Monofluorinated analogues of polybrominated diphenyl ethers as analytical standards: Synthesis, NMR, and GC-MS characterization and molecular orbital studies

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Biological Validation of a Sample Preparation Method for ER-CALUX Bioanalysis of Estrogenic Activity in Sediment Using Mixtures of Xeno-Estrogens

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The combined estrogenic effects of mixtures of environmental pollutants in the in vitro ER-CALUX (chemical activated luciferase gene expression) bioassay were examined to biologically validate a sample preparation method for the analysis of estrogenic compounds in sediment. The method used accelerated solvent extraction (ASE) and gel permeation chromatography (GPC) and was validated with respect to recovery of biological response taking mixture effects into account. Four mixtures of three to six xeno-estrogenic compounds (bisphenol A, 4-nonylphenol, (4,4′-dichlorodiphenyl)trichloroethane, (2,4′-dichlorodiphenyl)-trichloroethane, dieldrin, 4-*n*-octylphenol, α-chlordane, dibutylphthalate, (4,4′-dichlorodiphenyl)dichloroethylene, and 2,4,5-trichlorobiphenyl) were prepared. Experimentally determined mixture effects were well described by the concept of concentration addition (CA), as expected for similarly acting compounds. Observed estradiol equivalence factors of the mixtures (on average 1.2 ± 0.3) agreed very well with the value predicted according to CA. The sample preparation method was then applied to pure mixtures of standards and to sediment spiked with one of the mixtures. Recoveries of estrogenic compounds were estimated by determination of their mixture potencies in ER-CALUX and compared to the mixture effects predicted by CA. Recoveries of estrogenic activity were between 80 and 129%, indicating that the additive behavior of mixtures of xeno-estrogens is well conserved during sample preparation. Together with an average repeatability of 18.3%, low average limit of detection (2.6 ± 1.8 pg of EEQ/g), and coefficient of variance (3.5 ± 3.3%), this demonstrated the suitability of the sample preparation method for the analysis of mixtures of (xeno-)estrogenic compounds in sediment with the ER-CALUX assay.

Introduction

Bioassays are valuable supplements to available chemical−analytical techniques for the detection of estrogens, e.g. because of their high sensitivity for estrogenic hormones, their biological relevance by directly measuring activity via (part of) the mechanism of action, and their ability to measure the total estrogenic potency of mixtures present in a sample (*1*). Indeed, in real life, exposure to chemicals seldom is to a single compound but usually to mixtures of different chemicals. Therefore, in vitro bioassays have also often been used to assess combination effects of exposure to mixtures of chemicals (e.g. refs *2* and *3*).

Measurement of estrogenic activity in (extracts of) environmental samples with in vitro bioassays has been performed in different compartments, e.g. water, suspended particulate matter, several animal matrixes, and sediment (*1*, *4*, *5*). Several studies have focused on the validation of bioassays for the detection of (mixture) effects of (xeno-)estrogens and for the application to environmental and human samples (e.g. refs *1* and *6*−*8*)). However, although the need for validated methods is clear, the development and validation of sample preparation methods for bioassay analysis of estrogenic compounds in sediment have received less attention. Methods should recover all possible relevant compounds in a range of chemical and physical properties that is as wide as possible, because of the diversity in chemical and structural properties of compounds by now known to have estrogenic activity, such as steroids, alkylphenols, bisphenols, phthalates, and chlorinated hydrocarbons.

A sample preparation method for the bioassay analysis of estrogenic compounds in sediment was developed and chemically validated at our laboratories (data not shown). The method uses accelerated solvent extraction (ASE) with dichloromethane−acetone (3:1, v/v) in combination with a gel permeation chromatography (GPC) cleanup with dichloromethane as eluens. A total of 24 environmental pollutants were tested for estrogenic activity in the ER-CALUX bioassay (*e*strogen *r*esponsive *c*hemical *a*ctivated *lu*ciferase gene e*x*pression (*9*)), and their recovery from sediment spiked with these compounds after accelerated solvent or Soxhlet extraction in combination with gel permeation chromatography (GPC) cleanup was determined by chemical analysis (on average about 81−85%). However, the behavior of estrogenic compounds as a mixture in the extract during the sample preparation remained unknown.

In this paper, we report the biological validation of this sample preparation method, i.e., the determination of the recovery of the estrogenic activity in the ER-CALUX assay of mixtures of xeno-estrogens.

The first aim of this study was to evaluate whether mixture effects of xeno-estrogens in the ER-CALUX assay could be accurately predicted on the basis of concentrations and concentration−response relationships of the individual components. The concept of concentration addition (CA), introduced by Loewe and Muischneck in 1926 (*10*), assumes that chemicals act according to a similar mechanism and therefore states that equal levels of effects can be achieved by (partial) replacement of a component with other components in a mixture. The contribution of each component to the total effect of the mixture is considered to be proportional to its estrogenic potency and its concentration in the mixture. The CA concept has previously been shown to be valid for the prediction of mixture effects of estrogenic compounds in the yeast estrogen screen (YES assay) (*3*, *11*,

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TABLE 1. Composition of Four Mixtures of Xeno-Estrogens and of Spiking Solutions Used to Test Estrogenic Mixture Effects in ER-CALUX and Recovery of Estrogenic Activity after Sediment Extraction and Cleanup[a]

compd	concn in mixture stock solution (mM)	EC50 (mM of compd)	EEF[b]	concn[c] (nM of EEQ)	contribn to estrogenic potency[d] (%)	MIL[e] (%)
			Mixture 1			
BPA	5.5	0.25	1.6×10^{-5}	88.4	28.8	109.6
NP	5.7	0.14	2.4×10^{-5}	134.0	43.5	93.5
p,p′-DDT	16.9	0.82	3.5×10^{-6}	59.4	19.4	95.5
o,p′-DDT	3.1	0.39	8.2×10^{-6}	25.4	8.3	99.3
sum				307.2		
			Mixture 2			
dieldrin	21.6	4.13	8.3×10^{-7}	17.9	43.7	66.8
OP	7.0	4.42	1.0×10^{-6}	7.3	17.8	161.2
α-chlordane	11.7	2.79	1.4×10^{-6}	15.8	38.5	52.1
sum				41.0		
			Mixture 3			
DBP	45.8	6.34	6.8×10^{-7}	31.3	58.3	34.6
p,p′-DDE	36.4	7.20	4.2×10^{-7}	15.3	28.5	68.6
PCB29	13.4	8.32	5.3×10^{-7}	7.1	13.2	78.4
sum				53.7		
			Mixture 4			
dieldrin	10.8	4.13	8.3×10^{-7}	9.0	18.5	66.8
OP	3.5	4.42	1.0×10^{-6}	3.7	7.5	161.2
α-chlordane	5.8	2.79	1.4×10^{-6}	7.9	16.3	52.1
DBP	22.9	6.34	6.8×10^{-7}	15.7	32.3	34.6
p,p′-DDE	18.2	7.20	4.2×10^{-7}	7.7	15.8	68.6
PCB29	8.8	8.32	5.3×10^{-7}	4.7	9.6	78.4
sum				48.7		
			positive control			
E2	3.7×10^{-4}		1.0	367	100	100
			blank			
acetone	0		0	0	0	0

[a] Estrogenic equivalence factor (EEF) and maximum induction level (MIL) are given as parameters of estrogenicity. [b] EEF: estrogenic equivalence factor. EEF of compound x = $EC50_{E2}/EC50_X$. Value determined in same ER-CALUX experiment as in which mixture activity was assessed. [c] Calculated by multiplying the concentration in the mixture stock solution and EEF according to eq 9 (Materials and Methods). [d] Calculated as, with BPA as example, 88.4/307.2 × 100%. [e] MIL: maximum induction level relative to E2, defined in eq 5 (Materials and Methods).

12). Like the YES assay, the ER-CALUX assay is based on a receptor gene construct, with activation of the endogenous estrogen receptors as the sole molecular mechanism leading to response. We examined the appropriateness of the CA concept to predict combination effects of mixtures of estrogens in ER-CALUX by testing concentration−response curves of dilution series of four different fixed-ratio mixtures of xeno-estrogens in ER-CALUX that were compared to β-E2 as a reference compound.

The second aim of this study was to test the recovery of estrogenic activity during sample preparation. A mixture of four xeno-estrogenic compounds was tested in ER-CALUX assay to assess its combined effect. Then, the mixture was treated with part of or the complete sample preparation method before being tested in ER-CALUX assay to assess the recovery of the estrogenic activity during the treatment. In this way, it was examined if the combination effect of a mixture of estrogens in an environmental sample remains unaffected and representative for the sample during its extraction and cleanup.

Materials and Methods

Standards. 17-β-Estradiol (E2, >98% pure) and bisphenol A (BPA, >97% pure) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands), (2,4′-dichlorodiphenyl)-trichloroethane (*o,p′*-DDT, 97%), (4,4′-dichlorodiphenyl)-trichloroethane (*p,p′*-DDT, 98.7%), and (4,4′-dichlorodiphenyl)dichloroethylene (*p,p′*-DDE, 97%) from Dr. Ehrenstorfer (Augsburg, Germany), 4-nonylphenol (NP, 99.6%) and 4-octylphenol (OP) from Acros (Geel, Belgium), and α-chlordane (99.6%) and 2,4,5-trichlorobiphenyl (PCB 29, 99.99%) from Ultra Scientific (Wesel, Germany). Di-*n*-butylphthalate (DBP, 99%) was from from Sigma-Aldrich (Zwijndrecht, The Netherlands), and dieldrin, from LGC (Teddington, U.K.). Highly concentrated stock solutions (millimolar range) were prepared in acetone (ultra-resi analyzed, Mallinckrodt-Baker, Deventer, The Netherlands), out of which dilution series were prepared in dimethyl sulfoxide (DMSO, spectrophotometric grade 99.9%, Acros, Geel, Belgium).

Mixtures and Spiking Solutions. A total of 10 environmentally polluting compounds with estrogenic activity also used for the chemical validation were chosen for mixture experiments. Four different mixture stock solutions were prepared from solutions of the individual compounds. Each mixture stock solution contained compounds in millimolar concentrations and was composed in such way that, according to its individual estrogenic potency, each component would contribute equally to the overall estrogenic potency of the mixture (Table 1). Mixture 1 (BPA, NP, *p,p′*-DDT, and *o,p′*-DDT) consisted of four relatively potent xeno-estrogens with maximum induction levels equal to that of E2. Mixture 2 (dieldrin, OP, and α-chlordane) and mixture 3 (DBP, *p,p′*-DDE, and PCB29) consisted each of three slightly less potent xeno-estrogens with different maximum induction levels as compared to E2. To test the total estrogenic activity of a mixture of more components, mixtures 2 and 3 were combined to obtain a mixture of 6 xeno-estrogens (mixture 4). Dilution series of each mixture stock solution were prepared in DMSO for ER-CALUX measurements. In this way, the ratio of concentrations of individual compounds in the mixtures was equal for each dilution (so-called fixed-ratio mixtures). Highest concentrations tested in the ER-CALUX assay were a 1000-fold dilution of the stock solution

of mixture 1 and 500-fold dilutions of the stock solutions of mixtures 2−4.

Three spiking solutions were prepared for the experiments to assess the recovery of estrogenic activity during sediment extraction and cleanup. Mixture 1 was used as spiking solution containing a mixture of four potent xeno-estrogens. A spiking solution of E2 in acetone of approximately equal potency as mixture 1 (367 nM E2) was used as positive control, and the solvent acetone served as blank (Table 1).

ER-CALUX. ER-CALUX assay was performed with stably transfected T47D human breast cancer cells (T47D.Luc-cells) according to Legler et al. (*9*) with adaptations as described in ref *13*. T47D.Luc-cells were obtained from BioDetection Systems BV (Amsterdam, The Netherlands). A concentration series of E2 (10 concentrations between 0 and 100 pM) was included on each plate. To assess the estrogenic potency of mixtures, dilution series were tested in triplicate in at least two independent experiments. Estrogenic potencies of individual compounds were tested once in the same experiment.

Data Analysis. A 4-parametric sigmoidal model provided the best fit to the experimental data using a generalized least-squares approach. A sigmoidal standard curve with y representing luciferase activity in relative light units and x representing the concentration of compound was fitted for E2, each individual test compound and each mixture using the software program Slidewrite4.1 (Advanced Graphics Software, Carlsbad, CA):

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

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

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

$$y(\infty) = a_0 + a_1 \tag{3}$$

Median effective concentrations (EC50 values) were derived from the curves according to

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

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

$$\mathrm{MIL_X} = \frac{(y_X(\infty) - y_X(0))}{(y_{E2}(\infty) - y_{E2}(0))} \times 100\% \tag{5}$$

Quantification of estrogenic activity for the recovery experiments was done by interpolating luciferase activities caused by most highly diluted standards or extracts causing response between LOQ and EC50 in the estradiol standard curve and expressing them as pg of EEQ/20 μL of spike mixture (the amount used to spike 1 g of sediment) or pg of EEQ/g of sediment dry weight (dw).

Calculation of Expected Estrogenic Mixture Effects. For the mathematical representation of CA, toxic units (TU) can be used. The TU_i of compound (or mixture) i is the ratio of the actual concentration c of i and the concentration needed to cause a certain effect x (ECx_i):

$$\mathrm{TU}_i = c_i/\mathrm{EC}x_i \tag{6}$$

According to CA the overall TU of a mixture TU_{mix} is equal to the sum of all n TU_is in the mixture:

$$\sum_{i=1}^{n} \mathrm{TU}_i = \mathrm{TU}_{\mathrm{mix}} \tag{7}$$

In the case of estrogenic compounds, individual potencies of compounds are expressed relative to that of E2 by calculation of the estradiol equivalence factor (EEF value) for i at the median effective concentration (EC50) with the formula

$$\mathrm{EEF}_i = \mathrm{EC50}_{\beta-\mathrm{E2}}/\mathrm{EC50}_i \tag{8}$$

The concentration of a compound expressed in estradiol equivalents (EEQ) is then given by

$$\mathrm{EEQ}_i = c_i{}^* \mathrm{EEF_i} = \mathrm{TU}_i{}^* \mathrm{EC50}_{\mathrm{E2}} \tag{9, 10}$$

and, according to CA (eq 2),

$$\sum_{i=1}^{n} \mathrm{EEQ}_i = \mathrm{EEQ}_{\mathrm{mix}} \tag{11}$$

In this way, the effective concentration in EEQ of a mixture can be calculated solely on the basis of the individual concentrations and potencies of the mixture components. Concentration−response curves of individual compounds and mixtures in ER-CALUX and their 95% confidence intervals were calculated with the software program SlideWrite4.1. The 95% confidence intervals were used to visually assess the similarity between predicted and observed mixture concentration−response curves.

Recovery of Estrogenic Activity from Spiked Sediment. To assess recovery of estrogenic potency during sediment preparation, estrogenic activity of spiking solutions in ER-CALUX was tested in three stages of the procedure: (1) direct measurement of pure spiking solutions; (2) measurement after GPC cleanup of the solution; (3) after spiking of sediment with the solution, followed by ASE extraction and GPC cleanup. All recovery experiments were performed in triplicate/spiking solution.

Direct Measurement of Spiking Solution. Dilution series were prepared of mixture 1, positive control, and blank and tested in ER-CALUX.

GPC Cleanup of Spiking Solution. An 800 μL volume of dichloromethane (DCM, Suprasolv GC quality, Merck, Darmstadt, Germany) was added to 200 μL of spiking solution to a total volume of 1 mL and quantitatively injected on the GPC system (PL-gel, 10 μm, 300 × 25 mm, Polymer laboratories, 2 columns in serial connection) with 10 mL/min dichloromethane as eluens. Former research had shown 96% of the estrogenic activity in sediment extract to elute in the collected fraction (*14*). The eluate was split in two portions, one for ER-CALUX and one for chemical analysis. Totals of 50% of the extract of mixture 1 and of the blank were destined for each type of analysis, 20% of the positive control was destined for ER-CALUX, and 80% was for chemical analysis. Portions destined for CALUX analysis were evaporated until approximately 1 μL remained, taken up in DMSO, and tested.

Extraction and Cleanup of Sediment Spiked with Spiking Solution. Surface sediment was collected from a reference location (Oysterpit, Kamperland, The Netherlands) shown previously to have neglible estrogenic activity. Sediment was sieved (mesh size 63 μm), freeze-dried, and homogenized. Portions of 10 g were spiked with 200 μL of spiking solution and extracted with DCM−acetone (3:1, v/v)) with accelerated

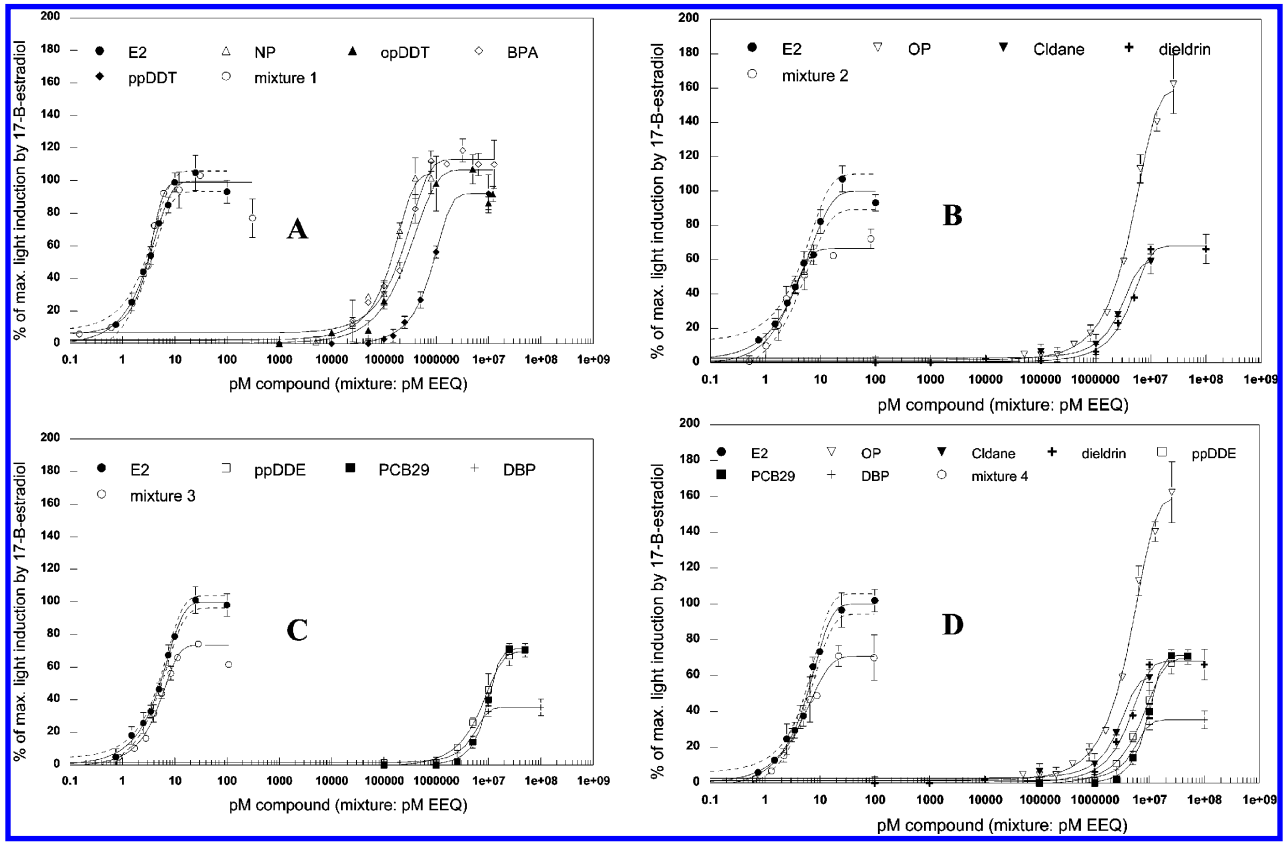

FIGURE 1. Concentration−response curves for 17-β-estradiol (E2) for four different mixtures of xeno-estrogens and their individual components in the ER-CALUX assay. Mixture components are 4-nonylphenol (NP), (2,4′-dichlorodiphenyl)trichloroethane (*o,p*′-DDT), bisphenol A (BPA), and (4,4′-dichlorodiphenyl)trichloroethane (*p,p*′-DDT) (mixture 1, A), 4-*n*-octylphenol (OP), α-chlordane (Clordane), and dieldrin (mixture 2, B), (4,4′-dichlorodiphenyl)dichloroethylene (*p,p*′-DDE), 2,4,5-trichlorobiphenyl (PCB29), and dibutylphthalate (DBP) (mixture 3, C), and a combination of mixtures 2 and 3 (mixture 4, D). Error bars represent standard deviations of triplicates of ER-CALUX measurements. Dashed lines represent 95% confidence intervals for the E2 curves. N.B.: Concentrations of individual components are expressed in pM of compound, mixture concentrations are expressed in pM of estradiol equivalents (EEQ).

solvent extraction (ASE, 3 extraction cycles, 50 °C, system pressure 2000 psi, ASE200, Dionex, Sunnyvale, CA). Extracts were evaporated until about 1 mL was left, quantitatively injected on GPC, and further treated as described above for GPC cleanup.

Confirmation of Recovery of Estrogenic Activity from Spiked Sediment by Chemical Analysis. To compare biologically and chemically determined recoveries in the same extract, recovery of E2 from positive controls was determined by chemical analysis. E2-d_4 (internal standard) was added, extracts were cleaned on HPLC (ODS2, Waters Spherisorb, 5 μm, 4.6 × 150 mm at 22 °C with methanol−water (65:35, v/v) as mobile phase), silylated, and analyzed on a GC with ion trap detector as described in ref *5*.

Of the set of xeno-estrogens in the mixture, *o,p*′-DDT and *p,p*′-DDT were chosen to be analyzed as indicators of the chemical recovery in the same extract. PCB103 was added (internal standard), and extracts were cleaned with Al_2O_3 deactivated with 15% water, eluted with petroleum ether−diethyl ether (95:5 v/v), and analyzed by gas chromatography combined with electron capture detection (GC-ECD).

Repeatability of the Method. Surface sediments from the reference location Oysterpit and from the harbor of the small town Zierikzee, The Netherlands, were freeze-dried, sieved, and homogenized and then extracted with ASE, cleaned with GPC, taken up in DMSO and tested in ER-CALUX for estrogenic activity in four independent experiments.

Results and Discussion

Estrogenic Activity of Single Compounds. All 10 compounds induced luciferase activity in the ER-CALUX assay in a concentration-dependent manner, indicating activation of endogenous estrogen receptors (Figure 1). Although all compounds have S-shaped curves, not all compounds reach the same MIL as E2. For OP and BPA, superagonistic behavior ($MIL_X > MIL_{E2}$) is observed. For dieldrin, α-chlordane, DBP, *p,p*′-DDE, and PCB29, partially agonistic behavior ($MIL_X < MIL_{E2}$) is found.

MILs and estrogenic potencies expressed as EEF values are given in Table 1. Potencies range between 2.4×10^{-5} for NP and 4.2×10^{-7} for *p,p*′-DDE, indicating that compounds are about 10^6 fold less potent than E2, the endogenous ligand of the estrogen receptor. Deviations in EEF values, due to interexperimental fluctuations, may influence the predicted mixture effects. To avoid this type of fluctuations, EEF values shown here are the result of single measurements of full concentration response series performed in the same experiments as in which the mixtures were tested. These EEF values were used to predict the estrogenic activity of the mixtures (Table 1). Mixtures were composed in such a way that each component would contribute equally to the overall effect. The calculated contribution and the total mixture potency are given in Table 1.

Estrogenic Activity of Fixed-Ratio Mixtures. According to the sigmoidal fit used, curve shapes are determined by four parameters: slope at EC50 concentration; EC50; minimum response level and maximum response level (these two taken together in MIL). Mixture behavior according to the CA concept should lead to concentration−response curves of the mixtures directly comparable with the corresponding E2 curves, with similar slopes, MIL values, and EC50 values, provided the mixture concentrations are

TABLE 2. Median Effective Concentrations (EC50), Estrogenic Equivalence Factors (EEF), and Maximum Induction Levels (MIL) of Four Mixtures of Xeno-Estrogens in ER-CALUX[a]

mixture	EC50 mix (pM of EEQ)	EC50 E2 (pM)	EEF[b]	MIL obsd	MIL[c] (% of calcd)
		mixture 1			
	3.1	3.5	1.1	95.6	96.6
	3.3	3.0	0.9	94.2	94.3
av			1.0	94.9	95.4
		mixture 2			
	3.2	4.4	1.4	68.6	87.9
	2.8	5.0	1.8	62.9	80.7
av			1.6	65.7	84.3
		mixture 3			
	4.9	5.3	1.1	61.6	123
	5.9	5.3	0.9	74.4	149
av			1.0	68.1	136
		mixture 4			
	4.5	6.0	1.3	73.4	117
	3.9	4.4	1.1	69.1	110
av			1.2	71.2	114
overall av			1.2 ± 0.3		107 ± 23

[a] Mixtures were tested in two independent experiments. The first row of results of each mixture corresponds with the concentration−response curves in Figure 1. [b] Calculated according to eq 8 (Materials and Methods), with mixture EC50 values expressed in pM of EEQ. [c] Observed MIL (maximum induction level) as percentage of MILs of mixture components weighted by their relative contribution to the estrogenic potency of the mixture.

expressed in pM EEQ (eqs 9 and 11). Thus, CA should give rise to visual overlap between the mixture curves and the E2 curves. Figure 1 shows the mixture curves (expressed in pM of EEQ), their corresponding E2 curves, and the concentration−response curves of the individual mixture components (expressed in pM of compound). As can be seen from the figure, all mixture curves are indeed (partly) overlapping with the corresponding E2 curves, with very similar slopes at steep parts of all curves.

EC50 values of mixtures and of E2 curves on the same plates and the EEF values derived from these values are provided in Table 2. Indeed, mixtures are, expressed in EEQs, equipotent to E2 and thus have EEF values very close to 1. Nonadditive (synergistic or antagonistic) mixture behavior would have led to a shift of the curves (respectively to the left or to the right), resulting in mixture EEF values deviating from 1 (respectively larger or smaller).

The MIL of mixture 1 is very similar to that of the E2 curve. However, mixtures 2−4 have lower MIL values (Table 2). These might be due to the presence of partial agonists with lower individual maximum responses in these mixtures. After all, one of the principles behind the CA concept is the requirement that components can replace each other to generate the same level of effect. Partial agonists only partially fulfill this requirement and thus cause the mixture curves to deviate from that of E2 at higher concentrations. The difficulty of predicting maximum effect levels of mixtures containing partial agonists with the CA model is also discussed by others (*3*, *11*). In our experiments, MILs of mixtures containing partial agonists could, however, roughly be estimated by calculation of the average MIL of the mixture components, weighted by their relative contributions to the total estrogenic activity of the mixture (last column in Table 2). In the bioassay analysis of estrogenic activity of environmental mixtures, quantification of the estrogenic response should be performed at an effect level lower than that of the component with the lowest maximum effect level to avoid disturbance of the quantification due to the presence of partial agonists. However, in environmental mixtures, the components contributing to the estrogenic activity, and therefore their maximum induction levels, are often unknown (*13*, *15*). Response quantification of ER-CALUX assay measurements

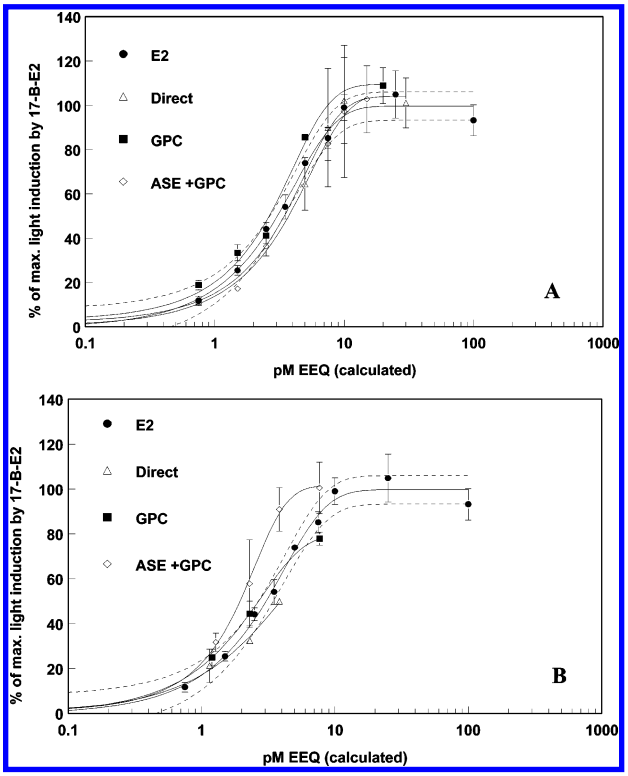

FIGURE 2. Dilution−response curves of estradiol (A) and a mixture of 4 xeno-estrogens (mixture 1; B) in ER-CALUX after direct analysis, analysis after GPC treatment, and analysis after spiking and extraction of sediment and cleanup with GPC (ASE + GPC). As concentrations are expressed in concentrations estradiol equivalents (EEQ), overlap with E2 calibration curves in quantifiable ranges implies that recovery of estrogenic activity is close to 100%. Error bars represent standard deviations of triplicates of ER-CALUX measurements. Dashed lines represent 95% confidence intervals for the E2 calibration curves.

should therefore always be performed in the lower half of the concentration−response curve (below EC50), in a response range in which a linear decrease of response is

TABLE 3. Recovery of Estrogenic Activity in ER-CALUX and Chemical Recovery after Direct Measurement of Solution (Direct), after GPC Cleanup (GPC), or after ASE extraction and GPC Cleanup of Spiked Sediment (ASE + GPC) before Measurement

		recovery: estrogenic activity in ER-CALUX assay		recovery: chem analysis	
name	compds	ng of EEQ/20 μL of spike[a]	%	ng/20 μL of spike	%
		Direct			
blank	acetone	$<0.9 \times 10^{-3}$			
positive control	E2	1.9 ± 0.2	94.7 ± 9.4	2.1 ± 0.2	107 ± 6
mixture 1	BPA, NP, *p,p′*-DDT, *o,p′*-DDT	1.6 ± 0.3	99.0 ± 18.5		
	p,p′-DDT			$9.9(\pm 0.8) \times 10^4$	82.7 ± 6.4
	o,p′-DDT			$2.2(\pm 0.2) \times 10^4$	102 ± 10
		GPC			
blank	acetone	$<0.4 \times 10^{-3}$			
positive control	E2	2.3 ± 0.8	117 ± 39	1.3 ± 0.1	63.0 ± 4.3
mixture 1	BPA, NP, *p,p′*-DDT, *o,p′*-DDT	1.7 ± 0.1	101 ± 8		
	p,p′-DDT			$10.0(\pm 0.0) \times 10^4$	83.2 ± 0.2
	o,p′-DDT			$2.1(\pm 0.0) \times 10^4$	95.7 ± 0.7
		ASE + GPC			
blank	acetone	$19.0(\pm 3.1) \times 10^{-3}$		$19.0(\pm 3.1) \times 10^{-3}$	
positive control	E2	1.6 ± 0.1	80.1 ± 5.3	1.6 ± 0.1	64.2 ± 8.8
mixture 1	BPA, NP, *p,p′*-DDT, *o,p′*-DDT	2.1 ± 0.4	129 ± 27		
	p,p′-DDT			$13.3(\pm 0.4) \times 10^4$	110 ± 4
	o,p′-DDT			$2.9(\pm 0.1) \times 10^4$	129 ± 7

[a] 20 μL of spike solution is the equal amount of solution as used for the spiking of 1 g of sediment. A.

obtained upon dilution of the extract. This has been demonstrated for sediment in ref *13*.

The 95% confidence interval of the E2 curve can be used as a criterion to assess the suitability of the CA concept statistically (*16*). Mixture 1, containing only full agonists, covers the entire range of effects within the 95% confidence interval. Mixtures 2 and 4 have their lower and steep curve parts in the interval as well. Mixture 3 slightly deviates from the interval, possibly due to the influence of the partial agonist DBP. Our results indicate that the combined effect of mixtures of xeno-estrogens in the ER-CALUX assay can be predicted and described appropriately by the CA concept. For the quantification of responses, however, one should be extra cautious for the presence of partial and/or superagonistic estrogens. This study confirms the suitability of CA to describe the effects of mixtures of similar acting xeno-estrogens in reporter gene assays as reported earlier (*3*, *12*, *17*, *18*).

Recovery of Estrogenic Activity during Extraction and Cleanup of Sediment. The behavior of mixtures in sediment extracts, an issue of even more relevance than that of pure mixtures, was assessed by biological validation of the sample preparation method. Mixture 1 was chosen for spiking the sediment, as this mixture consisted of four relatively potent xeno-estrogens that were full agonists and that were also used in the chemical validation of the same method (*15*). In addition to mixture 1, the sample preparation method was also validated for use in the ER-CALUX assay with E2 alone (positive control) and acetone (blank) (Table 1). First, each solution was tested directly; i.e, cells were exposed to solutions that had not gone through any extraction or cleanup ("direct", in Figure 2). Second, solutions were treated with GPC and tested in ER-CALUX ("GPC" in Figure 2), and third, sediment was spiked with the three solutions, extracted, cleaned with GPC, and tested in ER-CALUX ("ASE + GPC" in Figure 2). Recoveries of estrogenic compounds were estimated by determination of potencies in ER-CALUX and compared to the effects predicted by CA at 100% recovery. Due to the high spiking concentrations (E2, 2.0 ng of E2/g of dw; mixture 1, 1.7 ng of EEQ/g of dw), extracts could be diluted to generate a dilution−response curve. A 100% recovery, assuming additive mixture behavior, would in both cases lead to overlap of the curves with the E2 calibration curve in the same graph. Indeed, the curves of direct, GPC-treated, and ASE + GPC-treated E2 spikes are almost completely within the 95% confidence interval of the E2 calibration curve (Figure 2A). Comparably good results are found for the curves of direct and GPC-treated mixture 1 (Figure 2B), indicating high recoveries. However, the curve of ASE + GPC-treated mixture 1 is somewhat shifted to the left, indicating that during the whole procedure the mixture has gained some activity. This could possibly be due to e.g. the introduction of a slight contamination during the procedure, although this was not found in the nonspiked sediment (blanks) or in the E2-spiked sediment.

Average recoveries of estrogenic activity were calculated by interpolation of responses in the quantifiable range of the E2 calibration curve (Table 3). No activity was detected in the blanks, after direct and GPC measurements. The activity detected in ASE + GPC measurements is caused by estrogenic compounds in the sediment itself. However, this activity is negligible compared with activities in spiked sediment (about 1%). Recoveries of estrogenic activity of E2 and mixture 1 were all between 80 and 129%. There was no clear difference in recoveries between solutions that were tested directly, after GPC treatment, or after treatment with the complete sample preparation procedure. This indicates that differences in recoveries are more likely to be due to experimental fluctuations than to structural losses or intro-

duction of compounds during the sample preparation procedure.

The high recoveries of estrogenic activity are generally confirmed by chemically determined recoveries of E2, *o*,*p*′-DDT, and *p*,*p*′-DDT in the same extracts that were all between 63 and 129% (Table 3). The recovery of E2 during GPC and ASE + GPC is however lower compared to the recovery of estrogenic activities in the same extracts. Apart from normal fluctuations between the two measurements, this may be due a loss of E2 after splitting of the extracts that is not reflected by the recovery of the deuterated internal standard.

Repeatability of the Method. Two sediment samples differing in level of estrogenic activity, namely from reference location Oysterpit Kamperland (sediment from this location was also used for the spiking experiments) and from Zierikzee harbor (high activity), were extracted, cleaned, and analyzed in ER-CALUX in four independent experiments. Average estrogenic activity was 19.2 ± 2.6 pg of EEQ/g of dw (repeatability 13.7%) for Kamperland and 463 ± 106 pg of EEQ/g of dw (repeatability 23.0%) for Zierikzee harbor, with an average repeatability of 18.3%. The average limit of detection was 2.6 ± 1.8 pg of EEQ/g of dw. The average coefficient of variation was 3.5 ± 3.3%.

In conclusion, this study showed the additive behavior of mixtures of xeno-estrogenic compounds in the ER-CALUX bioassay. Additive mixture effects were well-conserved during the extraction and cleanup of sediment and could be described with the CA concept. The investigated method for the ASE extraction and GPC cleanup of estrogenic compounds in sediment for ER-CALUX bioassay analysis showed good recoveries, a low limit of detection, and good repeatability. The method, validated both chemically (data not shown) and biologically at our laboratories, can be applied to field samples. The availability of such validated methods for biomonitoring is becoming more and more important through the increase in the application of bioassays in (inter)-national surveys and monitoring programs for (xeno-)-estrogenic compounds in the aquatic environment.

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Supporting Information Available

Fit parameters for a sigmoidal fit of mixtures and individual compounds in Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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