Identification of dioxin-like and estrogenic compounds in sediment using CALUX® assay-directed fractionation combined with two-dimensional comprehensive GCxGC–ToF MS

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Introduction

Sediment can act as a sink for many persistent chemicals released in the aquatic environment, and may form a source of exposure to aquatic organisms. In the Netherlands, the Dutch delta acts as a sedimentary basin for the major rivers Rhine, Meuse and Scheldt. Contaminants discharged upstream tend to accumulate there. One group of contaminants which has received much attention consists of the endocrine disrupting compounds which are xenobiotic as well as natural chemicals that may interfere with the endocrine system, leading to disturbances in development and reproduction. Although the presence of endocrine disrupting (e.g. dioxin-like and estrogenic) activities has been reported in sediment samples, the responsible compounds are often still mainly unknown[1;2].

The dioxin responsive (DR-) and estrogen responsive (ER-) CALUX®-assays (Chemical Activated Luciferase Gene Expression) are mechanism-based, rapid and extremely sensitive *in vitro* reporter gene bioassays developed to assess dioxin-like and estrogenic activity[3;4]. They provide useful information about the total dioxin-like or estrogenic potential of complex mixtures of chemicals in environmental samples. They are especially useful if combined with instrumental analytical approaches, as *e.g.* in bioassay-directed fractionation. In this approach, bioassays are used to direct fractionation and chemical analysis in order to elucidate compounds responsible for the toxic activity found in a sample.

The present study was undertaken to elucidate dioxin-like and estrogenic chemicals in sediment from the harbor of the small town of Zierikzee in the Dutch delta area. Former research had shown high dioxin-like and estrogenic activity in sediment from this location[1]. DR- and ER-CALUX® assay were used to direct fractionation and chemical analysis of sediment extract. Active fractions were analyzed with comprehensive multidimensional GC×GC–Time-of-Flight Mass Spectrometry to elucidate responsible compounds.

Methods and Materials

Environmental sampling, extraction and clean-up
Surface sediments were sampled in Zierikzee harbor and Eastern Scheldt in April 2002 using a Van Veen grab. Sediment samples were stored at -20ºC in bottles of dark brown glass until further treatment. Samples were sieved (250 µm), homogenized and freeze-dried. Forty grams of dried sediment from each location were extracted with a mixture of dichloromethane and acetone (3:1, v/v) with Accelerated Solvent Extraction (3 extraction cycles, 50ºC, system pressure 2000 psi; ASE200, Dionex). The extract was evaporated to a volume of 1 ml and cleaned on a Gel Permeation Chromatography (GPC) system (PL-gel, 10µm, 50Å, 300x25 mm, Polymer Laboratories, 2 columns in serial connection, with 10 ml/min dichloromethane as eluent). Eluate was collected between 17-24 minutes after injection. The extract was divided into two portions. Five percent of the extract was evaporated and dissolved in 40 µl dimethylsulphoxide (DMSO) for CALUX® measurements. The other 95% was evaporated and taken up in 100 µl methanol:water (MeOH: H_2O; 1:1 v/v) for fractionation.
Fractionation
Fractionation of GPC-extract was performed using a Reversed Phase High Pressure Liquid Chromatography (RP-HPLC) system at 22ºC, with a C18 semi preparative column (Vydac 2TP510, 5µm, 10.0x250 mm; mobile phase initially 50% MeOH and 50% H_2O (4.7 ml/min), changing linearly to 100% MeOH in 50 min.) according to [5;6]. 1 ml MeOH:H_2O sediment extracts were injected and 30 fractions of 3 minutes were collected. After injection, the vial that had contained the MeOH:H_2O extract was rinsed with hexane to dissolve possibly present compounds too non-polar to dissolve in MeOH:H_2O prior to fractionation. This fraction (the non-polar residual fraction) was dissolved in 70 µl DMSO and tested with DR- and ER-CALUX®. HPLC-fractions were dried with N_2 at 40°C and split into three portions. Portion A (10% of the extract weight) was evaporated and dissolved in 40 µl of DMSO for Dr- and ER-CALUX® measurements, Portion B (45%) was used for target analysis of known estrogens with GC-MS/MS and for two-dimensional comprehensive GC×GC–ToF MS measurements. Portion C (45%) of fraction 42-63 min. was further fractionated into 8 fractions by Normal Phase HPLC as described by Fernandez et al.[7]. 30% of the eluate in each fraction was evaporated, dissolved in 35 µl DMSO and tested in DR-CALUX® for dioxin-like activity.
Biological and chemical analyses
CALUX® assays. DR- and ER-CALUX® measurements were performed according to [2, 3, 4], with adaptations as described elsewhere[1]. H4IIE.Luc and T47D.Luc cells were obtained from BioDetection Systems (Amsterdam, The Netherlands).
GC-MS/MS analysis of estrogenic hormones. Portions B of the RP-HPLC fractions were derivatized by silylation (Sil A reagent, Sigma-Aldrich) and analyzed for the presence of the natural estrogenic hormones 17β-and 17α- estradiol (β-E2 and α-E2), the metabolites estrone and estriol and the synthetic estrogen 17α-ethynylestradiol (EE2) on a GC-MS with ion trap detector in MS/MS mode as described in [5].
Two-dimensional comprehensive GC×GC–ToF MS. 1 µl of derivatized extracts of fractions 9-24 min and 42-63 min were splitlessly injected on a GCxGC-ToF-MS (Agilent GC G1530A equipped with a PTV Optic 2 ATAS injector (300°C, 2 min.) and a Leco Pegasus II Time of Flight Mass Spectrometer (acquisition rate 50Hz, mass range m/z 70-800, ion source 250°, transfer line 280°), with a Restek XTI-5 column in the first dimension (length 10m, 0.25 mm i.d., 0.25 µm film thickness) and a SGE BPX-50 column in the second dimension (length 1 m, 0.1 mm i.d., 0,1 µm

film thickness). Helium (constant pressure 200 kPa) was used as carrier gas. Oven temperature was held at 70° for 1 min., then increased with 5°/min to 360° and held at 360° for 20 min. Modulation was performed each 5 seconds using an air cooling system. Obtained chromatograms were represented as contour plots showing peak intensities on a color scale using Transform software.

Results and Discussion

Dioxin-like and estrogenic activity in total extracts

GPC-extracts of sediments from Zierikzee harbor and reference location Eastern Scheldt were tested in DR- and ER-CALUX® bioassays to assess tot total dioxin-like and estrogenic activities present. Sediment from Zierikzee showed high responses in both assays, compared with activities formerly assessed for Dutch sediments[1, 8, 9]. Very low dioxin-like and estrogenic activities were found for location Eastern Scheldt (Table 1).

Table 1: Dioxin-like and estrogenic activities (average ± standard deviation) in total and fractionated sediment extracts.

	Zierikzee harbor	Eastern Scheldt
Dioxin-like activity	(pg TEQ/g dw)	(pg TEQ/g dw)
Total extract	4914 ± 17	19.7 ± 2.8
Sum of fractions	5665 ± 526	4.0 ± 0.5
Recovery of fractionation (%)	115	20
Estrogenic activity	(pg EEQ/g dw)	(pg EEQ/g dw)
Total extract	592 ± 16	4.0 ± 0.1
Sum of fractions	827 ± 93	10.5 ± 0.7
Recovery of fractionation (%)	139	263

TEQ: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent; EEQ: 17β-estradiol equivalent; dw: dry weight.

Distribution of DR and ER agonistic activities after HPLC-fractionation

To reduce the complexity of the sediment extracts, GPC-extracts were fractionated according to polarity with RP-HPLC. For each sediment sample, 30 three-minute fractions were collected and tested with DR- and ER-CALUX® for dioxin-like and estrogenic activities. For location Eastern Scheldt both activities were again low for all fractions, confirming the suitability of this location as reference. For Zierikzee harbor, dioxin-like activity was only found in less polar fractions (from 30 min. onwards). This finding is in accordance with the non-polar properties of known aryl hydrocarbon receptor agonists, which elute from the column when the gradient in eluent composition has reached 100% MeOH. Estrogenic activity was found in two clusters of fractions. Most estrogenic activity (69%) was found in fractions with retention times (Tret) between 12 and 21 minutes. Due to their relatively polar nature, the natural estrogenic hormones (e.g. β-E2, log Kow 4.01), if present in sediment, would elute in this time window. A second cluster of estrogenic activity was observed in less polar fractions with Tret between 45 and 60 min. Recovery of the fractionation was calculated by dividing the activity summed over all fractions by the activity in the total extracts (Table 1). For location Zierikzee harbor recoveries indicated that no great losses of compounds had occurred during fractionation. However, deviations from 100% recovery might also result from the separation of interfering active compounds during fractionation. The large deviations from 100% recovery observed for Eastern Scheldt are probably due to the low activities present in this sediment, resulting in CALUX® measurements close to the limit of detection.

To enable chemical analysis of the clusters of active fractions, another batch of the same Zierikzee harbor sediment sample was extracted, cleaned and fractionated. This time, collected fractions were based on the relevant activity clusters. Dioxin-like and estrogenic activities in all fractions are shown in Figure 1. The observed activity profile was in good agreement with that of the first fractionation, thereby showing the reproducibility of the fractionation procedure. Although again dioxin-like activity was observed in less polar fractions, only a small portion of dioxin-like activity (6%) and even only 1% of the estrogenic activity were left behind in the vial during the injection and therefore detected in the non-polar residual fraction. This indicates that, notwithstanding the relatively polar starting conditions (MeOH:H_2O; 1:1 v/v), the applied fractionation procedure covered almost all relevant compounds.

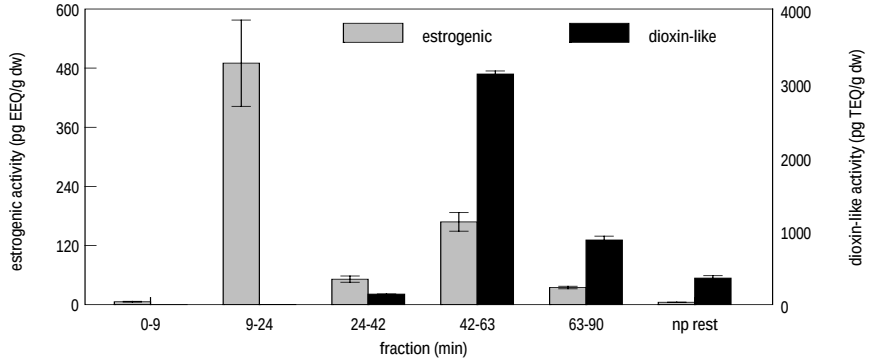

Figure 1: Dioxin-like and estrogenic activities in RP-HPLC-fractions of sediment extract from Zierikzee harbor. Activities (average ± standard deviation) shown are expressed in picograms 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) Equivalents (TEQ) or Estradiol Equivalents (EEQ) per gram dry weighted (dw) sediment. np rest: non-polar residual fraction (see text).

Chemical analysis of estrogenic hormones

Most estrogenic activity was observed in fraction 9-24 min. of location Zierikzee harbor. To investigate the involvement of the hormones β-E2, α-E2, estrone, estriol and the synthetic estrogen EE2 in this activity, dedicated target analyses for these compounds were performed with GC-MS/MS. β-E2, α-E2 and estrone were found to be present in this fraction. Their measured concentrations were multiplied with their relative estrogenic potencies to derive estrogenic activities caused by the presence of each individual compound. Together, they were able to account for about half of the estrogenic activity in fraction 9-24. Estriol and EE2 were not present in detectable concentrations. The presence of estrogenic hormones in sediments has been reported before [10, 11].

Two-dimensional comprehensive GC×GC–ToF MS measurements

Two-dimensional comprehensive GC×GC was used to analyze derivatized extracts of fractions 9-24 min. and 42-63 min. for ER-agonistic compounds (fraction 9-21 min.) and DR- agonistic compounds (fraction 42-63 min.). For fraction 9-24 min. a very complex chromatogram was obtained, indicating the presence of thousands of compounds in this fraction. By selecting characteristic ions for the derivatives of the estrogenic hormones, the presence of α-E2, β-E2 and estrone was confirmed, again indicating their possible (partial) responsibility for the estrogenic activity observed in this fraction. In accordance with the GC-MS/MS analysis, neither EE2 nor estriol were detected. The contour plot of the characteristic ions for the estrogenic hormones in fraction 9-24 min. is shown in Figure 2.

The GCxGC chromatogram of fraction 42-63 min. showed even greater complexity than the one of fraction 9-21 min. Because the identification of active compounds in such a complex chromatogram is very difficult, it was decided to further fractionate the non-derivatized portion C of the same fraction with NP-HPLC. For this fractionation a recovery of 128 ± 6 % of the dioxin-like activity was achieved. Most activity (73%) was found in a fraction in which, if present, polycyclic aromatic hydrocarbons (PAHs) are known to elute[7]. As a lot of these compounds are known to be DR agonistic (e.g. [12]), the GCxGC chromatogram was searched for PAHs. Indeed, a number of PAHs were identified in the extract of fraction 42-63 min. Examples of PAHs detected are shown in the contour plot of Figure 3. PAHs shown are all known to have dioxin-like potencies with TEF (2,3,7,8-tetrachlorodibenzo-*p*-dioxin Equivalence Factor; half maximum effect

concentration of dioxin / half maximum effect concentration of compound) values between 2.3 E-8 for fluoranthene and 1.6 E-3 for benzo(k)fluoranthene[12]. Also considering the relative high peak intensities of these compounds in the chromatogram, this suggests that PAHs may play an important role in the occurrence of dioxin-like activity in sediment from Zierikzee harbor.

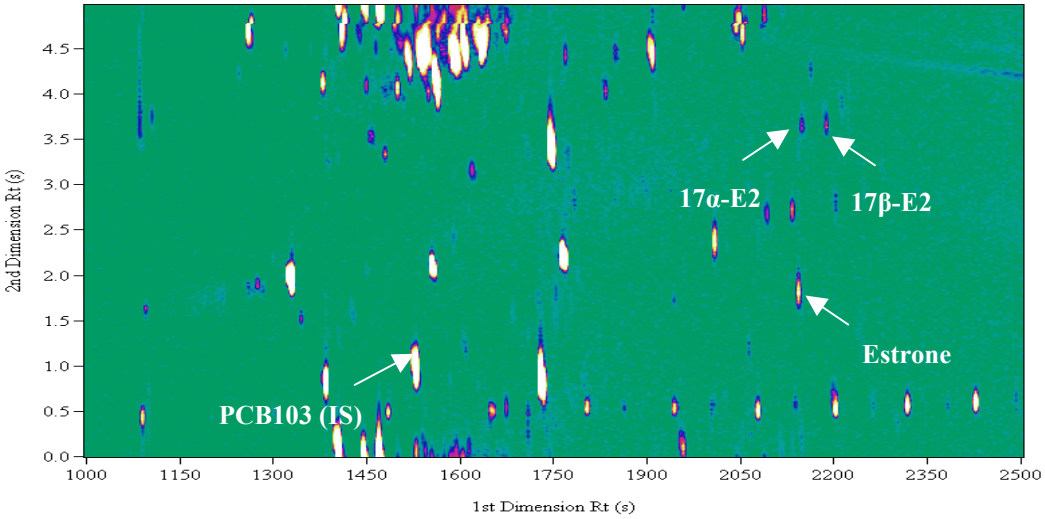

Figure 2: GCxGC-ToF MS separation of fraction 9–24 min., showing the characteristic ions m/z 285 of 17α-estradiol (17α-E2) and 17β-estradiol (17β-E2), m/z 342 of estrone and m/z 254x0.1 of 2,2',4,5',6-pentachlorobiphenyl (PCB 103) that was used as internal standard (IS).

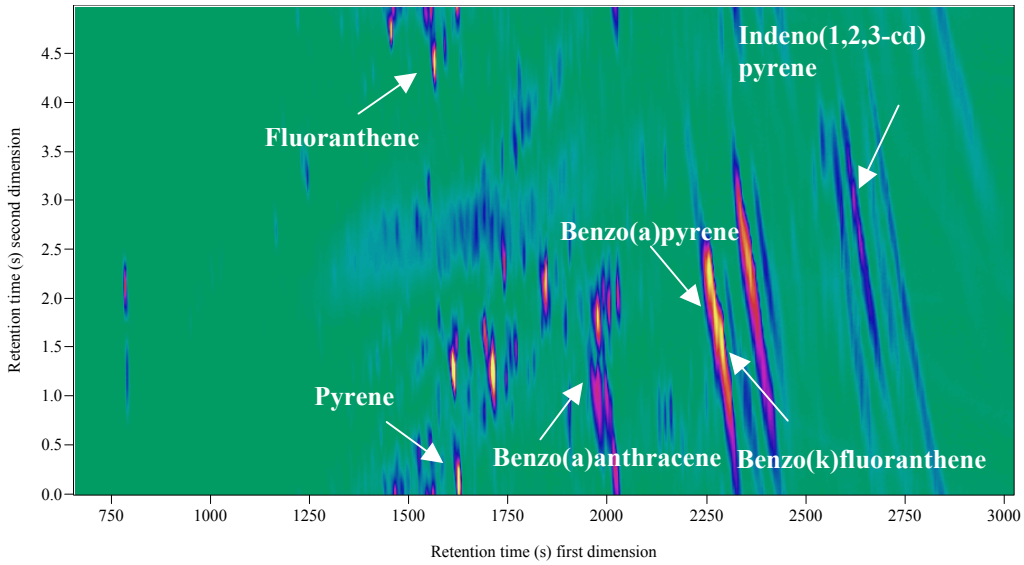

Figure 3: GCxGC-ToF MS separation of fraction 42-63 min., showing characteristic ions for several PAHs (m/z 152+166x0.4+178x0.4+202x0.4+228x0.1+252x0.4+126x0.4+276+278).

Conclusion

DR- and ER-CALUX® assays were successfully applied to direct the identification of dioxin-like and estrogenic compounds in sediment from the harbor of the small town Zierikzee in the Dutch delta. RP- and NP-HPLC fractionations were used to reduce the complexity of the sediment extract and to gain insight in groups of compounds possibly responsible for the observed activities. 17-β-estradiol and its metabolite estrone were the main identified contributors to the estrogenic activity. Using two-dimensional comprehensive GC×GC, polycyclic aromatic hydrocarbons were identified as compounds probably responsible for the majority of the observed dioxin-like activity. Future research will focus on the elucidation of unknown estrogenic compounds in fraction 42-63 min.

Acknowledgements

The authors thank Freek Ariese for the provision of sediment samples, Kees Swart and Petra Booy for their technical assistance and Pim Leonards for his advice on the use of NP-HPLC.

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