealed two estrogenic fractions. Chemical analysis of candidate compounds in these sediment fractions suggested alkylphenols and estrone as candidate compounds responsible for the observed estrogenic effect. Alkylphenol concentrations could partially explain the estrogenicity of the fractions. However, xenoestrogens below the analytical detection limit or non-targeted estrogenic compounds have probably also contributed to the sample's estrogenic potency. The results indicated the suitability of the tg(*cyp19a1b*-GFP) fish embryo for an integrated chemical-biological analysis of estrogenic effects.

1. Introduction

Numerous studies have shown that a large number of manmade chemicals represent potential oestrogen receptor (ER) agonists (Colborn et al., 1993; Frye et al., 2012; Maffini et al., 2006) capable to activate oestrogen signalling pathways thus interfering with oestrogen-regulated processes. In the aquatic environment these chemicals are of high concern due to their interference with sexual differentiation and reproduction in fish and other aquatic vertebrates (Kidd et al., 2007; Scholz and Klüver, 2009). Effluents from wastewater treatment plants, sewage discharges and stormwater runoff from rural areas represent major sources of these xenoestrogens (Nakada et al., 2006; Racz and Goel, 2010; Yang et al., 2012). Ultimately they can also accumulate in sediments since many xenoestrogens are moderately hydrophobic with a log K_{ow} (octanol–water partition coefficients) typically in the range of 3–5 (Racz and Goel, 2010). Thus, they provide a source of continuous exposure for aquatic organism (*via* sediment contact or through desorption into the water column).

Many estrogenic compounds in the aquatic ecosystems are typically present at or even below the analytical detection limit. However, even these low concentrations can be of biological relevance given the potential combined effect of oestrogen agonists (Silva et al., 2002). Thus, effect-based monitoring approaches play a key role in the detection of estrogens in the environment. However, the detection of estrogenicity by biological test systems is often hampered by the complexity of environmental mixtures. Toxic components may mask the estrogenic response (Hollert et al., 2005). One of the approaches to address this challenge is the application of effect-directed analysis (EDA), an approach that applies

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fractionation techniques together with biotesting in order to reduce sample complexity, to make masked effects detectable and to support the identification of causative chemicals (Brack, 2003). There are numerous studies reporting the use of *in vitro* assays to assess estrogenicity of riverine sediment samples (Grund et al., 2011; Schmitt et al., 2012; Wang et al., 2012). The use of such *in vitro* assays in EDA approach has been shown relevant in identifying environmental ligands of steroid receptors including ER (Creusot et al., 2014, 2013). However, *in vitro* assays cannot reflect the complexity of an organism due to limitations of pharmacokinetic and pharmacodynamic processes. Vertebrates are difficult to use for EDA approaches, because of ethical reasons, the limited number of sample material, and the high sample numbers to analyse. However, the use of embryos of zebrafish (*Danio rerio*) provides an alternative to experiments with adult animals. In contrast to cellular *in vitro* models, the use of fish embryos combines the complexity of a full organism with the simplicity and reproducibility of cellular assays. Zebrafish embryos have been already shown to be compliant with the EDA approach as reported in a study on developmental toxicants in soil sample from a municipal landfill site (Legler et al., 2011).

For the assessment of estrogenic activity, alterations in expression of oestrogen-regulated genes are frequently used as end-points (Jung et al., 2012). In transgenic zebrafish strains, quantification of oestrogen-sensitive genes is facilitated by fluorescence or luminescence of reporter genes (Brion et al., 2012; Chen et al., 2010; Ji et al., 2012; Legler et al., 2000). Recently, embryos of the tg(*cyp19a1b*-GFP) strain have been successfully used for the assessment of the estrogenic activity of synthetic or natural hormones, industrial chemicals and mixtures of these (Brion et al., 2012; Petersen et al., 2013). This transgenic strain contains a construct of the promoter region of the ER-regulated zebrafish brain aromatase gene (*cyp19a1b*) linked to green fluorescent protein (GFP). The brain aromatase is responsible for the conversion of androgens to estrogens and its expression is restricted to the radial glial cells (Menuet et al., 2005). GFP expression in the tg(*cyp19a1b*-GFP) strain perfectly mimics the expression of the endogenous *cyp19a1b* (Tong et al., 2009; Vosges et al., 2010). It has been shown that the estrogenic regulation of the *cyp19a1b* requires oestrogen-responsive element (ERE and 1/2 ERE) and a glial x-responsive element (GxRE) recruiting glial-specific transcription factors (Le Page et al., 2008; Menuet et al., 2005). This results in a high brain-specific upregulation of the *cyp19a1b* gene in fish (especially in embryos) exposed to agonist ligands of ER or compounds that are metabolised into estrogenic metabolites (*e.g.*, pro-estrogenic compounds, aromatisable and non-aromatisable androgens) (Brion et al., 2012; Mouriec et al., 2009).

In this study, we present the first application of the tg(*cyp19a1b*-GFP) embryos for the assessment of the estrogenic activity in the EDA of an environmental sample. EDA was conducted on the extract of a sediment sample from the Czech river Bilina, for which a previous analysis (Schmitt et al., 2010) using the yeast oestrogen screening assay (YES) had identified estrogenic effects. Given the known estrogenic activity of this sediment sample it was used as a proof-of-principle to evaluate the suitability of embryos of a transgenic zebrafish reporter strain for the identification of estrogenic effects and compounds in sediments by EDA.

2. Materials and methods

2.1. Chemicals

A list of suppliers of analytical standards, chemicals and corresponding CAS number used during the exposure experiments can be found in the Table S1.

2.2. Effect-directed analysis

2.2.1. Sediment sampling

The sediment sample was obtained from a sampling campaign at the city of Most from the river Bilina, Czech Republic. Further details about the site and the sample processing were already described by Schmitt et al. (2010).

2.2.2. Sample preparation

Chemicals from the sediment sample were extracted with pressurised liquid extraction, followed by a clean-up with gel permeation chromatography and fractionation with normal phase HPLC. In each step of sample preparation an aliquot of the sample was removed and analysed for the presence of estrogenic compounds using embryos of the tg(*cyp19a1b*-GFP) zebrafish strain. Prior to analysis of estrogenic effects, aliquots had been exposed to different dilutions (see below) to identify the highest non-toxic concentration. Toxicity tests had been performed as described by Knöbel et al. (2012) except that exposures were conducted in crystallisation dishes. In those aliquots where no lethal or sublethal effect was shown, the oestrogen screening assay was performed (see Fig. 1).

2.2.2.1. Pressurised liquid extraction. Fifty grams of the sediment sample were subjected to pressurised liquid extraction (PLE) in an accelerated solvent extraction (ASE) apparatus (Dionex ASE 200 Idstein, Germany). In brief, the sample was split up into portions of 10 g and mixed thoroughly with 4.2–4.5 g of anhydrous Na_2SO_4, filled into a 33 mL stainless steel extraction cell and extracted with acetone and dichloromethane (DCM) (35:65 volume ratio) at a temperature of 50 °C and a pressure of 1500 psi. The extraction was performed in three cycles of static extraction for 10 min. Finally, the extraction cell was purged for 100 s with nitrogen. From the total extraction volume of 450 mL corresponding to 50 g sediment equivalents (SEQ), an aliquot of 10 mL, corresponding to 1.1 g SEQ was removed, dried under nitrogen and redissolved in 1.5 mL of dimethylsulfoxide (DMSO). Fish embryos were exposed to dilutions of 0.01, 0.1 and 1% of this DMSO sediment extract. Since malformations and oedema were observed for >20% of all embryos in the 1% dilution, we did not analyse estrogenic activity for this concentration. The remaining 440 mL (48.9 SEQ) from the initial PLE extract were concentrated and redissolved in 10 mL of DCM for further extraction by gel permeation (Fig. 1).

2.2.2.2. Gel permeation chromatography clean-up. The sediment extract was subjected to a clean-up by gel permeation chromatography (GPC) in order to eliminate macromolecules (*e.g.* humic acid) and elemental sulfur. The clean-up method was conducted according to the EPA 3640 test method using a Bio-Beads S-X3 gel column with a DCM (5 mL/min) mobile phase (EPA, 1994). The injection volume was 1 mL and the collection window was set to 22.2–45.5 min (of 66 min total duration). The collected fractions from the subsequent runs were poured together, dried under nitrogen and redissolved in 10 mL of *n*-hexane. One mL of *n*-hexane extract was dried in a gentle stream of nitrogen and redissolved in 1 mL of DMSO which corresponds to 4.89 SEQ. Dilutions of 0.01, 0.05% of this cleaned-up extract was used for analysis of estrogenic activity, since dilutions of 0.1, 0.5 and 1% caused mortality.

2.2.2.3. Normal-phase HPLC fractionation. Extracts corresponding to 44.01 g SEQ were loaded onto a HPLC by two consecutive injections of 4.5 mL. HPLC fractionation composed of three solvent delivery modules (Pro Star 210, Varian, Palo Alto, CA, USA), a fraction collector (Pro Star fraction collector 701, Varian), and a diode array detection system (Pro Star PDA detector 330, Varian) operated from 200 to 400 nm. For the fractionation a