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Evaluation of an extraction method for a mixture of endocrine disrupters in sediment using chemical and in vitro biological analyses

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Abstract Aquatic sediments are contaminated by a wide diversity of organic pollutants such as endocrine-disrupting chemicals (EDCs) which encompass a broad range of chemical classes having natural and anthropogenic origins. The use of in vitro bioassays is now widely accepted as an alternative method for their detection in complex samples. However, based on the diversity of EDC chemical properties, their common extraction is difficult and comprehensive validation of extraction methods for a bioanalysis purpose is still weakly documented. In this study, we compared the performance of several organic solvents, i.e., acetone, methanol, dichloromethane, heptane, dichloromethane/acetone (50:50, *v*/*v*), dichloromethane/methanol (50:50, *v*/*v*), heptane/acetone (50:50, *v*/*v*), and heptane/methanol (50:50, *v*/*v*), to extract a diversity of active chemicals from a spiked sediment matrix using pressurized liquid extraction. For this purpose, we defined a mixture of 12 EDCs with a wide range of polarity ($2 < \log Kow < 8$) (i.e., estrone, 17β-estradiol, bisphenol A, *o*,*p*′DDT, 4-*tert*-octylphenol, fenofibrate, triphenyl phosphate, clotrimazole, PCB-126, 2,3,7,8 TCDD, benzo[k]fluoranthene, and dibenzo[a,h]anthracene). Working concentrations of each individual compound in the mixture were determined as equipotent concentrations on the basis of the concentration-addition (CA) model applied to in vitro estrogenic, dioxin-like, and pregnane X receptor (PXR)-like activities. Extraction efficiencies based on both chemical and biological analyses were assessed in triplicate in artificial blank sediment spiked with this mixture and in natural sediment contaminated by native EDCs. In both spiked and natural sediment, MeOH/DCM yields the best recovery while heptane was the least efficient solvent. Our study provided the validation of a sediment extraction methodology for EDC bioanalysis purposes, which can be used for comprehensive environmental contamination characterization.

Keywords Pressurized liquid extraction · EDC · Mixture effect · In vitro bioanalytical tools

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Introduction

Sediment is the sink for thousands of chemical pollutants. Among them, the so-called endocrine-disrupting chemicals (EDCs) have received increasing attention due to their capacity to interfere with the hormonal system. EDCs which may possibly cause adverse effects (i.e., reproduction and/or development) on exposed organisms (Desbrow et al. 1998; Jobling and Tyler 2003) encompass a broad range of chemical classes (steroid, alkylphenols, pesticides, pharmaceuticals, surfactants, plasticizers, etc.) and have natural and/or anthropogenic origins (Hotchkiss et al. 2008). However, regarding the wide chemical diversity of EDCs, the comprehensive assessment of

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environmental contamination by these compounds is still challenging.(Dévier et al. 2011).

Targeted chemical analysis is a sensitive and selective tool to assess environmental concentrations of such compounds in order to evaluate potential risk of exposed organisms. However, such risk assessment is mainly based on individual priority pollutants through comparison between the measured environmental concentration and the predicted non-effect concentration (i.e., hazard assessment), hence providing only a partial scope of environmental contamination complexity. Bioanalytical strategies based on effect-based tools (EBTs) are expected to address such a challenge (Altenburger et al. 2015). Among EBTs, bioassays, such as nuclear receptor-based in vitro reporter gene assays, provide sensitive, specific, and integrative tools to assess contamination by EDCs. They allow quantifying biological toxic-equivalent (bio-TEQ) loads in environmental extracts that integrate the contribution of all available bioactive compounds present in examined samples. A broad range of such bioanalytical tools have been developed in the last decade and applied to analysis of environmental samples. While most of them target estrogenic and dioxin-like activities, other signaling pathways have also been recently considered in the bioanalysis of environmental samples such as glucocorticoid, progesterone, androgenic, or pregnane X receptor (PXR) pathways (Van der Linden et al. 2008; Hill et al. 2010; Creusot et al. 2010, 2014). The occurrence of such activities in environmental samples underlines the need to consider other pathways than those mediated by estrogen (ER) and aryl hydrocarbon (AhR) receptors.

The recovery of the broadest range of active compounds in organic extraction is a critical step in bioanalysis as they represent a diversity of chemical classes and physico-chemical properties (e.g., hydrophobicity, polarity, sorption coefficients, etc.) which makes their common extraction difficult (Seiler et al. 2008).

In most validation studies, extraction methods have focused on specific chemical classes of known individual active compounds, such as estrogenic (Jeannot et al. 2002; Kinani et al. 2008; Arditsoglou and Voutsa 2008) or dioxin-like compounds (Helaleh et al. 2005; Kishida et al. 2010; Wang et al. 2010a, b). However, the development of optimized extraction methods that have been validated for both chemical and bioassay analyses of a larger range of EDCs in sediment has not been frequently reported. So far, such an approach has been assessed only for estrogenic (Houtman et al. 2007) and dioxin-like activities (McCant et al. 1999).

Several procedures have been described for the extraction of EDCs in solid matrices (sediment, sludge, organisms). They include Soxhlet extraction (Khim et al. 1999), microwave-assisted extraction (Labadie and Hill 2007), ultrasonication-assisted extraction (Kinani et al. 2008), supercritical fluid extraction (SFE) (Lye et al. 1999), steam distillation, and pressurized liquid extraction (PLE) (Andreu et al. 2007). PLE has recently been developed and has been successfully used for the rapid and automated extraction of a broad range of chemical classes including steroids (Nieto et al. 2008), pharmaceuticals (Jelic et al. 2009; Radjenovic et al. 2009), pesticides (Schafer et al. 2008; Wang et al. 2010a, b), PAHs (Hollert et al. 2002; Burkhardt et al. 2005), PCDD/Fs (Kiguchi et al. 2006), PCBs (Focant et al. 2001), and alkylphenols (Petrovic et al. 2002). In its principle, working at high temperatures and pressures facilitates desorption and diffusion of organic compounds from the solid matrix. Hence, PLE is often more efficient than Soxhlet or other extraction methods. In addition, it reduces solvent consumption in comparison to other extraction tools.

The aim of this study was to evaluate the PLE method for the extraction of a broad range of chemicals having various physico-chemical properties and having several biological activities. Thus, we designed a mixture of 12 EDCs belonging to different chemical classes and selected as having agonist effect in in vitro estrogenic, dioxin-like, and PXR bioassays (Table 1). The additive effect was assessed in each bioassay by using the concentration-addition (CA) model (Supporting Information), allowing to determine the "working" concentrations of each component of the mixture. This mixture was then used to spike artificial blank sediment, and extraction recoveries by using different extraction solvents were determined on the basis of both chemical (individual concentrations) and in vitro biological (toxic equivalents) analyses. Finally, extraction performances were compared to those obtained using natural sediment sampled at a contaminated river site.

Materials and methods

Chemical and reagents

All the chemicals of the mixture (Table 1), dimethyl-sulfonide (DMSO), and luciferin were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Analytical standards (Table 4) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), LGC-standard (Molsheim, France), and Cluzeau (Sainte-Foy-La-Grande, France). Dichloromethane (DCM), methanol (MeOH), heptane, and acetone (HPLC reagent grade, Merck) used for PLE extraction were purchased from VWR. Acetonitrile, dichloromethane, isooctane, and methanol (HPLC reagent grade, Scharlau) used for purification before chemical analysis were from ICS (Belin-Béliet, France).

Mixture design

In order to establish recoveries based on biological analysis, we prepared a mixture of 12 EDCs including ligands for estrogen receptor (ER), aryl hydrocarbon receptor (AhR), and

Table 1 Identity, provider, purity, log Kow, and biological target of individual chemicals used for the spiking mixture

Compound	CAS	Provider	Purity (%)	Log Kow[a]	Major biological target
Estrone (E1)	53-16-7	Sigma-Aldrich	≥99 %	3.43	ER
17β-Estradiol (E2)	50-28-2	Sigma-Aldrich	≥98 %	3.94	ER
Bisphenol A (BPA)	80-05-7	Sigma-Aldrich	97 %	3.64	ER
4-*tert*-Octylphenol (4tOP)	140-66-9	Sigma-Aldrich	97 %	5.5	ER
*o,p'*DDT	789-02-6	Sigma-Aldrich (Fluka)	–	6.79	ER
Fenofibrate	49562-28-9	Sigma-Aldrich	≥99 %	3.26	PXR
Triphenyl phosphate (TPP)	115-86-6	Sigma-Aldrich	≥99 %	4.7	PXR
Clotrimazole	23593-75-1	Sigma-Aldrich		6.26	PXR
Benzo[k]fluoranthen (BkF)	207-08-9	Sigma-Aldrich	≥98 %	6.11	AhR
Dibenzo[a,h]anthracen (DaA)	53-70-3	Sigma-Aldrich		6.11	AhR
PCB 126	40186-72-9	LGC-Standard	≥99 %	6.98	AhR
2,3,7,8 TCDD	1746-01-6	LGC-Standard	≥99 %	6.92	AhR

[a] Taken from LogKow win Syracuse Research Corporation (Syrres), http://www.syrres.com/esc/physprop.htm

PXR, with log Kow ranging between 2 and 8 (Table 1). The concentration of each chemical used in the mixture was determined by using the concept of concentration addition (CA) assuming a similar mode of action of each mixture component. The methodology used for mixture effect assessment, in vitro biological activity of individual chemicals, and definition of the "working" concentrations in the mixture is provided in Supporting Information.

Spiking procedure of blank sediment

Because the calculation of mixture recovery based on biological activity requires a non-active matrix, we prepared an artificial blank sediment as previously described (Kinani et al. 2008) according to OECD recommendations (OECD-218 2004). Briefly, brown peat (5 %), $CaCO_3$ (0.1 %), kaolin (20.0 %), and quartzic sand (75 %) (HN38, 50–200 μm) were mixed. Culture medium (294.0 mg of $CaCl_2$, $2H_2O$, 123.2 mg of $MgSO_4$, $7H_2O$, 64.7 mg of $NaHCO_3$, and 5.7 mg of KCl in 1 L of deionized water) was then added to this composition in a medium/sediment ratio of 4:1, *v*/*v*. This mixture was incubated for 10 days at 23.0 °C with a continuous ventilation of the water column. The volume of medium was supplemented three times a week, in order to compensate for evaporation. The obtained blank sediment (COT, 10 %) was then sieved at 1 mm, freeze-dried, homogenized, and stored at −20 °C in an amber glass bottle before analysis.

The mixture of 12 EDCs was prepared in acetone. The quantity of this mixture to be used for the spiking process was determined in order to obtain maximal biological response for each bioassay at the highest tested concentration of the organic extract. The spiking process (Fig. 1) consisted of drowning the freeze-dried sediment with the spiking mixture in an amber glass bottle for one night at room temperature. This enabled the simulation of the partition process between the dissolved and particulate phases. Then, further rolling and evaporation under a fume hood were conducted for 6 h. Finally, the bottle was left under the fume hood overnight for total evaporation of acetone. Then, the bottle was rolled for 1 h to complete homogenization. The freeze-dried and spiked sediment was then stored at −20 °C before extraction. In order to evaluate the potential loss or contamination during this procedure, an experimental control was also performed consisting of handling the mixture without sediment (Fig. 1).

Natural sediment

The performance of the method to extract environmental EDCs was also tested using river sediment. For this purpose, sediment from the Reveillon River (France) where estrogenic, anti-androgenic, PXR-like, and dioxin-like activities have been previously characterized was selected (Kinani et al. 2010). The sediment was collected with a grab, sieved at 1 mm, freeze-dried, homogenized, and stored at −20 °C in an amber glass bottle before analysis (Creusot et al. 2013).

Extraction procedure

To optimize extraction conditions of a broad range of active chemicals, we evaluated the extraction performance of non-polar (heptane, DCM) and polar solvents (acetone, MeOH) and their mixture (50:50, *v*/*v*) (heptane/acetone, DCM/acetone, MeOH/DCM, and MeOH/heptane). Extraction was carried out using an ASE 350® apparatus (Dionex, France) with 10-mL extraction cells filled with a mixture of sediment/sand (50:50, *w*/*w*) (approximately 5 g of freeze-dried sediment in each cell). Extraction conditions were as follows: extraction

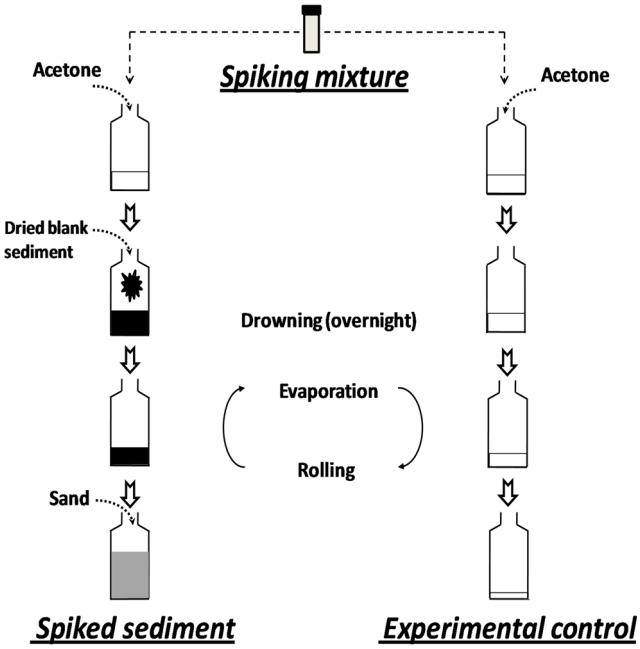

Fig. 1 Procedure used for spiking the blank sediment

temperature, 75 °C; extraction pressure, 1500 PSI; preheating period, 5 min; static extraction, 5 min; number of extraction cycles, 3; final extraction volume, 25 mL; flush volume, 60 % of the cell volume; and nitrogen purge, 60 s. Procedural blanks were obtained using sand alone.

Chemical analyses

Quantitative analysis of compounds was performed using GC-MS (PAHs), GC-ECD (PCB, BDE, and OCPs), UPLC-MS/MS (steroids, alkylphenols, and bisphenol A), and RRLC-MS/MS (fenofibrate, clotrimazole, and pharmaceuticals) methods, as previously described by Creusot et al. (2013).

Biological analysis

Estrogenic, PXR-like, and dioxin-like activities were assessed by using three in vitro bioassays based on cultured permanent cell lines, namely MELN (luciferase reporter gene assay coupled to ER, Balaguer et al. 1999), HG5LN-hPXR (luciferase reporter gene assay coupled to human PXR, Lemaire et al. 2006), and PLHC-1 (EROD assay, Louiz et al. 2008) cell lines, respectively. Details on routine cell culture and testing conditions were previously described (Kinani et al. 2010; Creusot et al. 2010). Cells were exposed in microtiter plates to dilution ranges of standard chemicals or sediment extracts for 16 h for MELN and HG5LN-hPXR assays or 4 and 24 h for PLHC-1 assay. Luciferase (MELN, HG5LN-hPXR) and EROD (PLHC-1) assays were then performed exactly as previously described (Creusot et al. 2013; Louiz et al. 2008).

Data analysis

Bioassay data modeling

All dose-response curves were modeled with the Hill equation model by using RegTox 7.5 Microsoft Excel Macro, freely available at http://www.normalesup.org/~vindimian/fr_index.html. This allowed calculating effective concentrations for each compound alone (i.e., ECx, the concentration of compound or extract leading to x % effect). When a chemical or environmental extract yielded an incomplete dose-response curve, the Hill parameter for maximal response was fixed to 100 %. The relative potencies (REP) of individual compounds were calculated as the ratio of the EC20 of the reference compound to that of the reference compound in a given bioassay. Bioassay-derived toxic equivalents (bio-TEQ) were calculated as the ratio of EC20 of the reference compounds on the EC20 of the environmental extracts.

Recoveries calculation

Extraction recoveries of the spiked sediment were established from both chemical and biological analyses. For each extraction condition, the whole process (i.e., from extraction to analyses) was carried out in triplicate (three independent experiments). To determine extraction recovery based on chemical analysis, concentrations of the mixture components in the spiked sediment were compared to measured concentrations in the spiking solution. For biological analysis, bio-TEQs of the spiked-sediment extract were compared to those measured in the spiking mixture.

Statistical analyses

Statistical analyses were assessed using the freely available software R (http://www.r-project.org/). In order to compare the CA-prediction effect to the measured effect (see Supporting Information), we first applied the non-parametric Shapiro-Wilk test on residues in order to address the normality of the distribution of the residues. We then processed to a Student test on the mean of the residues in order to assess if it differed significantly from 0 ($n = 15$–18; degree of freedom $= n - 1$; $\alpha = 0.05$). For a p value <0.05, we can conclude that the CA-prediction effect differed significantly from the experimental effect.

Quality assurance

All glassware was cleaned with detergent and rinsed with ultrapure water, followed by heating at 450 °C overnight. Procedural blanks (glass material and solvents) were performed for both chemical and biological analyses. Limits of detection (LODs) in chemical analysis were evaluated with

signal/noise ratios equal to or greater than 3. LODs of biological analyses were based on the EC5 of the extract. Finally, no biological activities were detected in non-spiked artificial sediment (blank matrix).

Results and discussion

Establishment of the spiking mixture

The experimentally established dose-response curves of the mixture of the 12 EDCs showed that the measured effect slightly and significantly differed from the prediction of the effect by the CA model. The difference was slight for estrogenic and AhR-24 h ($p < 0.05$) while it was more significant for PXR-like and AhR-4 h activities ($p < 0.01$) (Fig. 2, see also Supporting Information for statistical analyses). These results suggest a more than additive mixture effect on PXR and AhR-4 h activities, and to a lesser extent on estrogenic activity, at low effective concentrations. Moreover, experimental and predicted response curves fitted better at higher mixture concentrations. Interestingly, a very recent study demonstrated that, due to a large ligand-binding pocket, PXR can stably bind binary mixtures of weakly active chemicals, such as E2 and transnonachlor, which leads to synergistic activation of target genes mediated by this receptor (Delfosse et al. 2015). Such synergistic effect was demonstrated in different cell models, including the HG5LN-hPXR cells that were used in the present study; it is thus likely that such a phenomenon may have contributed to the observed divergence between observed and CA-predicted curves in our study.

Extraction recoveries of the mixture in the spiked blank sediment

Several studies based on PLE methods have reported minor influence of pressure, static time, and flush volume and major effect of temperature and solvent on the extraction performance of organic contaminants (Schafer et al. 2008; Nieto et al. 2008; Olivella 2005). Here, we evaluated the effect of the solvent composition on the extraction performance while the other ASE parameters were fixed. The temperature of 75 °C was chosen as this temperature is commonly used by other authors and in the literature with the aim to limit the loss of thermo-labile chemicals. The recoveries derived from chemical and biological analyses after complete extraction of the mixture components from the spiked sediment are shown in Table 2; dose-response curves allowing to calculate the bio-TEQ of spiked sediments are presented in Fig. 3. In the experimental control (Fig. 1), the recovery rates based on

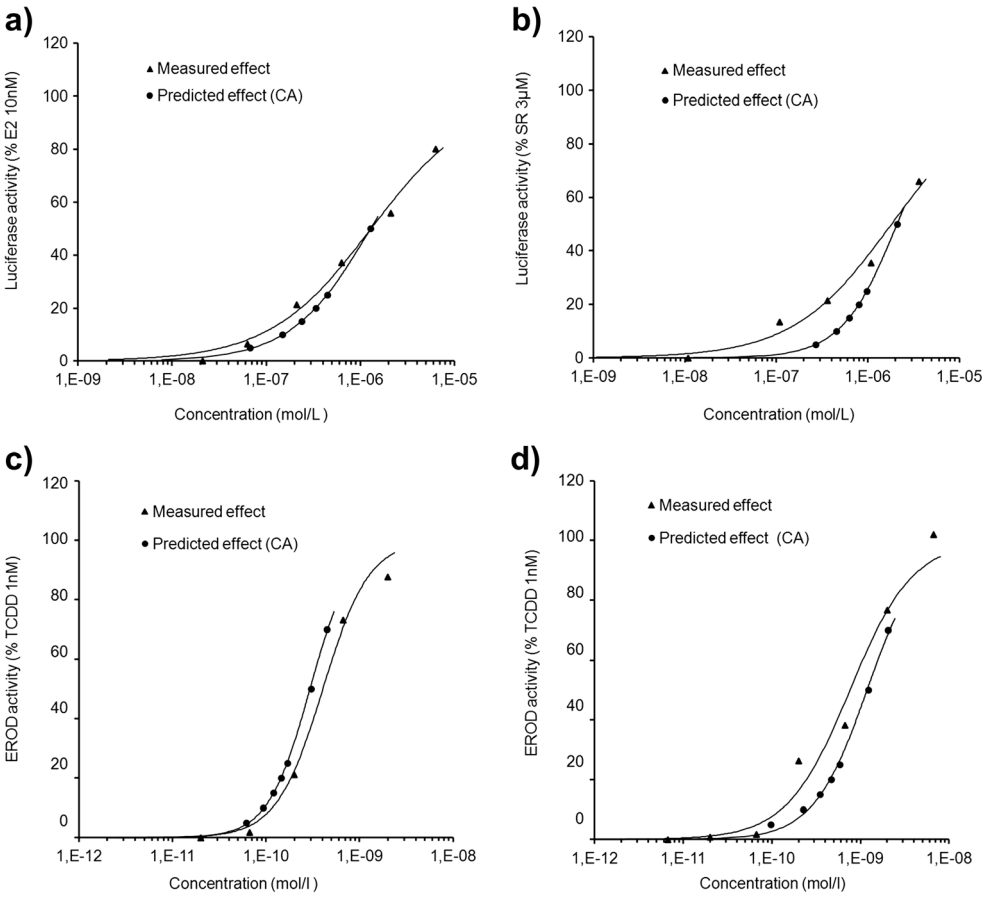

Fig. 2 Dose-response curves of measured and predicted in vitro activities of the spiking mixture: **a** estrogenic activity, **b** PXR-like activity, **c** AhR activity after a 24-h exposure (dioxin-like activity), **d** AhR activity after a 4-h exposure (PAH-like activity)

biological activities were always higher than 80 %, suggesting that the pre-extraction conditions did not lead to a considerable loss of the mixture components.

Estrogenic chemicals

For estrogenic compounds, biological and chemical analyses showed overall good recovery rates. Based on in vitro bio-TEQs, all the tested solvents yielded a recovery higher than 80 % except for acetone (64 %) and heptane (56 %). Mixtures of MeOH/DCM and heptane/MeOH provided the best recoveries (Table 2). Based on individual concentrations, recoveries were always higher than 75 % except for E1 (50 %) and E2 (62 %) with heptane/acetone and for E1 (26 %), E2 (22 %), and BPA (17 %) with heptane. Heptane is a non-polar solvent whereas E1, E2, and BPA are mid-polar compounds (log Kow 3–4). This could explain the weak extraction recoveries for biological and chemical analyses using this solvent. Up to now, assessment of the extraction performance of estrogenic chemicals in solid matrices has often been restricted to steroids or alkylphenols (Arditsoglou and Voutsa 2008; Ahn et al. 2007). Hence, these studies reported the use of polar solvents (i.e., MeOH, acetone, or MeOH/acetone) (Nieto et al. 2008; Petrovic et al. 2002; Jeannot et al. 2002) for the extraction of estrogenic compounds although less polar estrogenic compounds were often not considered. Nevertheless, our results were in accordance with the study by Houtman et al. (2007) reporting the efficient use of a mixture of polar and non-polar solvents for the extraction of estrogenic chemicals with a wide range of polarity.

PXR activators

For PXR-like active chemicals, biological and chemical analyses showed similar and acceptable extraction recoveries, except for clotrimazole with DCM/acetone (46 %), heptane/acetone (22 %), DCM (10 %), and heptane (7 %) as assessed by chemical analysis (Table 2). Clotrimazole is a potent PXR ligand, and its low extractability could explain the weak extraction recovery of PXR-like activity observed for heptane and DCM. Adsorption of clotrimazole to plastic or glassware has been previously reported (Peschka et al. 2007). However, Huang et al. (2010) obtained good recovery of this compound (70 %) in spiked sludge using ultrasonication-assisted extraction with acidified methanol while this recovery decreased with less polar solvent. Thus, both absorption and solvent polarity may have contributed to a weaker recovery of clotrimazole in our study.

Nevertheless, PXR-like activity presented better extraction recovery rates as compared to that yielded by chemical analyses of clotrimazole and fenofibrate. This could suggest that some of the mixture components other than the typical PXR activators (e.g., E2, BPA, 4tOP) may also have contributed to the observed PXR-like activity in the spiked sediment, although they are predicted as being non-active on PXR at these

Table 2 Extraction recoveries (%) in spiked blank sediment based on biological (BIO) and chemical analyses (CHEM). In vitro bioassay-based recoveries were calculated as the ratio of the Bio-TEQ in the spiked-sediment extracts to that of the spiking mixture (means ± SD, $n = 3$)

Activity	Analysis	Compounds	Extraction solvent								
			MeOH	Acetone	MeOH/DCM	Hept/MeOH	DCM/Ac	Hept/Ac	DCM	Heptane	Exp. control
Estrogenic	BIO	Mixture	80 ± 3.7	64 ± 1.2	93 ± 15	110 ± 14.4	86 ± 4	77 ± 19	80 ± 3.1	56 ± 6	85
	CHEM	E1	77 ± 38	78 ± 5	68 ± 5	74 ± 16	94 ± 31	50 ± 19	82 ± 10	26 ± 2	106
		E2	89 ± 16	89 ± 23	103 ± 4	91 ± 19	104 ± 6	62 ± 32	90 ± 1	22 ± 8	66
		4tOP	78 ± 1	99 ± 17	76 ± 2	88 ± 27	96 ± 4	98 ± 12	83 ± 19	84 ± 19	102
		BPA	75 ± 11	105 ± 10	86 ± 4	97 ± 14	112 ± 36	105 ± 19	85 ± 16	17 ± 15	86
		*o,p'*DDT	74 ± 11	83 ± 2	144 ± 0	85 ± 12	99 ± 5	73 ± 17	122 ± 4	73 ± 6	102
PXR-like	BIO	Mixture	74 ± 2	79 ± 32	97 ± 15	117 ± 24	76 ± 16	75 ± 7	61 ± 5	62 ± 3	88
	CHEM	Fenofibrate	66 ± 26	64 ± 16	98 ± 5	91 ± 16	59 ± 29	82 ± 26	92 ± 21	66 ± 19	56
		Clotrimazole	53 ± 15	81 ± 3	70 ± 14	64 ± 0	46 ± 1	22 ± 9	10 ± 2	7 ± 1	57
		TPP	72 ± 1	108 ± 7	88 ± 0	102 ± 18	94 ± 17	79 ± 1	68 ± 3	73 ± 4	92
PAH-like	BIO	Mixture	86 ± 4	73 ± 1	76 ± 9	88 ± 7	84 ± 1	72 ± 6	76 ± 4	71 ± 7	82
	CHEM	DaA	92 ± 11	124 ± 9	85 ± 5	77 ± 25	95 ± 19	85 ± 16	96 ± 13	34 ± 4	86
		BkF	85 ± 1	107 ± 8	87 ± 1	78 ± 23	94 ± 22	88 ± 13	92 ± 11	36 ± 4	82
TCDD-like	BIO	Mixture	100 ± 2	90 ± 11	66 ± 1	95 ± 4	80 ± 16	76 ± 25	86 ± 2	110 ± 17	80
	CHEM	PCB 126	99 ± 3	117 ± 19	87 ± 2	99 ± 3	103 ± 21	115 ± 9	107 ± 7	87 ± 10	80
		TCDD	72 ± 30	73 ± 28	79 ± 3	61 ± 13	76 ± 9	92 ± 16	66 ± 16	45 ± 14	68

concentrations as defined by the addition model. This hypothesis is, however, supported by the fact that the CA-predicted effect based on all the PXR ligands occurring in the mixture underestimated the measured effect (Fig. 2). Moreover, a synergistic action on this receptor may have occurred (Delfosse et al. 2015). Altogether, the best recoveries for both chemical and biological analyses were obtained with MeOH/DCM and Hept/MeOH, as observed for the estrogenic activity.

Dioxin-like chemicals

For dioxin-like compounds, extraction recoveries based on biological analysis were always higher than 70 % for both persistent (AhR-24 h) and non-persistent (AhR-4 h) dioxin-like activities (Table 2). However, chemical analyses showed slightly different trends. Overall recoveries were also higher than 70 % except for DaA (24 %), BkF (36 %), and TCDD (45 %) using heptane while good recoveries were still observed for PAHs using acetone. Several studies have previously reported a greater performance of acetone or MeOH than non-polar solvents for the extraction of PAHs (Oluseyi et al. 2011), PCBs, PCDD/DFs (Kishida et al. 2010), or other persistent organic pollutants (organochlorinated pesticides, PBDEs) (Camino-Sánchez et al. 2011). However, the weaker extraction performance of PAH and TCDD using heptane remains unclear.

Extraction performance of environmentally occurring EDCs: application to contaminated sediment

It is obvious that using spiked blank sediment insufficiently reflects the binding and complex interaction of compounds within natural sediment. For instance, spiking compounds to sediment samples may result in a weaker binding to organic or particulate matter than it is the case in natural samples leading to higher recoveries (de Boer et al. 2001). Therefore, it was necessary to test our extraction method using natural sediment. For this purpose, we selected sediment sampled at a river site that has been previously shown to be contaminated by multiple EDCs (Kinani et al. 2010; Creusot et al. 2013). This sample was subjected to the different extraction conditions, as described above using a spiked matrix.

Significant activities were detected in all bioassays, and dose-response curves were established (Fig. 4) and allowed calculating bio-TEQs (Table 3). Overall, there

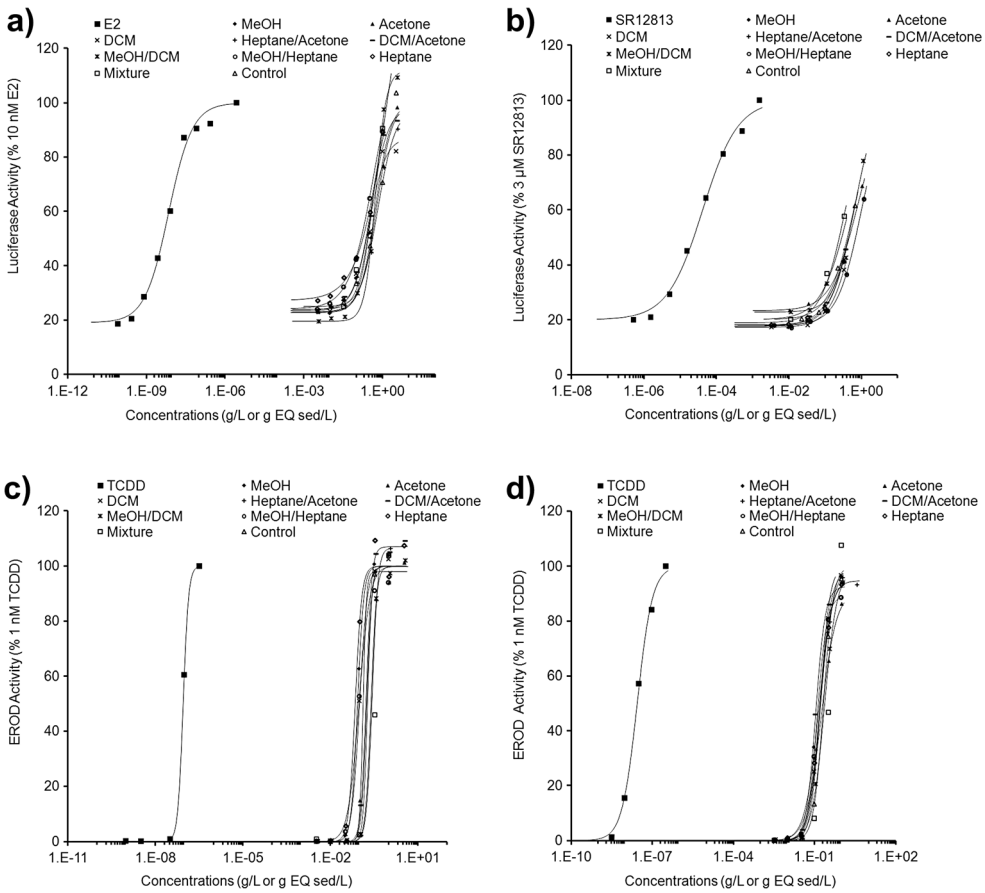

Fig. 3 Dose-response curves of spiked blank sediment extracted with different solvents: **a** estrogenic activity, **b** PXR-like activity, **c** AhR activity after a 24-h exposure (dioxin-like activity), **d** AhR activity after a 4-h exposure (PAH-like activity). No activity could be detected in the non-spiked blank sediment extract

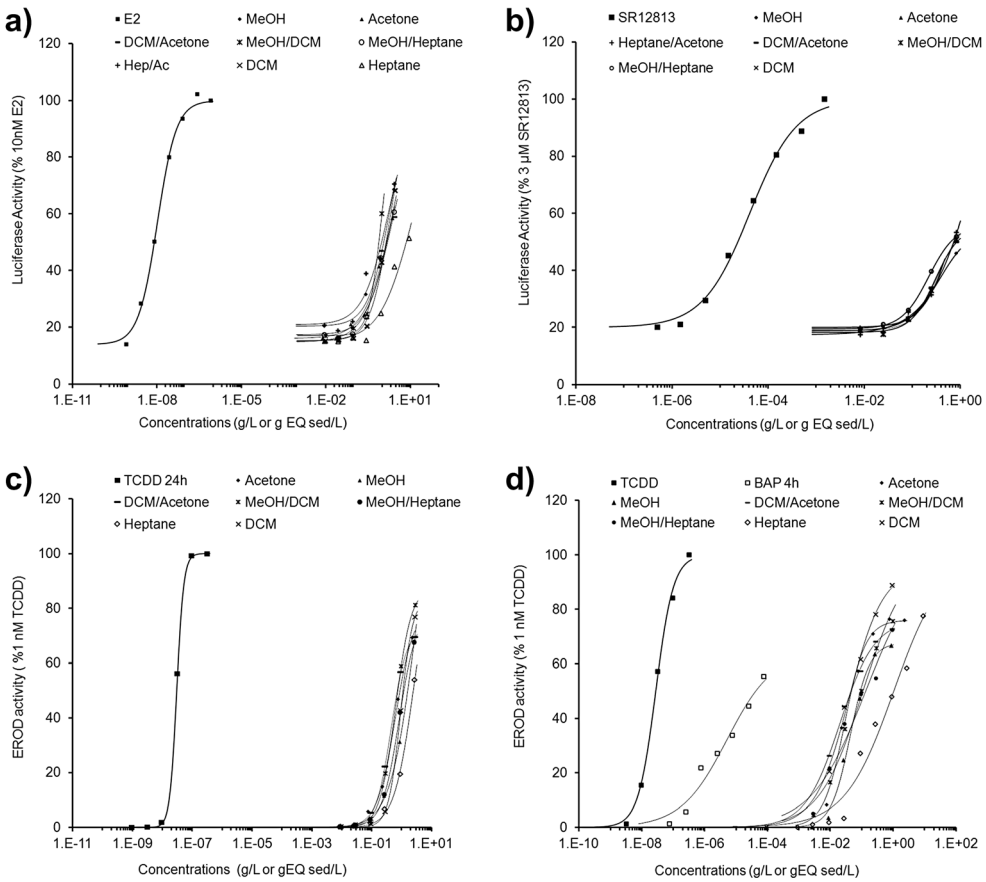

Fig. 4 Dose-response curves of natural sediment extracted with different solvents: **a** estrogenic activity, **b** PXR-like activity, **c** AhR activity after a 24-h exposure (dioxin-like activity), **d** AhR activity after a 4-h exposure (PAH-like activity)

were no differences between the different solvents used for the extraction. Nevertheless, in accordance with the results obtained for the spiked sediment, heptane was the less efficient solvent while MeOH/DCM offered the most efficient extraction for the three biological activities, especially for the PXR-like activity (Table 3).

Results of the chemical analyses (Table 4) showed high levels of PAHs, alkylphenols, and BPA (µg/g d.w. range); moderate levels of OCPs, PCBs, and PBDEs (ng/g d.w. range); and weak levels of steroids (<ng/g d.w. range) while no pharmaceuticals (e.g., antibiotics, anticancer drugs) could be detected (data not shown). The diversity and quantity of pollutants were similar to those previously reported (Kinani et al. 2010; Creusot et al. 2013), highlighting a continuous contamination of this river over time. Overall, as previously noted by the biological analysis, the extraction performance of the tested solvents was almost equivalent between solvents. Nevertheless, some differences were observed. The polar solvent MeOH allowed the best extraction of OCPs, PCBs, and PBDEs, as previously reported in other studies (Camino-Sánchez et al. 2011), while polar/non-polar solvent mixtures allowed the best efficient extraction for PAHs (Kiguchi et al. 2006), as did MeOH/DCM in our study.

It is noticed that the mixture of Hept/MeOH also leads to a good extraction performance for both biological and chemical analyses (Tables 3 and 4). Since dichloromethane is known to

Table 3 Bio-TEQ values in a natural sediment extracted by different solvent conditions (means ± SD, $n = 3$)

	LOD	MeOH	Acetone	MeOH/DCM	Hept/MeOH	DCM/Ac	Hept/Ac	DCM	Heptane
Estrogenic (ng E2-EQ/g d.w.)	0.017	8.8 ± 1.0	8.0 ± 1.8	9.7 ± 1.8	8.0 ± 1.8	8.6 ± 0.8	7.8 ± 0.8	6.2 ± 1.7	1.4 ± 0.6
PXR-like (µg SR-EQ/g d.w.)	0.185	31.0 ± 5.4	28.8 ± 2.4	50.6 ± 11.3	46.4 ± 3.4	34.9 ± 4.8	28.5 ± 5.5	35.6 ± 5.4	n.d.
PAH-like (µg BaP-EQ/g d.w.)	1.5	25.4 ± 6.5	36.6 ± 4.8	46.2 ± 2.5	46.4 ± 2.9	31.1 ± 4.7	54.5 ± 2.3	32.6 ± 4.4	11.0 ± 2.4
TCDD-like (ng TCDD-EQ/g d.w.)	8.4	37.3 ± 7.9	37.2 ± 1.1	34.9 ± 8.9	32.3 ± 7.3	26.8 ± 4.7	31.3 ± 4.5	33.5 ± 5.1	14.1 ± 3.9

LOD limit of detection in nanograms of standard/gram d.w., *n.d.* not detected

Table 4 Concentrations of native EDCs in the natural sediment (means ± sd, $n = 3$)

		LOD	MeOH	Acetone	MeOH/DCM	Hept/MeOH	DCM/Ac	Hept/Ac	DCM	Heptane
PAHs	Naphthalene	0.4	10 ± 10	9 ± 7	2 ± 3	20 ± 9	21 ± 9	5 ± 0.3	12 ± 4	31 ± 7
	Acenaphthylene	0.9	17 ± 0.8	15 ± 5	17 ± 3	10 ± 3	24 ± 7	20 ± 5	15 ± 3	11 ± 3
	Acenaphtene	n.a.	7 ± 5	12 ± 10	0.1 ± 0.4	9 ± 7	11 ± 3.4	10 ± 3	6 ± 3	10 ± 1
	Fluorene	0.4	56 ± 32	34 ± 8	49 ± 15	35 ± 13	42 ± 9	42 ± 9	32 ± 9	40 ± 14
	Phenanthren	4.3	445 ± 29	352 ± 36	479 ± 44	339 ± 80	37 ± 1	441 ± 104	378 ± 64	435 ± 13
	Anthracen	3.4	56 ± 24	74 ± 30	60 ± 22	43 ± 11	37 ± 32	266 ± 345	57 ± 14	36 ± 47
	Fluoranthen	6.7	1020 ± 19	1004 ± 153	1264 ± 606	870 ± 99	109 ± 54	1265 ± 476	898 ± 45	1059 ± 252
	Pyrene	8.1	886 ± 26	667 ± 249	903 ± 171	733 ± 93	852 ± 58	1083 ± 316	701 ± 93	889 ± 207
	Benzo[a]anthracen	6.6	950 ± 53	792 ± 157	904 ± 98	684 ± 178	858 ± 76	884 ± 39	738 ± 158	643 ± 191
	Triphenlylen + chrys	4.9	880 ± 39	751 ± 167	817 ± 88	660 ± 146	757 ± 44	1081 ± 518	673 ± 77	514 ± 166
	BbF + BjF + BkF	2.2	1556 ± 6	1316 ± 289	1450 ± 352	1121 ± 150	1347 ± 310	1110 ± 533	1157 ± 342	613 ± 60
	BeP	8.4	579 ± 86	540 ± 85	545 ± 57	446 ± 102	520 ± 39	610 ± 91	479 ± 38	402 ± 108
	BaP	2.9	698 ± 108	686 ± 108	704 ± 18	536 ± 139	647 ± 29	543 ± 87	619 ± 52	451 ± 149
	Per	1.8	211 ± 48	187 ± 21	209 ± 16	156 ± 45	193 ± 4	324 ± 192	172 ± 37	93 ± 44
	IP	3.2	380 ± 36	506 ± 84	487 ± 72	365 ± 99	499 ± 36	412 ± 254	436 ± 28	274 ± 91
	DaA + DaC	4.9	88 ± 6	103 ± 10	112 ± 7	87 ± 26	108 ± 6	225 ± 180	87 ± 3	53 ± 18
	BP	3.1	213 ± 205	459 ± 79	436 ± 66	330 ± 76	423 ± 32	454 ± 2	369 ± 7	273 ± 81
	∑ PAHs (ng/g)		7830 ± 403	7498 ± 1228	8435 ± 1066	6427 ± 1174	7707 ± 538	9022 ± 1217	6818 ± 980	5801 ± 1599
PCBs	CB 50 + 28	0.4	0.7 ± 0.0	0.4 ± 0.2	0.8 ± 0.5	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	2 ± 1	0.4 ± 0.1
	CB 52	0.4	1 ± 2	0.3 ± 0.3	2 ± 0.9	0.3 ± 0.3	1 ± 0.8	1 ± 0.4	2 ± 1	1 ± 0.4
	CB 101	0.4	5 ± 0.3	2 ± 0.8	4 ± 1.8	2 ± 0.3	3 ± 2	0.6 ± 1.1	4 ± 3	3 ± 0.7
	CB 118	0.5	8 ± 0.3	4 ± 2	5 ± 0.5	3 ± 0.5	5 ± 2	5 ± 0.3	4 ± 0.4	5 ± 0.8
	CB 153	0.4	11 ± 0.4	4 ± 2	5 ± 0.5	5 ± 1.0	8 ± 3	7 ± 0.2	6 ± 0.8	6 ± 2
	CB 138	0.4	13 ± 3	6 ± 3	9 ± 3	6 ± 0.7	10 ± 3	8 ± 0.4	7 ± 0.6	8 ± 2
	CB 180	0.4	9 ± 0.2	4 ± 1	6 ± 2	4 ± 0.5	6 ± 2	5 ± 0.4	4 ± 2	6 ± 1.2
	∑ PCBs (ng/g)		41 ± 9	20 ± 9	30 ± 5	21 ± 3	35 ± 11	27 ± 3	27 ± 3	30 ± 7
PBDEs	BDE 47	0.5	4 ± 0.1	2 ± 0.8	3.7 ± 1.2	2.3 ± 0.2	2.7 ± 0.8	3.0 ± 0.3	2 ± 0.2	3 ± 0.5
	BDE 119	1.3	5 ± 0.6	3 ± 0.6	2.8 ± 2.5	2.4 ± 0.2	2.3 ± 2.0	2.2 ± 0.1	2 ± 1	3 ± 0.1
	BDE 99	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BDE 153	n.a.	0.8 ± 1	0.5 ± 0.7	< LOD	< LOD	< LOD	0.9 ± 0.4	< LOD	0.6 ± 0.1
	∑ PBDEs (ng/g)		10 ± 0.3	5 ± 2	6 ± 4	4.7 ± 0.3	5 ± 1	5 ± 0.8	4 ± 1.2	7 ± 0.7
OCPs	HCB	0.4	1.7 ± 0.3	6 ± 0.9	2 ± 1.7	0.9 ± 0.2	1.2 ± 1.1	0.6 ± 0.0	0.4 ± 0.0	1.4 ± 1.4
	Gamma-HCH	0.3	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
	Heptachlor	0.1	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.0 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
	Heptachlor epoxide	0.2	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
	2,4′DDE	0.4	0.8 ± 0.2	0.3 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	0.5 ± 0.0	3 ± 0.5
	cis Chlordane	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	trans Nonachlor	0.4	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.2
	4,4′DDE + dieldrin	10.1	9 ± 9	9 ± 0.5	14 ± 5	5 ± 6	6 ± 0.4	7 ± 6	8 ± 0.8	1.0 ± 0.9
	2,4′DDD	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	4,4′DDD	3.6	8 ± 1	4 ± 0.4	5 ± 2	5 ± 0.4	5 ± 2	5 ± 0.8	3 ± 0.0	2.7 ± 0.8
	2,4′DDT	0.8	1.0 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	1 ± 0.4	0.9 ± 0.3	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.3
	4,4′DDT	1.3	11 ± 8	3 ± 0.0	2 ± 1	3 ± 2.1	4 ± 3.4	2 ± 0.1	1.2 ± 0.5	0.6 ± 1.1
	Mirex	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	∑ OCPs (ng/g)		35 ± 4	23 ± 4	25 ± 11	21 ± 5	22 ± 5	17 ± 5	16 ± 1	12 ± 2
Alkylphenols	NP1EO	20	158 ± 16	98 ± 26	123 ± 18	83 ± 29	141 ± 60	142 ± 16	79 ± 6	21 ± 10
	NP2EO	0.3	31 ± 6	22 ± 6	27 ± 1	16 ± 4	28 ± 12	36 ± 5.3	9 ± 4	6 ± 0.5
	4-NP	19	783 ± 75	813 ± 96	666 ± 140	713 ± 231	764 ± 66	597 ± 176	791 ± 127	714 ± 194
	OP	n.a.	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	BPA	8	152 ± 13	270 ± 62	177 ± 102	163 ± 36	86 ± 13	83 ± 18	150 ± 50	54 ± 20

Table 4 (continued)

		LOD	MeOH	Acetone	MeOH/DCM	Hept/MeOH	DCM/Ac	Hept/Ac	DCM	Heptane
	NP1EC	0.3	8 ± 3	3.3 ± 2	3.4 ± 0.7	10 ± 3	2 ± 1.4	n.d.	3 ± 1.3	n.d.
	∑AKPs (ng/g)		1132 ± 101	1206 ± 128	996 ± 198	984 ± 233	1021 ± 116	857 ± 205	1030 ± 188	796 ± 223
Steroids	Estriol	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Norethindrone	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Testosterone	0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
	17α-Ethynylestradiol	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Estrone	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	17α-Estradiol	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	17β-Estradiol	0.2	0.6 ± 0.8	0.2 ± 0.3	0.5 ± 0.5	0.2 ± 0.3	0.3 ± 0.4	<LOD	n.d.	n.d.
	Levonorgestrel	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Progesterone	0.6	5 ± 1	5 ± 2	4 ± 0.9	5 ± 1.7	4 ± 0.8	5 ± 0.3	1.1 ± 0.2	0.3 ± 0.0
	Mestranol	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	∑ steroids (ng/g)		6 ± 0.1	5 ± 2	6 ± 2	6.0 ± 0.5	4.8 ± 1.5	5.2 ± 1.9	1.2 ± 0.2	0.3 ± 0.0

LOD limit of detection in nanograms of standard/gram d.w., *n.a.* not assessed, *n.d.* not detected

be hazardous for the environment and human health (Bonnard et al. 2014), a mixture of Hept/MeOH should thus be recommended although heptane is usually two times more expensive than DCM. A cost-benefit study may help in selecting the best method as regards risks associated with the use of such a hazardous solvent.

Conclusion

By using a systematic methodological approach based on chemical and bioanalytical analyses, we validated an extraction methodology for the assessment of a broad range of known and unknown active chemicals occurring in the sediment. Good extraction recoveries were obtained using artificial sediment spiked with a defined mixture of active chemicals while a mixture of non-polar and polar solvents allowed the best extraction. Also, extraction of native EDCs in natural sediment having multiple in vitro activities confirmed that a mixture of polar and non-polar solvent, especially MeOH/DCM, allowed the best extraction performance, which supports its use for comprehensive assessment of organic contaminants in sediment using a bioanalytical approach. This study, in line with previous reports (Houtman et al. 2004), recommends the use of bioassay-derived quantitative response (e.g., bio-TEQ) to validate the sample preparation process and stresses the need for harmonization of sample preparation methods to provide a comparable scope of environmental contamination by EDCs. Beyond method validation, such approach revealed a possible involvement of a mixture effect other than additive interactions between the mixture components, especially for PXR-like activity, whereas the concentration-addition concept is often the rule in environmental hazard assessment. Altogether, our results confirmed the need to use a set of complementary in vitro bioanalytical tools to perform a comprehensive assessment of chemical quality of complex environmental matrices such as sediment.

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