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Effect directed analysis of riverine sediments—The usefulness of *Potamopyrgus antipodarum* for *in vivo* effect confirmation of endocrine disruption

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ABSTRACT

In vivo tests are not commonly used in effect directed analysis (EDA) approaches. In the present study, a novel methodology was developed whereby *Potamopyrgus antipodarum*, which is known to be sensitive to endocrine disrupting compounds, was used as test organism. Field sediments from a polluted site in the north of Belgium were extracted and fractionated using three coupled and automatically switched normal-phase HPLC columns. Part of the fractions were spiked to artificial sediments and tested in a sediment contact test with *P. antipodarum*. The other part was used for an *in vitro* effect confirmation with the ER-LUC and anti-AR CALUX assays. Two of the six tested fractions stimulated the reproduction of the snails, while two others inhibited the reproduction. The fractions that caused an increase in reproduction also showed an increased estrogenic potency in the ER-LUC assay. Chemical analysis revealed that one of the most prominent compounds in those fractions was bisphenol-A, which has already been reported to have a stimulating effect on the reproduction of *P. antipodarum* by other authors. Due to the fact that previous studies have shown that this snail is also present in the field at this certain site, it was possible to directly link the results with effects that were observed in the field. This study indicates that effect directed analyses, supported by *in vivo* biotests, are very useful tools in order to identify the compounds that cause adverse effects on organisms or even population level.

1. Introduction

One of the biggest challenges in evaluating the toxicity of complex field samples is the identification of single compounds or groups of compounds that could be responsible for adverse effects on biological communities in the environment. Within the last years, several different approaches were developed, which use biotests as diagnostic tools in the so-called effect directed analysis (EDA) or toxicity identification evaluation (TIE) (Samoiloff et al., 1983; Brack, 2003; Brack et al., 2007). These approaches are characterized by a step-by-step assessment of toxicity of environmental samples.

In the beginning, the whole sample (or an extract) is tested with one or more appropriate biotests in order to screen the toxicity of a specific sample. Biotests that are used in this first screening should preferably be cheap and easy to use high-throughput tests, such as most *in vitro* test using bacteria or specialised cell lines. If the sample appears to be toxic in this first screening, it will be fractionated, in order to narrow the compounds, which could be potentially responsible for the observed effects. Fractionation techniques differ, depending on the nature of the sample (water, sediment, pore water etc) and the aim of the study. The fractions are then tested again with the same biotests as used before. Afterwards the most toxic fractions are analysed by instrumental analytical techniques such as GC–MS or LC–MS in order to detect possible key pollutants. They finally need to be confirmed, e.g. by testing uncontaminated standards at concentrations measured in the fractions or in dilution series around this value. In the end, there should be preferably a small number of toxicants, proven to be responsible for the observed effects in the biotests. In this whole procedure, the step from the whole sample assays to the tests with the single fractions is crucial. There is still a need to extend the range of biotests, especially the more environmentally relevant *in vivo* biotests that can be used for both whole samples as well as extracts (Hecker and Hollert, 2009). This is most problematic for the toxicity assessment of sediments, since only very few available *in vitro* assays can be performed on whole sediment samples (Weber et al., 2006). There are only few studies using *in vivo* whole sediment tests for EDA or TIE (Phillips et al., 2009) pointing out that there is a need for those studies.

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Within this study, an *in vivo* sediment contact test with the mudsnail *Potamopyrgus antipodarum* (Duft et al., 2007), has been adapted in order to test extracted sediments and their fractions via exposure to spiked artificial sediments. This particular snail has already been described as being very sensitive to endocrine disrupting compounds (Duft et al., 2003), whereby even an estrogen receptor (Stange et al., 2009) and vertebrate like steroids (Gust et al., 2010) were detected in the snails' tissue. As sampling site, we selected a rather polluted site in the north of Belgium, which was already described and investigated in previous studies (Weiss et al., 2009; Schmitt et al., 2010a; Sormunen et al., 2010). The fact that the raw sediment of this site has already been tested with *P. antipodarum* (Schmitt et al., 2010a) and that a small population of this species was even present in the same river, gives the unique opportunity to link observed effects directly to field populations (Schmitt et al., 2010b). The final goal is the development of an endocrine endpoint specific *in vivo* test that has strong links to field conditions and that could form an important step for the integration of EDA in environmental risk assessment. This paper will provide suggestions for a suitable test organism and test design.

2. Material and methods

2.1. Sampling site and preparation

Samples were taken at the site, called Eenhoorn (Een) at the river Groot Schijn in the northern part of Belgium. This river is suspected of point source pollution from both industrial as well as domestic wastewater. Further details about this site were already given in previous studies (Schmitt et al., 2010a; Sormunen et al., 2010). Sediment samples were taken with a core sampler measuring 10 cm in diameter. Three replicates were taken along a river section and homogenised on site. Sediments were dried and sieved (63 μm) directly after sampling and the extraction and fractionation procedure was carried out subsequently.

2.2. Extraction and fractionation

Freeze-dried, sieved and homogenised sediment samples, equal to 5 g sediment equivalence (SEQ) were extracted with pressurized liquid extraction using an accelerated solvent extraction device (Dionex ASE 200, Sunnyvale, CA). Dichloromethane:acetone 3:1 (v:v) served as extraction solvent, and the Dionex ASE 200 was operated with a pressure of 103 bar; three cycles lasting 10 min each and a temperature of 50 °C. Extracts were purified with accelerated membrane assisted clean-up (AMAC) similar as described in detail by Streck et al. (2008). Briefly, analytes were separated from matrix components using a semi permeable membrane made of polyethylene (80 μm Polymer Synthese Werk, Rheinberg, Germany) and Dionex ASE 200 in a dialysis like process. Aliquots of the extract equivalent to 10 g of sediment were dissolved in 1 mL dichloromethane and processed by AMAC with 16 cycles lasting 5 min using hexane:dichloromethane 1:1 (v:v) at 40 °C and 35.5 bar followed by further 16 cycles using dichloromethane:acetone 7:3 (v:v). All extracts were evaporated to dryness, re-dissolved in hexane:dichloromethane 9:1 (v:v) and fractionated on a normal phase multi step HPLC with three HPLC columns, a nitrophenylpropyl silica (250 mm × 21 mm, 5 μm Nucleosil 100-5 NO_2) and a cyanopropyl silica column (250 mm × 21 mm, 5 μm Nucleosil 100-5 CN, both from Macherey-Nagel, Düren, Germany), and a porous graphitised carbon column (Hypersil PGC, 50 mm × 10 mm, 7 μm, Thermo Fisher Scientific, Waltham, MA., USA) as it was described by Lübcke-von Varel et al. (2008). Fractions eluting first contain compounds which are known to contribute only for a minor part or even not at all to the total estrogenic or anti-androgenic activity (e.g. diaromatic chlorinated compounds or low molecular weight polycyclic aromatic hydrocarbons) (Nesaretnam and Darbre, 1997; Pliskova et al., 2005) and were finally discarded. Fractions eluting later from the nitrophenylpropyl silica and the cyanopropyl silica column, which can contain more polar compounds, e.g. hydroxylated polyaromatic compounds or steroids, were processed further for chemical or biological analysis. These fractions are numbered as fractions 12–17.

2.3. Sediment contact test

P. antipodarum were collected from the small river l'Homme close to Han-sur-Lesse in the south of Belgium (the Ardennes), six months prior to the experiments. This area is mainly a recreational area with no heavy industry, and low population density. Hence chemical pollution should be limited. Snails were bred in 10 L glass aquaria containing 6–8 L fully reconstituted water (pH-value 7.9–8.4, conductivity: 500–600 $\mu S\,cm^{-1}$) at 16 °C and at a light regime (L:D) of 16:8 hours. Snails were fed daily on commercially available fish diet as described by Duft et al. (2007).

The sediment contact test was carried out in a static system under the same constant conditions as used for the snail culture. 1 L glass beakers were used as test vessels. They contained 50 g of artificial sediment and 600 mL reconstituted water each. The artificial sediment consisted of 97.5% quartz sand and 2.5% fine-grounded beech leaves (*Fagus sylvatica*). The dried sediment fractions were dissolved in 50 mL dichloromethane and the artificial sediment was spiked with 25 mL dissolved fractions (equal to 2.5 g SEQ), by ensuring that the whole sediment matrix is covered with the dissolved fractions. Two replicates were carried out for each fraction. Beside the fractions, also a solvent control, spiked with 25 mL pure dichloromethane was included. The solvent was evaporated over night and the reconstituted water was added the next day. 30 adult snails with a shell height of 3.5 ± 1 mm were randomly inserted to each test vessel. After four weeks of exposure the snails' growth, reproduction (expressed as number of embryos) and mortality were analysed according to Duft et al. (2007).

2.4. Anti AR-calux

Anti-androgenic potencies of the Een fractions were determined in the AR-CALUX® bioassay (BioDetection Systems, Amsterdam, The Netherlands). The AR-CALUX bioassay is a reporter gene assay consisting of a human osteoblast cell line that carries a luciferase gene under transcriptional control of an androgen responsive element (Sonneveld et al., 2005). Dihydrotestosterone (DHT, Sigma–Aldrich, The Netherlands) and flutamide (FLU, Sigma–Aldrich, The Netherlands) were used as androgenic and anti-androgenic reference compounds, respectively. The assay was performed according to the original publication by Sonneveld et al. (2005) and AR antagonistic responses (exposure for 24 h) were expressed as relative amounts of FLU equivalents per g of sediment dw. Triplicates of a dilution series in DMSO of the sediment fractions were analysed in the presence of the EC_{50} of DHT (2×10^{-10} M). The amount of FLU equivalents (FEQ) in the sample was determined by interpolating the response of the sample into the concentration–response curve of the reference compound. To exclude the possibility that cytotoxicity was responsible for the inhibition, those samples exhibiting anti-androgenic activity were measured again in the presence of an excess of DHT, which should lead to an increase in luciferase and thus light production. A minimum of 10% inhibition was set as limit of detection for antagonistic androgenicity. The most diluted extract still containing activity higher than the limit of detection (LOD) was used for quantification to avoid possible matrix effects.