Available online at www.sciencedirect.com
ScienceDirect
Science of the Total Environment 386 (2007) 134–144
Science of the Total Environment
An International Journal for Scientific Research into the Environment and its Relationship with Humankind
www.elsevier.com/locate/scitotenv

Sample preparation method for the ER-CALUX bioassay screening of (xeno-)estrogenic activity in sediment extracts

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Received 28 January 2007; received in revised form 24 May 2007; accepted 5 June 2007
Available online 9 July 2007

Abstract

The application of bioassays to assess the occurrence of estrogenic compounds in the environment is increasing in both a scientific and statutory context. The availability of appropriate validated methods for sample pre-treatment and analysis is crucial for the successful implementation of bioassays. Here, we present a sample preparation method for the bioassay screening of estrogenic activity in sediment with the in vitro Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER-CALUX®) assay. The method makes use of an Accelerated Solvent (ASE) or Soxhlet extraction with a mixture of dichloromethane and acetone (3:1, v/v), followed by clean up of the extract by Gel Permeation Chromatography (GPC). Recoveries of a panel of 17 pollutants differing largely in physical–chemical properties from spiked sediment were determined and appeared to be on average about 86%. Furthermore, the estrogenic potencies of all test compounds were individually assessed by determination of concentration–response relationships in the ER-CALUX assay. Concentration dependent estrogenic potency was found for 14 of the 17 compounds, with potencies of about 10^5 to 10^7 fold lower than the natural estrogenic hormone 17β-estradiol. Anti-estrogenic potency was assessed by testing combinations of estradiol and individual test compounds, but was found for none of the compounds. The low estrogenic activity of the test compounds in the spiking mixture was well recovered during GPC treatment of the pure mixture, but did not contribute significantly to the background estrogenic activity present in the spiked sediment. Application of the method to field samples showed that estrogenic activity can be found at different types of locations, and demonstrated that levels between locations may vary considerably over relatively short distances.

Keywords: Sediment; Estrogen; Extraction

1. Introduction

Contamination with organic chemicals is one of the impacts human activities have on the aquatic environment. Compounds like polyhalogenated aromatic

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doi:10.1016/j.scitotenv.2007.06.002

hydrocarbons, pesticides, surfactants and their degradation products are ubiquitous nowadays. Due to sorption processes, organic chemicals may accumulate in sediments. This can lead to the exposure of sediment-dwelling aquatic organisms. A very wide range of natural as well as anthropogenic chemicals, such as steroids, alkylphenols, bisphenols, phthalates and chlorinated hydrocarbons, have been found to be weakly estrogenic (Klotz et al., 1996; Andersson et al., 1999; Blair et al., 2000). These compounds differ greatly in physical–chemical properties and chemical structures. Exposure to compounds with an estrogenic mode of action is associated with the occurrence of endocrine disruptive effects in wild populations of male fish, such as high frequency of intersex gonads (Gray and Metcalfe, 1997; Kirby et al., 2004) and the induction of the female yolk protein vitellogenin (Jobling et al., 1998; Kirby et al., 2004).

Chemical analysis of (xeno)-estrogens in environmental matrices is essential to assess exposure concentrations of individual compounds, e.g. for (trend) monitoring and for risk assessment purposes. However, with this type of analysis alone, biological effects cannot be predicted, and mixture effects and contributions of unknown compounds with similar modes of action to the overall effect cannot be taken into account. Therefore, several in vitro bioassays for estrogenicity, based on the mechanism of activation of the estrogen receptor, have been developed as tools to address the above mentioned issues (Routledge and Sumpter, 1996; Murk et al., 2002). These assays can be used to determine estrogenic potencies of individual compounds and for measuring the total estrogenic activity of complex mixtures of compounds. Bioassays can thus be used to determine total estrogenic activities in (extracts of) environmental samples, without the necessity of knowing all compounds present that contribute to the activity. The Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER-CALUX) assay is an example of a rapid and very sensitive in vitro reporter gene assay for estrogenic activity (Legler et al., 1999). It has been used for the assessment of estrogenic potency in sediment with detection limits in the low pg Estradiol Equivalents/g dry sediment range (Legler et al., 2003; Houtman et al., 2004a).

Due to the added value of bioassay measurements to chemical monitoring, the application of bioassays in a statutory context is nowadays gaining attention. On a national level, the Dutch government has committed the use of bioassays in the regulation of disposal of contaminated dredged material into coastal waters (Ministerie van Verkeer en Waterstaat, 2004) and is exploring the possibilities of the application of bioassays in the monitoring of ecological quality within the European Water Framework Directive (European Commission, 2005; Maas and Van den Heuvel-Greve, 2005), in which sediment is expected to receive more attention in time (SedNet, 2004). In addition, countries as the United Kingdom, Ireland, France, Italy, the USA, Canada and Australia have implemented the use of bioassays for environmental monitoring in law or other regulations, as reviewed by Van den Heuvel-Greve et al. (2005). On the international level, the OSPAR Commission for the Protection of the Marine Environment of the North-East Atlantic has recommended the optimization and integration of the assessment of biological effects and chemical monitoring (OSPAR Commission, 2000).

The development and validation of methods is crucial for the successful implementation of bioassays, especially when estimating the contribution of known chemicals to estrogenic activity measured in bioassays. Although several studies have focused on the use of bioassays for the detection of (mixture) effects of (xeno)-estrogens (Payne et al., 2000; De Boever et al., 2001; Murk et al., 2002; Legler et al., 2002b), the development of sample extraction and preparation methods for both chemical and bioassay analysis of these compounds in sediment has received less attention.

Several differences between bioassay analysis and chemical (target) analysis should be considered in the development of sample preparation methods for bioassay analysis. First, the use of internal standards, common practice in chemical analysis to correct for low recoveries of target compounds, is difficult. Because bioassays measure the combined activities of all compounds in a sample, the activity of the internal standard compound cannot be distinguished from the activity of the other compounds present. Recoveries can therefore best be determined “externally” in separate samples or positive controls. The method should therefore provide recoveries high enough to get a good impression of the total activity present in the sample without correction for the recovery of an internal standard. Furthermore, as bioassays are often applied to samples in which unknown compounds cause the activity, sample preparation methods should recover a broad range of compounds from a sample, to ensure the inclusion of as many active compounds as possible. This is especially important in the analysis of estrogenic activity, as estrogenic compounds are very diverse in chemical and structural properties (Blair et al., 2000). Chemical analysis is often focused on optimal recoveries of a small number of target compounds. In contrast, sample preparation methods for bioassay analysis of (xeno-)estrogens should retain as many

possibly relevant compounds covering a range of chemical and physical properties that is as wide as possible. Nevertheless, compounds interfering with the analysis should be removed. In addition, this type of "broad range" sample preparation method may allow the use of the same extract for the characterization of other toxic activities with other assays.

Here, we present an extraction and clean up method developed for the analysis of (xeno-)estrogenic compounds in sediment with the ER-CALUX assay. Estrogenic compounds were extracted from sediment with Accelerated Solvent Extraction or Soxhlet extraction. Both methods were tested with two different solvent combinations, to cover a wide range of polarities. Gel permeation chromatography (GPC) was used to remove sulphur and biogenic macromolecules (e.g. humic acids) from the extract. Previous work showed that such a clean up of sediment extracts is necessary to avoid disturbance of the ER-CALUX response by non-specific or cytotoxic effects of matrix components (Houtman et al., 2004a). First, the recovery of a series of pollutants, spiked to sediment samples in environmentally relevant concentrations, was chemically determined. The panel of spiking compounds was composed in such a way that its components represented the main classes of compounds known nowadays to have xeno-estrogenic or other endocrine disrupting potential. Non- or slightly estrogenic compounds were also included to extend the range of chemical and physical properties in the spiking mixture. Among the spiking compounds were priority compounds according to OSPAR and the European Water Framework Directive (Blair et al., 2000). Secondly, the estrogenic and anti-estrogenic properties of these compounds were determined to investigate if their presence in the spiked sediment would evoke a response in the ER-CALUX assay. Finally, the applicability of the method was investigated in a small field study with sediment samples from Dutch freshwater and marine locations.

2. Materials and methods

2.1. Solvents, standards and spiking mixture

All solvents were pro-analysis quality or better and were purchased from JT Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany) unless stated otherwise.

Standards of 17β-estradiol (E2), bisphenol A (BPA), γ-hexachlorocyclohexane (lindane), and tetrachlorobisphenol A (TCBPA) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands), 2,4′-dichlorodiphenyltrichloroethane (*o*,*p*′-DDT), 4,4′-dichlorodiphenyl trichloroethane (*p*,*p*′-DDT) and 4,4′-dichlorodiphenyl dichloroethylene (*p*,*p*′-DDE) from Dr. Ehrenstorfer (Augsburg, Germany), and benz(*g*,*h*,*i*)perylene, α-chlordane, β-endosulfan, 2,4,5-trichlorobiphenyl (PCB29), 1,2′,3,4,5′-pentabromobiphenyl (PBB101) and 1,2′,3,3′,4,5′-hexabromobiphenyl (PBB153) from Ultra Scientific (Wesel, Germany). 1,1′,3,3′-Tetrabromodiphenylether (BDE47) and 1,1′,3,3′,4-pentabromodiphenylether (BDE 99) were obtained via the EU FIRE project from Professor A. Bergman, Stockholm University, Sweden. 4-Nonylphenol (NP) and 4-octylphenol (OP) were obtained from Acros (Geel, Belgium), and dieldrin from LGC (Teddington, UK). All standards were at least 97% pure, except TCBPA and dieldrin for which no purity data were available. From the standards, highly concentrated stock solutions (mM range) were made in acetone. The concentrations of the stock solutions depended on the solubility of the individual compounds and on the available amount of pure compounds. Dilution series in dimethylsulfoxide (DMSO, spectrophotometric grade, Acros, Geel, Belgium) were prepared from the stock solutions for the measurement of (anti)-estrogenic activity.

The recovery after the sample preparation was determined by chemical analysis of a panel of compounds that were spiked to sediment. A spiking mixture was prepared to spike the sediment with the compounds. Concentrations in the mixture were chosen according to their occurrence in the environment, but at levels at least 10 fold the signal-to-noise ratio as determined by GC–ECD and GC–MS in the unspiked sediment. Spiking concentrations of the individual compounds in sediment are shown in Table 1. To prevent that the same compounds originally present in the sediment sample would interfere with determined recoveries, deuterated or ^{13}C labeled analogues were used for the following compounds: *p*,*p*′-DDT-d8, *o*,*p*′-DDT-d8, and *p*,*p*′-DDE-d8 from Dr. Ehrenstorfer (Augsburg, Germany), α-chlordane-^{13}C, dieldrin-^{13}C, and β-endosulfan-d4 from C.N. Schmidt (Amsterdam, The Netherlands), BPA-d16 from Sigma-Aldrich (Zwijndrecht, The Netherlands). A similar spiking mixture containing all compounds in their non-deuterated and non ^{13}C labeled forms, at similar concentrations, was prepared and used for the analysis in the ER-CALUX assay. The reference compound E2 was not a component of the mixture, but was tested separately.

2.2. Sample pre-treatment

Wet surface sediment was collected from a reference location (Oysterpit, Kamperland, The Netherlands) and sieved (mesh size 63 μm). Fifty grams of this fraction

were spiked with 1 mL of the spiking mixture in acetone. After addition of 100 mL demineralized water, the slurry was shaken for 72 h, freeze-dried and homogenized. To assess background levels of individual compounds, the whole procedure was repeated with unspiked sediment. In this way, concentrations of compounds originally present in the used sediment and for which no deuterated or ^{13}C labeled analog was used in the mixture, could be subtracted from the concentrations measured in spiked sediment.

2.3. Extraction with Soxhlet or ASE

Spiked and unspiked sediments were extracted with two methods, respectively Soxhlet and Accelerated Solvent Extraction (ASE 200; Dionex, Sunnyvale, CA, USA). Each extraction method was performed using two different solvent combinations, hexane:acetone (3:1, v/v) and dichloromethane (dcm):acetone (3:1, v/v), to result in four different methods that were mutually compared. Soxhlet extraction was performed for 16 h at 70 °C with the hexane:acetone mixture or at 50 °C with the dcm:acetone mixture respectively. ASE extractions were performed at 50 °C, 2000 psi, with three extraction cycles. Soxhlet, as well as ASE extractions, were performed in triplicate. Extracts were evaporated until approximately 1 mL of the residual solvent was left.

2.4. Clean up with gel permeation chromatography

GPC clean up was performed on polystyrene-diphenylbenzene copolymer columns (PL-gel, 10 μm, 50 Å, 300×25 mm, Polymer Laboratories, 2 columns in serial connection, with a 2 mL injection loop, and with 10 mL/min dcm as eluens, T=18 °C). Chromatograms were recorded with a Waters 900 photodiode array detector (λ=200–350 nm). The elution profile of individual compounds was assessed by injection of 2 mL of standard solutions (concentration 0.5–10 mg/L). The elution profile of sediment matrix was assessed by injection of

Table 1
Composition of the spiking mixture, estrogenic potencies of its components (expressed as median effective concentrations (EC50), estradiol equivalence factors (EEF) and maximum induction levels (MIL)) and their calculated contributions to the estrogenic activity of the mixture

Compound	Log Kow[1]	Concentration	Concentration	EC50	EEF[2]	MIL[3]	Contribution
		μM	ng/g sediment	μM	–	%	pg EEQ/g sediment
Reference compound							
17β-estradiol (E2)	3.94	–[4]	–[4]	4.6 (±1.0) E-6	1	100	–[4]
Xeno-estrogens							
Bisphenol A (BPA)	3.64	3.85	19.26	0.08±0.00	$4.4\ (\pm0.5)\times10^{-5}$	144±44	0.93
α-Chlordane	6.26	0.76	6.85	2.2±0.9	$1.5\ (\pm0.1)\times10^{-6}$	55±5	0.01
p,p′-DDE	6.00	0.92	6.61	7.5±0.4	$4.9\ (\pm0.9)\times10^{-7}$	63±8	0.00
p,p′-DDT	6.79	0.79	6.10	2.3±2.0	$2.4\ (\pm1.6)\times10^{-6}$	94±2	0.01
o,p′-DDT	6.79	0.77	5.97	0.09±0.03	$4.9\ (\pm2.4)\times10^{-5}$	96±5	0.28
Dieldrin	5.45	0.80	6.64	3.6±0.7	$8.8\ (\pm0.7)\times10^{-7}$	70±5	0.00
β-Endosulfan	3.50	1.36	11.89	2.2±0.6	$2.1\ (\pm1.2)\times10^{-6}$	50±2	0.02
4-Nonylphenol (NP)	5.99	2.72	13.13	0.10±0.01	$4.0\ (\pm0.1)\times10^{-5}$	117±5	0.60
4-Octylphenol (OP)	5.50	2.91	13.12	1.3±0.0	$2.8\ (\pm0.0)\times10^{-6}$	143±26	0.04
PCB 29	5.69	0.81	4.56	7.0±1.9	$5.5\ (\pm0.3)\times10^{-7}$	85±10	0.00
TCBPA	6.22	8.96	71.78	8.3±0.6	$6.2\ (\pm0.4)\times10^{-7}$	91±26	0.03
Non- or slightly estrogenic compouds							
Benz(*g,h,i*)perylene	6.70	9.79	59.17	>1.0	$<2.70\times10^{-6}$	–[5]	0.00
Lindane	4.26	0.90	5.73	15±7	$2.9\ (\pm0.5)\times10^{-7}$	25±3	0.00
PBB 101	8.21	0.36	4.30	>0.1	$<4.3\times10^{-4}$	–[5]	0.00
PBB 153	9.10	0.31	4.30	>0.6	$<4.3\times10^{-6}$	–[5]	0.00
BDE 47	6.77	0.41	4.39	3.9±0.5	$8.9\ (\pm1.2)\times10^{-7}$	35±1	0.00
BDE 99	7.66	0.35	4.32	2.5±0.2	$1.4\ (\pm0.1)\times10^{-6}$	8±0	0.00
Calculated sum of estrogenic activity							1.85

[1]Estimated value according to Episuite v1.67, U.S.EPA.
[2]EEF: Estrogenic Equivalence Factor. EEF of compound X calculated with Eq. (5) (Materials and methods).Values are averages of two independent experiments.
[3]MIL: maximum induction level relative to E2, calculated with Eq. (6) (Materials and methods).
[4]Values of 17β-estradiol are given for comparison. 17β-estradiol was not a component of the spiking mixture, but was tested separately.
[5]Maximum induction level was not achieved.

2 mL of the extract equivalent of ca. 2 g dry weight of a standard internal reference marine sediment available at our laboratories (extracted by Soxhlet extraction, with hexane:acetone 3:1 v/v as solvent at 70 °C).

The recovery of compounds after GPC clean up was assessed in triplicate by injection of 2 mL of a mixture of the spiking compounds (individual concentrations about 20 μg/L). Based on the results of the elution profile tests, the fraction 17–24 min after injection was chosen to be collected and internal standard PCB112 was added to obtain a concentration of 20 μg/L to correct for possible losses after the GPC cleaning step. The eluate was evaporated until approximately 1 mL remained, 4 mL of ethyl acetate was added and the extract was evaporated further till ca. 200 μL remained. To test the influence of sediment matrix on the elution and recovery of compounds during GPC clean up, the experiment was repeated in triplicate with 2 mL of the spiking mixture being enriched with the extract equivalent of ca. 2 g dry weight of the same sediment extract as used in the assessment of the elution profile. Background levels of the same compounds as present in the spiking mixture were determined in unspiked sediment extract that was injected on GPC and analyzed. In both cases PCB112 (20 μg/L) served as internal standard.

Clean up of the extracts of spiked sediment was performed by quantitative injection of the extract, and collection of the fraction 17–24 min.

2.5. Chemical analysis

Analysis of compounds was performed with gas chromatography with electron capture detection (GC–ECD) and gas chromatography with mass selective detection (GC–MSD) in the positive (PCI) and negative (NCI) chemical ionization mode. Compounds containing chlorine or bromine atoms were analyzed with GC–ECD or GC–NCI–MS. Other compounds were analyzed with GC–PCI–MS. The GC–ECD measurements were performed on a HP GC–ECD 6890 with a 15 m Varian CP-Sil 8 CB low bleed column (0.15 mm i.d., 0.30 μm film thickness). The oven temperature program was as follows: initial temperature 90 °C for 3 min, increasing with 30 °C/min to 215 °C, which was held for 40 min, then at a rate of 5 °C/min to 270 °C, which was held for 30 min. The injector temperature was 250 °C and the detector temperature was 300°. GC–MSD measurements were performed on a HP GC 6890 with a HP 5973 network quadrupole mass selective detector, equipped with a 50 m CP-Sil 8 column (0.25 mm i.d., 0.25 μm film thickness). The oven temperature program was: initial temperature 70 °C for 3 min, increasing at 30 °C/min to 210°, was held there for 5 min, increased then with 10 °C/min to 310 °C and was held there for 17 min. An additional 10 min at this temperature served for cleaning the column. The PCI and NCI modes were used with CH_4 as CI gas, in the PCI mode at a flow of 0.8 mL/min and an ion source temperature of 250°, and in the NCI mode at a flow of 1.2 mL/min and an ion source temperature of 150 °C. Quantification was performed by external calibration using a calibration series of 3 concentrations. E2 was determined with GC–MS/MS according to Houtman et al. (2004b).

2.6. (Anti-)estrogenicity testing in ER-CALUX

The in vitro ER-CALUX assay was performed with stably transfected T47D human breast cancer cells (T47D.Luc-cells) according to Legler et al. (1999) with adaptations as described in Houtman et al. (2004a). ER-CALUX cells were obtained from BioDetection Systems B.V., Amsterdam, The Netherlands. Estrogenic potencies of individual compounds were tested in triplicate in at least two independent experiments and a concentration series of E2 (ten concentrations between 0 and 100 pM) was included on each plate. Sigmoidal standard curves with the y value representing luciferase activity in relative light units and the x value representing the concentration of compound) were fitted for E2 and each individual test compound using the software program SlideWrite 4.1 (Advanced Graphics Software, Carlsbad,CA, USA), according to Eq. (1).

$$y = a_0 + \frac{a_1}{1 + e^{\frac{-(x-a_2)}{a_3}}}. \quad (1)$$

Background activity $y(0)$ and maximum response $y(\infty)$ were calculated as:

$$y(0) = a_0 + \frac{a_1}{1 + e^{\frac{a_2}{a_3}}} \quad (2)$$

$$y(\infty) = a_0 + a_1. \quad (3)$$

Median effective concentrations (EC50 values) were derived from the concentration–response curve of each individual compound according to:

$$x\left(\frac{1}{2}y_\infty + \frac{1}{2}y_0\right) = -a_3 \ln\left(\frac{2}{\left(1 + \frac{1}{\left(1+e^{\frac{a_2}{a_3}}\right)}\right)} - 1\right) + a_2. \quad (4)$$

For comparison of estrogenic potency between E2 and the other compounds, the estrogenic activity of compound *X* was expressed relative to that of E2 by calculation of the estradiol equivalence factor (EEF value) for *X* with Eq. (5).

$$\mathrm{EEF}_X = \frac{\mathrm{EC50}_{\mathrm{E2}}}{\mathrm{EC50}_X}. \quad (5)$$

Maximum induction levels (MILs) of concentration–response curves of compound *X* relative to that of that of E2 were calculated as:

$$\mathrm{MIL}_X = \frac{(y_X(\infty) - y_X(0))}{(y_{\mathrm{E2}}(\infty) - y_{\mathrm{E2}}(0))} * 100\%. \quad (6)$$

Anti-estrogenicity of compounds was tested by co-exposure of the cells to a fixed concentration of E2 (3 pM, approximately 2/3 of EC50) and differing concentrations of test compounds (ranging between 0.1 and 100 μM). The concentration of DMSO present in the exposure medium during these measurements was 0.2% (v/v). Anti-estrogenic activity in this study was defined as the capacity of a compound to significantly inhibit the luciferase activity in the ER-CALUX assay induced by 3 pM E2. Combined activity of the test compound and E2 was interpolated in the E2 calibration curve and expressed as pM EEQ. In addition, the combined activity was compared to the expected combined activity according to the Concentration Addition concept (Loewe and Muischnek, 1926). Tamoxifen, a compound known to be anti-estrogenic in breast cancer cells, was used as positive control for anti-estrogenic activity. Significance of deviation from additivity was evaluated with Student's *t*-test in Slide Write 4.1.

2.7. Estrogenic activity of the spiking mixture after sample preparation

Estrogenic activity was tested in the GPC treated spiking mixture and in the extract of spiked sediment. ASE dcm:acetone 3:1 (v/v) extracts of 5 g of spiked sediment and 100 μL spiking mixture (the equivalence of 5 g of spiked sediment) were dissolved in triplicate portions of 1 mL dcm and cleaned with GPC as described above for spiked sediment, evaporated, dissolved in 50 μL DMSO and tested with the ER-CALUX assay for estrogenic activity.

2.8. Application to field samples

Surface sediments (about 2 kg) from five locations in The Netherlands were collected using a Van Veen grab, freeze-dried, sieved (mesh size 250 μm) and homogenized. Portions of 5 g were extracted with ASE and dcm:acetone (3:1, v/v), cleaned with GPC, taken up in 50 μL DMSO and tested in ER-CALUX for estrogenic activity.

3. Results and discussion

3.1. GPC clean up

The suitability of GPC for the removal of matrix components from sediment extracts for bioassay analysis was evaluated by the assessment of the elution profile and the recovery of a panel of environmentally relevant test compounds (Table 1). In addition, the separation that could be achieved between the most important matrix constituents and those compounds was evaluated.

The GPC elution profile of the test compounds is shown in Fig. 1. A dark-coloured fraction, containing the sediment matrix components, eluted between 11 and 19 min (Fig. 1). This implies a partial co-elution of matrix and some of the test compounds. Elemental sulphur (as S_8) eluted between 25 and 28 min after injection. It was decided that the fraction eluting between 17 and 24 min after injection should be collected to recover all test compounds, and at the same time to remove all sulphur and most of the matrix.

The recoveries of the test compounds after GPC were generally very good, both in the absence (on average 97±13%) and presence (on average 109±14%) of sediment extract. No influence on recovery of the test compounds was observed for the physical–chemical properties polarity and vapour pressure (data not shown), neither for the presence nor the absence of sediment matrix. Using GPC, a wide range of compounds could be separated from matrix components that would otherwise interfere with chemical and bioassay analysis. As GPC is a non-destructive technique, this clean up method enables the analysis of less persistent estrogenic compounds. This is important because of the observed occurrence of less persistent estrogenic compounds in sediments (Houtman et al., 2004a).

3.2. Extraction and clean up using extracts of spiked sediment

The recoveries of test compounds from spiked sediment after complete extraction and clean up with four different extraction methods combined with GPC clean up are visualized in Fig. 2. In general, comparable recoveries were found for both ASE and Soxhlet extractions and both solvent combinations. The average recoveries were 86±19% (ASE, dcm:acetone) 84±29%

(ASE, hexane:acetone), 87±18% (Soxhlet, dcm:acetone) and 89±28% (Soxhlet, hexane:acetone).

BPA was the only compound demonstrating a differential recovery depending on the solvent used, being only recovered after extraction with dcm:acetone (60%). As several xeno-estrogenic compounds, as well as the natural estrogenic hormones, have polarities close to that of BPA, this demonstrates the need of a more polar solvent for the extraction of (xeno-)estrogenic compounds. The endogenous estrogenic hormone E2, of which the recovery was investigated only with ASE and dcm:acetone (3:1, v/v), was recovered from sediment with a comparable efficiency as BPA (64%, Fig. 2). Also for less polar compounds, such as benz(*g*,*h*,*i*)perylene, PBB101 and 153 and BDE99, acceptable recoveries were found after extraction with dcm:acetone, indicating that this combination of solvents efficiently extracts compounds with a very wide range of polarities. This is an advantage if, apart from estrogenic activity, other toxic potencies need to be investigated in the same extract, such as androgenic activity, dioxin-like toxicity or mutagenicity (Vondrácek et al., 2001; Houtman et al., 2004a; Klamer et al., 2005).

3.3. Method evaluation

The present study used chemically determined recoveries of compounds extracted from spiked sediment to assess suitable sample preparation methods for the analysis of xeno-estrogenic activity in sediment. Acceptable recoveries were obtained for compounds differing largely in physical–chemical properties and structures. Our results thus indicate that extraction with dcm:acetone (3:1 v/v), performed either by Soxhlet or ASE extraction, is a good choice for the extraction of (xeno-)estrogenic compounds from sediment for bioassay analysis.

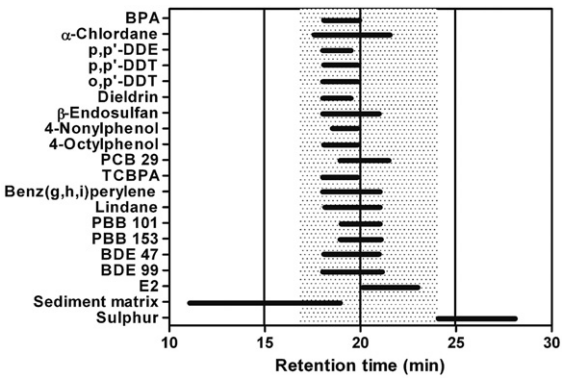

Fig. 1. Elution profile of a selection of environmental pollutants during Gel Permeation Chromatography treatment of sediment extract. Collection of the eluent in the time window 17–24 min after injection yields an extract from which sediment matrix and sulphur are largely removed.

Of course, one should be aware that not all compounds extracted from sediment with this combination of organic solvents might be bioavailable to organisms that are exposed to the investigated sediment. Therefore, concentrations or activities of compounds detected in extracts from sediment samples prepared with the discussed method could be considered as "worst case" values.

One difficulty in experiments with spiked sediment is to ensure that the binding of substances spiked to sediment accurately reflects the binding of compounds to (weathered) sediment samples from the environment. For example, addition of spiking compounds to sediment samples directly prior to extraction, common practice for aqueous samples such as water, may result in less strong binding of the compounds to the organic fraction of the sediment than is the case in real life samples and thus lead to higher recoveries (de Boer et al., 2001). One approach to partially circumvent this problem is to compare concentrations of compounds in unspiked sediment detected with different sample preparation methods (de Boer et al., 2001). However, in this approach, determination of recoveries is not possible. Therefore, the present study aimed to mimic binding of spiking compounds more realistically by letting the spiking mixture partition between the sediment and water in the slurry during a period of 3 days. Subsequently, the samples were freeze-dried. In this way, recoveries could be determined and extraction methods could be mutually compared.

The extraction method used in our further research was ASE extraction with dcm:acetone (3:1 v/v).

3.4. (Anti-)estrogenic activity of individual test compounds

All compounds from the selected panel were tested in the ER-CALUX assay for estrogenic and anti-estrogenic potencies. Fourteen compounds induced the estrogen receptor mediated production of luciferase in a concentration dependent manner. However, to what extent organisms exposed to these compounds in sediment are at risk for estrogenic effects not only depends on estrogenic potency, but e.g. also on the bioavailability and toxicokinetics of the compounds. These aspects are not fully covered by an in vitro test system.

Examples of concentration–response curves of a selection of compounds are depicted in Fig. 3. The EC50 values (Table 1) ranged from 0.10±0.0 μM for the most potent compound (NP), to 15±7 μM for the least

potent xeno-estrogen in this study (lindane). EEF values (Table 1) were between 4.9 (± 2.4)$\times 10^{-5}$ for *o*,*p*′-DDT, and 2.9 (± 0.5)$\times 10^{-7}$ for lindane. No estrogenic activity was observed for benz(*g*,*h*,*i*)perylene, PBB101 and PBB153. However, highest available test concentrations were relatively low for these compounds (at or below 1 μM). Therefore, it cannot be excluded that these compounds may be estrogenic at higher concentrations. Comparable estrogenic activities in the ER-CALUX assay have been reported earlier for BPA, α-chlordane, *o*,*p*′-DDT, dieldrin, β-endosulfan, NP, OP, BDE 47 and BDE 99 (Legler et al., 1999, 2002a; Meerts et al., 2001). Our results also confirm the estrogenic activities of *p*,*p*′-DDE, *p*,*p*′-DDT and lindane previously shown in other in vitro assays (Jobling et al., 1995; Payne et al., 2000, 2001; Okubo et al., 2004). PCB29 was also found to be estrogenic in vitro, an effect that had not been demonstrated earlier. However, the suspicion of PCB29 (or metabolites thereof) to be estrogenic in vivo has been raised before in common tern embryos (Nisbet et al., 1996).

Concentration–response curves of the estrogenic compounds tested show typical sigmoidal shapes (Fig. 3), with higher concentrations asymptotically approaching a maximum level of effect, reflected by the maximum induction level (MIL; Table 1). Full agonistic behaviour (with $MIL_X \approx MIL_{E2}$) was observed for *o*,*p*′-DDT, *p*,*p*′-DDT, NP, PCB 29 and TCBPA. Dieldrin, *p*,*p*′-DDE, lindane, β-endosulfan, BDE 47 and BDE 99 had maximum induction levels that were significantly lower than that of E2, identifying them as partial agonists. The mechanistic basis of partial agonism has been discussed

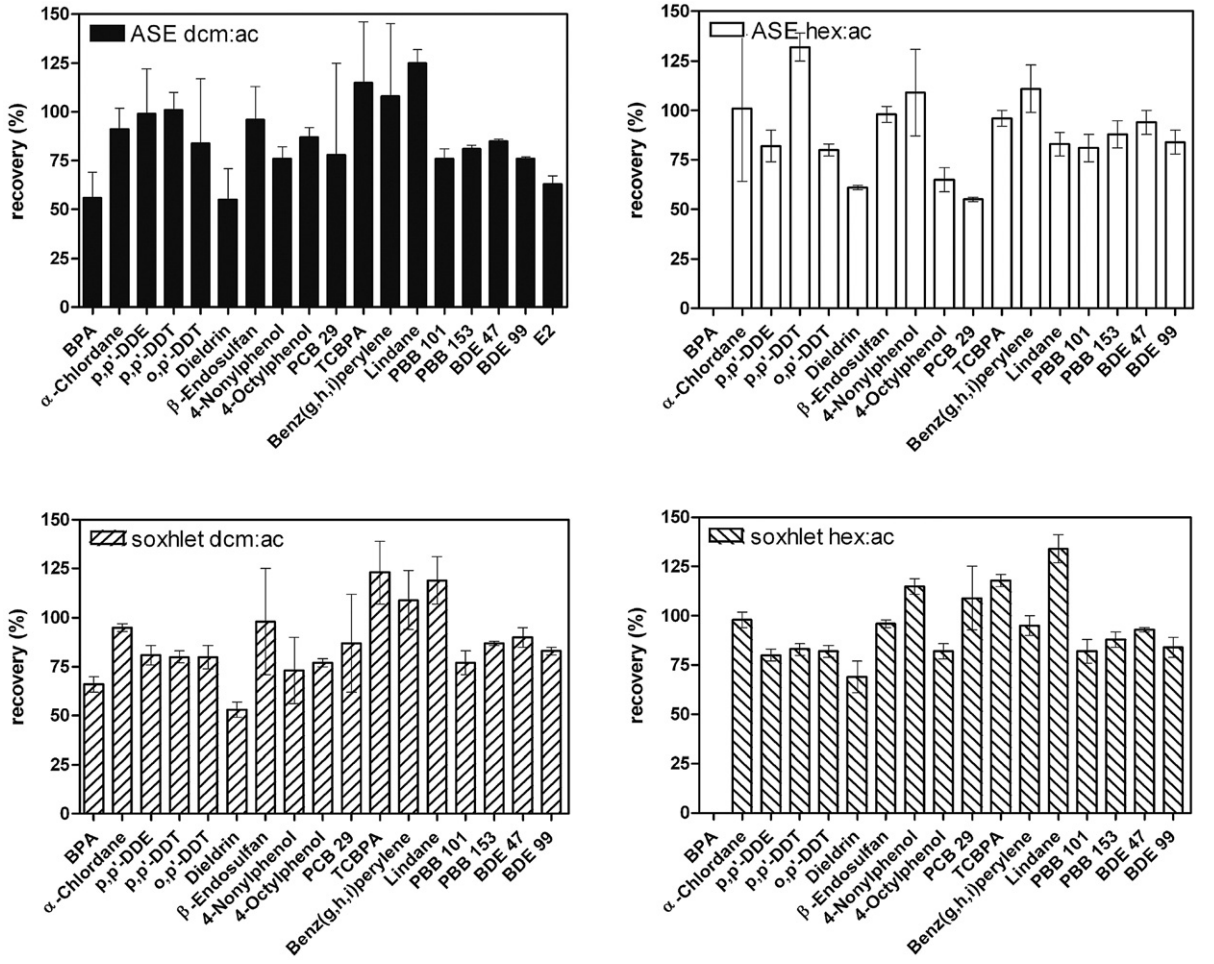

Fig. 2. Recoveries of a selection of environmental pollutants from spiked sediment that has been extracted with Accelerated Solvent Extraction (ASE) or Soxhlet extraction with dichloromethane (dcm):acetone (ac) (3:1 v/v) or hexane (hex):acetone (3:1 v/v). The extract was cleaned with Gel Permeation Chromatography. Abbreviations: BPA: bisphenol A; PCB: polychlorinated biphenyl; TCBPA: tetrachlorobisphenol A; PBB: polybromobiphenyl; BDE: bromodiphenylether; E2: 17β-estradiol.

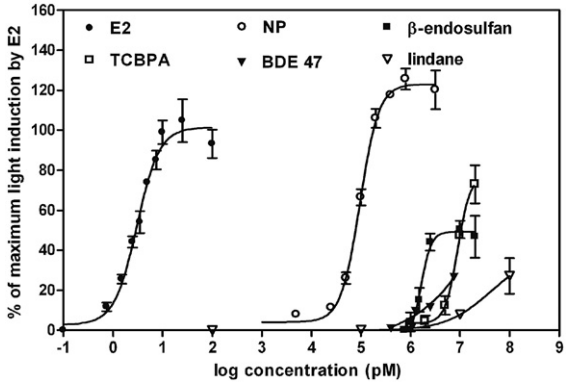

Fig. 3. Concentration–response curves in the ER-CALUX assay for reference compound 17β-estradiol (E2), full agonists 4-nonyl phenol (NP) and tetrachlorobisphenol A (TCBPA), and partial agonists β-endosulfan, 1,1′,3,3′-tetrabromodiphenylether (BDE 47) and lindane.

by Kong et al. for the partial agonist genistein (Kong et al., 2003). In this study, super-agonistic behaviour was observed for BPA and OP, although standard deviations in MIL were considerable. The mechanism behind this phenomenon remains to be elucidated, although effects on luciferase stability or stimulation of the expression of the estrogen receptor or co-activation factors by super-agonistic estrogens have been postulated as possible explanations (Legler et al., 1999).

Anti-estrogenic activity of the compounds was determined by exposure of the ER-CALUX cells to a fixed concentration of E2 (3 pM) in combination with different concentrations of test compounds. No inhibition of induction of luciferase activity was observed for any of the test compounds (data not shown), indicating none of the test compounds to be anti-estrogenic at the concentrations tested. However, it is possible that some of the compounds might behave as anti-estrogens at higher concentrations, as has been reported for micromolar concentrations of higher chlorinated PCBs (Pliskova et al., 2005). Combinations of E2 and estrogenic test compounds, depending on the applied concentration, often resulted in activities that were higher than that of E2 alone. Previous studies have shown that the estrogenic effects of mixtures of xeno-estrogens in the ER-CALUX assay can be described with the concept of Concentration Addition (Houtman et. al., 2006). Indeed, when comparing the combined activities of E2 and the estrogenic compounds in this study with their expected activities according to Concentration Addition, a very good agreement was found, with the observed activities on average being 101±19% of the predicted ones (data not shown).

3.5. Estrogenic activity of the spiking mixture

The theoretical contribution of each compound of the spiking mixture to the total estrogenic activity of the mixture was calculated as the product of the concentration and EEF value of each compound (Table 1). The total activity of the mixture was calculated as (with C_n the concentration of compound n and EEF_n its EEF value):

$$\text{Total activity} = \sum_{n=1}^{k} \text{EEQ} = \sum_{n=1}^{k} C_n \times \text{EEF}_n. \qquad (7)$$

Due to the low potencies of the xeno-estrogens in the mixture, the total activity of the mixture was thus calculated to be only 1.85 pg EEQ/20 μL (the equivalent of 1 g of spiked sediment). After GPC treatment and concentration of the pure spiking mixture in DMSO the activity was measured in the ER-CALUX assay, resulting in a value of 1.7±0.2 pg EEQ/20 μl spiking mixture, which shows the high average recovery of estrogenic activity (91±13%). The estrogenic activity of the spiking mixture, however, was too low to increase the estrogenic activity of spiked sediment above the background concentration of estrogenic activity present in unspiked sediment (19.0±0.3 pg EEQ/g dw). In a previous study, using a spiking mixture with higher concentrations of xeno-estrogens, high recovery of estrogenic activity after the extraction and clean up of sediment was demonstrated (Houtman et al., 2006).

The present study shows, in addition to the good recovery of estrogenic activity after GPC treatment, that, due to their low potencies in comparison with natural estrogenic hormones, only high concentrations of (mixtures of) xeno-estrogens in environmental samples are capable to evoke an estrogenic response in the ER-CALUX assay. And indeed, low concentrations of potent natural estrogenic hormones, and not the suspected xeno-estrogens, have been reported to be main contributors to estrogenic activity in several studies (Desbrow et al., 1998; Peck et al., 2004). Nevertheless, effective internal concentrations of xeno-estrogens could be achieved at lower environmental levels than expected solely based on the EEF, e.g. due to persistence, bioaccumulation and differences in sensitivity between the human T47D cells and other tissues or species and between life stages. Furthermore, exposure to mixtures instead of single compounds could lead to the onset of effects at concentrations at which the individual compounds would not be effective (Silva et al., 2002). These aspects together have led to the awareness that the presence and the possible effects in

the environment of mixtures of xeno-estrogenic compounds, albeit of weak potency in comparison with E2, deserve due attention (Silva et al., 2002; Charles et al., 2002).

3.6. Application of the method to field samples

The sample preparation method was applied to sediment samples from four locations in a small field study. Locations differed in e.g. marine or fresh water environment and in industrial and recreational pressure. The application illustrated the suitability of the method for the sample preparation of fresh water as well as salt-water sediments. The range of activities was comparable with those of former monitoring studies carried out at our laboratories (Legler et al., 2003; Houtman et al., 2004a), although in those studies different sample preparation methods were applied. Highest activity was found at location Zierikzee harbour (469±10 pg EEQ/g dw), a small harbour with pleasure boating and some old industry as main activities. The river Dommel, a small freshwater river where estrogenic effects in male fish have been observed before (Houtman et al., 2004b; Vethaak et al., 2005) showed less activity (81.6±5.4 pg EEQ/g dw), comparable with the industrial harbour of Terneuzen (69.4±4.2 pg EEQ/g dw). The sampling location in marine national park the Eastern Scheldt (4.0±0.1 pg EEQ/g dw), situated only a few kilometres out of Zierikzee harbour, showed a more than hundred fold lower estrogenic activity than Zierikzee harbour. Previous studies with Zierikzee sediment have identified the presence of the natural hormones E2 and E1 as main contributors to estrogenic activity (Houtman et al., 2006). These measurements confirm that estrogenic compounds can be found at different types of locations, and demonstrate that levels between locations may vary considerably over relatively short distances.

4. Conclusions

The availability of appropriate validated methods for sample pre-treatment and analysis is crucial for the successful implementation of bioassays both in a scientific and statutory context. In this study, a sample preparation method was presented for the bioassay analysis of estrogenic activity in sediment samples. With this method, good recoveries of spiked compounds covering a broad range of physical–chemical properties were achieved (on average 86%). The xeno-estrogens in the spiking mixture showed their estrogenic potency in the ER-CALUX assay, albeit with potencies of 10^5 to 10^7 fold lower than the natural estrogenic hormone E2. The combined estrogenic activity of the spiking mixture was well recovered during GPC treatment, but was too low to contribute significantly to the background estrogenic activity of the unspiked sediment. The sample preparation method can be applied to screen sediment samples for estrogenic activity possibly caused by a wide variety of contaminants as was shown in the small field study.

Acknowledgements

The authors thank Petra Booij, Bert van der Horst, Yoni van Houten and Ike van der Veen of the Institute for Environmental Studies for their technical assistance. Parts of this work were funded and assigned by the National Institute of Coastal and Marine Management, Dutch Ministry of Transport, Public Works and Water Management.

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