Determination of dioxin- and estrogen-like activity in sediment extracts using *in vitro* CALUX assays

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1. Introduction

There has been increasingly more evidence over recent years of endocrine disruption or reproductive changes in humans and organisms in the environment. Many classes of chemicals, including persistent organohalogens, have been indicated as potential endocrine disruptors. In order to identify and quantify the possible presence of endocrine disruptors, we and others have developed chemical activated luciferase gene expression (CALUX) systems. The CALUX system uses *in vitro* recombinant receptor and/or reporter gene assays for the rapid screening of complex mixtures in environmental samples[1,2,3]. In this paper we present some applications of the estrogen receptor- (ER) and aryl hydrocarbon receptor (AhR)-mediated CALUX systems to assess estrogen- and dioxin-like activity in extracts of dredged sediments.

2. Methods

Sediment extracts
Moderately polluted Rotterdam Harbour sediment samples of 400 g were extracted at RWS-RIKZ, Haren, Netherlands with hexane/acetone (3:1 v/v) in a Soxhlet apparatus for 24 hours. The hexane/acetone fraction is evaporated, during which time the extract is filtered three times over a soxhlet extraction thimble. Following evaporation, the remaining hexane extract is subjected to sulphur removal and clean up over a silica column. Precipitates remaining in the Soxhlet thimble are also extracted for three hours with acetone to collect more polar, water-soluble compounds. This acetone fraction is not further cleaned up. Samples in both the hexane and acetone fractions are evaporated and taken up in 500 μl dimethylsulfoxide (DMSO) (hexane fraction) or 500 μl ethanol (acetone fraction) so that 1 μl of solvent represents 40 mg of sediment. Sediment

samples from other freshwater and marine locations within the Netherlands (Table 1) were extracted by ALcontrol B.V., Hoogvliet, Netherlands by extensive shake extraction of 10 g samples with 50 ml acetone followed by an additional 50 ml petroleum ether. The extract is then subjected to sulphur removal and clean up over an aluminum oxide silica column. Extracts are evaporated and taken up in DMSO so that 1 µl of DMSO represents 100 mg of sediment.

Cell culture and transfection

For AhR-mediated CALUX (CALUX) assays, H4IIE rat hepatoma cells stably transfected with pGudluc1.1, a luciferase reporter gene containing upstream dioxin response elements[4], are exposed to tetrachlorodibenzo-p-dioxin (TCDD) and sediment extracts and assayed for luciferase production as described separately in Murk *et al.*[1]. Cells are exposed to maximal 0.4% solvent.

For estrogen receptor-mediated (ER-CALUX) assays, MCF-7 breast adenocarcinoma cells are transiently transfected with the recombinant yeast Gal4-regulated human estrogen receptor (Gal4-HEGO) and luciferase reporter gene (17m5-G-Luc) plasmids (a kind gift from Dr. P. Chambon, INSERM, France). Transient transfection is carried out according to the calcium phosphate precipitation method[5] following methods reported in Zacharewski *et al.*, 1995[6]. Cells are exposed to maximal 0.3% solvent. Luciferase is corrected for transient transfection efficiency by co-transfecting the plasmid pCH110 and assaying ß-galactosidase[5]. For both assays, cells are exposed for 24 hours after which cells are harvested in cell lysis reagent and samples of 20 µl are assayed for luciferase in an automatic 96-wells luminometer.

Determination of toxic equivalents

To determine toxic equivalents in sediment extracts, a standard line of TCDD is used for the CALUX and 17ß-estradiol (E_2) for the ER-CALUX. The light units produced by the undiluted or 5x diluted sediment extract is interpolated within the linear range of the standard line. The corresponding concentration of TCDD or E_2 is then calculated as ng TCDD-equivalents (TEQ) or E_2-equivalents (E_2-EQ) per kg sediment. In order to compare results of the CALUX with the ER-CALUX, values are corrected for equivalent amounts of lysis reagent and µl sediment extract dosed.

3. Results

The TCDD standard line of the CALUX assay in the stable H4IIE.pGudluc 1.1 cell line has a detection limit of less than 1 pM and an EC_{50} value of 10 pM (data not shown)[1]. The E2 standard line in the ER-CALUX assay with MCF-7 cells transiently transfected with Gal4-HEGO and 17m5-G-Luc has a detection limit of 6 pM and an EC_{50} value of approx. 70 pM (Figure 1).

MCF-7 cells exposed in the ER-CALUX assay to the more polar acetone fraction of extracts representing merely 40 mg of Rotterdam Harbour sediment demonstrated significant luciferase induction relative to control ($p<0.05$) (Figure 2a). The calculated E_2-EQ for this sample is 1166 ng/kg, indicating the presence of compounds that can bind to and activate the estrogen receptor. Exposure of MCF-7 cells to the hexane

fraction, however, did not result in significant induction. In the CALUX assay for dioxin-like activity, both the hexane and acetone fractions of Rotterdam Harbour sediment extracts significantly induced luciferase induction relative to control ($p<0.05$) (Figure 2b). Luciferase induction by extracts representing 40 mg sediment corresponded with a TEQ of 1.8 ng/kg for the acetone fraction and 1.2 ng/kg for the hexane fraction.

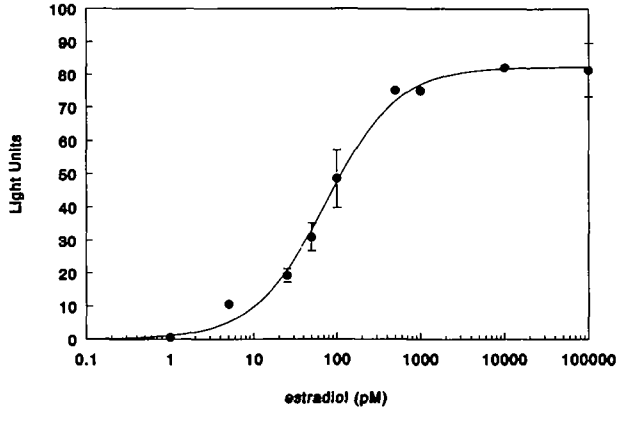

Figure 1: Luciferase induction (light units) in MCF-7 cells transiently transfected with Gal4-HEGO and 17m5-G-Luc following 24 hour exposure to 17ß-estradiol.

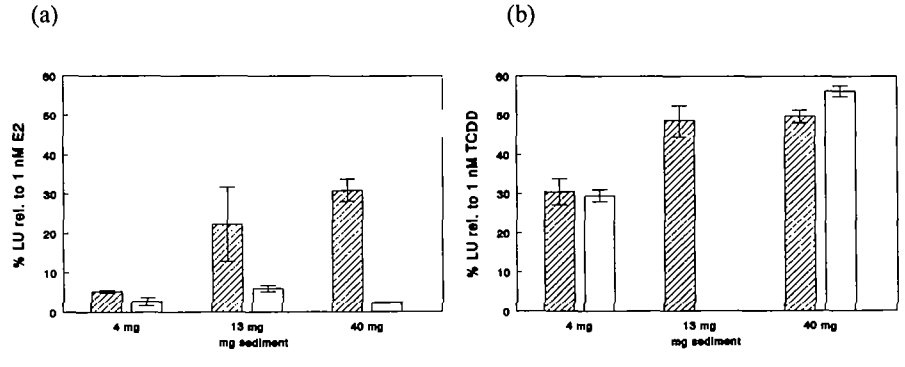

Figure 2: Percent luciferase induction (Light Units) (a) relative to 1 nM estradiol in ER-CALUX assay with MCF-7 cells transiently transfected with Gal4-HEGO and 17m5-G-Luc and (b) relative to 1 nM TCDD in CALUX assay with H4IIE cells stably transfected with pGudluc1.1. Cells were exposed for 24 hours to the acetone (dashed bars) and hexane (open bars) fraction of moderately polluted Rotterdam Harbour sediment extracts.

Luciferase induction by a number of freshwater and marine extracts demonstrates a general trend whereby high TCDD-equivalents (TEQ > 18 ng/kg) as measured in the CALUX assay correspond with high estradiol-equivalents (E_2-EQ > 88 ng/kg) as measured in the ER-CALUX assay (Table 1). In general, sediment locations with low to middle-range TEQs (0.3 to 15 ng/kg) correspond with E_2-EQs below the detection limit, with the exception of the marine Waalsoorden Bay sediment. In this sediment sample, a high E_2-EQ (61 ng/kg) was calculated though the corresponding TEQ was low (0.81 ng/kg).

Table 1: TCDD equivalents (TEQ ng/kg) as determined in the CALUX assay and Estradiol equivalents (E_2-EQ ng/kg) as determined in the ER-CALUX assay for freshwater and marine sediment extracts. (<d.l. = below detection limit)

Freshwater sediments:			Marine sediments:		
Location	TEQ ng/kg	E_2-EQ ng/kg	Location	TEQ ng/kg	E_2-EQ ng/kg
Dronter Lake	2.32	<d.l.	Rhine delta st.72	16.86	53.58
Veluwe Lake	0.30	<d.l.	Rhine delta st.19	21.18	413.92
Marker Lake	3.36	<d.l.	Rhine delta st.126	18.43	88.96
Ijzerdoorn	11.39	<d.l.	Rhine delta st.89	10.23	59.31
Blauwe kamer sand	2.31	<d.l	Rhine delta st.114	15.74	<d.l.
Blauwe Kamer mud	11.49	<d.l.	IJmuiden st. B	39.74	359.94
Amerongen clay	10.07	<d.l.	Harlingen st.17	14.15	48.79
Amerongen sand	6.38	<d.l.	Harlingen st.10	14.29	<d.l.
Ijssel Lake sand	1.09	<d.l.	Den Helder st.5	17.21	<d.l.
America Harbour	25.08	239.59	Waalsoorden Bay	0.81	61.12
North Sea Canal	30.99	437.73	Terneuzen	5.57	<d.l.
			Vlissingen-E.st. 6	20.07	<d.l.
			W. Scheldt st. B.17	1.15	<d.l.

4. Discussion

The presence of endocrine disrupting compounds in all environmental compartments addresses the need for screening and risk evaluation of these types of substances. *In vitro* CALUX recombinant receptor/reporter gene assays for measuring the dioxin- and estrogenic-like activity of complex mixtures can provide a rapid, cost-effective and accurate screening tool. In addition, this type of bioassay can give an indication of the mechanism of action, as well as accounting for synergistic, antagonistic and additive interactions between compounds in complex mixtures.

In this study, the CALUX and ER-CALUX assays were used to test sediment extracts for dioxin- and estrogen-like activity. Chemical (GC-MS) analysis of TCDD-equivalents in these extracts has not yet been carried out, though levels of standard PCBs, PAHs and chlorinated pesticides have been measured. Locations with a high CALUX-TEQ (> 18 ng/kg), such as Amerika Harbour, Rhine delta stations 19 and 126, and IJmuiden have a high PCB 153 concentration (11-17 µg/kg), as well as high total PAH levels (EPA Σ16 PAHs range from 7 to 147 mg/kg). The North Sea Canal sample showed high TEQs (31 ng/kg) though low PCB and PAH concentrations. However, chemical analysis revealed high levels of chlorinated pesticides (total DDD = 20 µg/kg) which may explain both elevated TEQ and E_2-EQ values.

In general, elevated TEQs calculated with the CALUX assay corresponded with high estradiol-equivalents in the ER-CALUX assay. The extraction method must be considered when comparing results, however. In the case of Rotterdam Harbour sediment extractions, estrogenic activity was found only in the more polar (acetone) fraction as compared to the more non-polar (hexane) fraction. In Table 1, freshwater and marine sediments are extracted to collect the organochlorine (non-polar) fraction, which gives rise to the possibility that more polar estrogenic substances may have been omitted. Future plans include testing fractions of varying polarity for estrogenic and dioxin-like activity.

Future plans also include the development of stable estrogen receptor-mediated cell lines. The use of stable transfectants such as the H4IIE.pGudluc1.1 cell line is more efficient, reproducible and has less internal variation. Stable ER-CALUX cell lines form the basis for future research on the potential effects of present levels of (anti-)estrogens on reproductive success in aquatic organisms in the Netherlands. Biological *in vivo* validation of developed *in vitro* reporter gene assays is necessary. Screening of (anti)-estrogenic substances both in *in vivo* bioassays and recombinant reporter gene assays *in vitro*, as well as correlation with population effects observed in the field, can provide information necessary for more realistic risk evaluation of (anti-)estrogenic substances.

5. Acknowledgements

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6. References

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