TOXICOLOGICAL PROFILING OF SEDIMENTS USING IN VITRO BIOASSAYS, WITH EMPHASIS ON ENDOCRINE DISRUPTION

Corine J. Houtman,*† Peter H. Cenijn,† Timo Hamers,‡ Marja H. Lamoree,† Juliette Legler,†
Albertinka J. Murk,‡ and Abraham Brouwer†
†Institute for Environmental Studies, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
‡Toxicology Section, Wageningen University, P.O. Box 8000, 6700 EA Wageningen, The Netherlands

Abstract—In vitro bioassays are valuable tools for screening environmental samples for the presence of bioactive (e.g., endocrine-disrupting) compounds. They can be used to direct chemical analysis of active compounds in toxicity identification and evaluation (TIE) approaches. In the present study, five in vitro bioassays were used to profile toxic potencies in sediments, with emphasis on endocrine disruption. Nonpolar total and acid-treated stable extracts of sediments from 15 locations in the Rhine Meuse estuary area in The Netherlands were assessed. Dioxin-like and estrogenic activities (using dioxin-responsive chemical-activated luciferase gene expression [DR-CALUX] and estrogen-responsive chemical-activated luciferase gene expression [ER-CALUX] assays) as well as genotoxicity (UMU test) and nonspecific toxic potency (*Vibrio fischeri* assay) were observed in sediment extracts. For the first time, to our knowledge, in vitro displacement of thyroid hormone thyroxine (T_4) from the thyroid hormone transport protein thransthyretin by sediment extracts was observed, indicating the presence of compounds potentially able to disrupt T_4 plasma transport processes. Antiestrogenic activity was also observed in sediment. The present study showed the occurrence of endocrine-disrupting potencies in sediments from the Dutch delta and the suitability of the ER- and DR-CALUX bioassays to direct endocrine-disruption TIE studies.

Keywords—Endocrine disruption   Sediment toxicity   Bioassay   Toxicity identification

INTRODUCTION

Organic chemicals released in the environment (e.g., via industrial and domestic effluents) may accumulate in sediments. Therefore, sediment can act as a sink for many persistent chemicals that are released in the aquatic environment, and it 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 here. One group of contaminants that has received much attention consists of the endocrine-disrupting compounds (EDC), which are xenobiotic as well as natural chemicals in the environment that may interfere with the normal functioning of human and wildlife endocrine systems. Exposure to EDCs may lead to adverse health effects, such as increased rates of specific cancers and reproductive system abnormalities [1,2]. The amount of evidence that effects observed in mainly aquatic wildlife are, indeed, the result of exposure to EDCs is still growing (e.g., for the feminizing effects of alkylphenolic chemicals in different fish species [3–6]). Also, several Dutch studies indicate disturbance of endocrine functioning in organisms exposed to contaminated sediments [7,8].

Although instrumental analysis can be used to identify and quantify known EDCs, hazard evaluation based on chemical monitoring and known toxic potencies of compounds is complicated, because EDCs are highly distinct compounds structurally, interactions between substances in mixtures have to be taken into account, and responsible active compounds often are still mainly unknown [e.g., 9]. Mechanism-based in vitro

bioassays in which specific biological effects are directly estimated can provide valuable information about the expected total toxic potency of the complex mixture of chemicals in an environmental sample. Bioassays can be especially useful if they are combined with instrumental analytical approaches, such as in toxicity identification and evaluation (TIE). In TIE approaches, bioassays are used to direct fractionation and chemical analysis to elucidate compounds responsible for the toxic activity found in a sample. To enable direction of a TIE for endocrine disruption, bioassays should meet several criteria: They should be sensitive, require a minimal amount of sample, generate response through a specific biological mechanism involved in endocrine functioning, be compatible with clean up requirements of chemical analysis, and be biologically relevant.

In the present study, we performed a survey of toxic potency occurring in sediment, with emphasis on endocrine disruption. Sediment samples were collected at 15 different locations in the Dutch Rhine Meuse estuary. Although detailed information about the type of contamination was not available, locations differed in their degree of exposure to agricultural and household discharge and vicinity regarding industrial activity. This sampling approach was used to obtain a general view regarding the presence of potential toxic activities in the Dutch Delta area. The study had three main goals.

Our first goal was to apply several in vitro bioassays to investigate four different types of endocrine disruption: Dioxin-like activity, estrogenic activity, antiestrogenic activity, and thyroid hormone displacement capacity. In addition, two bioassays for non–endocrine-disrupting potency, genotoxicity and nonspecific toxic potency, were applied to obtain a more general impression of toxic potencies in aquatic sediments and

to assess if these types of toxicity possibly mask endocrine-disruptive endpoints.

The dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX) assay was used to detect arylhydro-carbon (Ah) receptor–mediated, dioxin-like activity. Although according to the traditional opinion this receptor does not belong to the endocrine system, many chemicals acting via this receptor do, directly or indirectly, show endocrine activity [10]. Known Ah receptor–agonistic compounds, such as dioxins and polychlorinated biphenyls (PCBs), have therefore been listed as suspected endocrine disrupters in several reports (e.g., [11,12]). The DR-CALUX assay uses a rat hepatoma H4IIE cell line stably transfected with firefly (*Photinus pyralis*) luciferase gene under the control of dioxin-responsive elements [13]. Exposure of the cells to Ah-receptor agonists induces the production of luciferase, which is quantified by measuring light production after addition of the substrate luciferin.

To detect estrogenic and antiestrogenic activity, the estrogen-responsive, chemical-activated luciferase gene expression (ER-CALUX) assay was applied. The ER-CALUX assay uses human T47D breast adenocarcinoma cells expressing endogenous estrogen receptor α and β, which are stably transfected with an estrogen-responsive luciferase reporter gene [14]. Exposure of these cells to (xeno)estrogens results in the induction of luciferase production.

Interference with thyroid hormone (T_4; $3,3',5,5'$-tetraiodothyroxine) transport in plasma was measured in an in vitro T_4-transthyretin (TTR) binding competition assay. In this assay, the potency of a sample to compete with the radioligand $[^{125}I]T_4$ for binding the transport protein TTR is tested. Competitive protein binding is determined by measuring the amount of radioactive T_4 displaced from the protein after incubation [15].

In the UMU test, expression of SOS response umu-C genes induced by genotoxic activity in environmental samples was detected in the genetically engineered bacterial strain *Salmonella typhimurium* (TA1535/pSK1002). In this bacterial strain, the induction of the SOS repair system by genotoxic agents can be measured by a photometric determination of the β-galactosidase enzyme activity [16].

Nonspecific toxic potency was measured in the *Vibrio fischeri* assay, a nonspecific toxicity assay measuring bioluminescence (coupled to cell respiration) of the marine bacterium *V. fischeri* based on the Microtox® assay as developed by Bulich [17]. The assay was adapted to fast and small volume testing by Hamers et al. [18]. Assays were performed with nonpolar total as well as acid-treated stable extracts to get an idea regarding the chemical stability of compounds causing response in the bioassays.

Our second goal was to determine if the selected bioassays for endocrine disruption are suitable to direct TIE research for EDCs in sediments. The three assays for endocrine disruption used were evaluated according to the criteria outlined above.

Finally, our third goal was to determine if one or more of the selected locations showed high responses in one or more bioassays, caused by unknown compounds. This would identify possible locations for further TIE research.

MATERIALS AND METHODS

Environmental sampling

Surface sediments were sampled from 15 locations in the Dutch Delta (Fig. 1) from the March 19 to March 22, 2001,
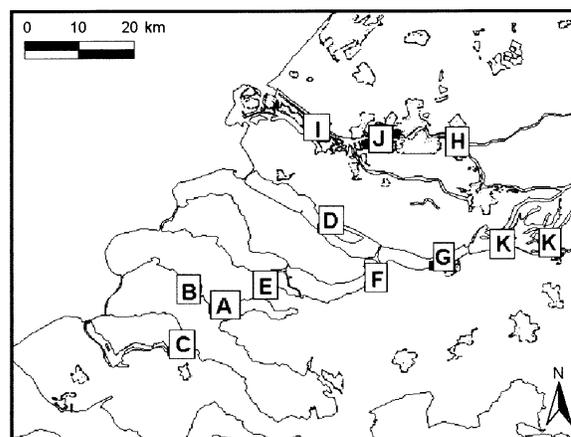

Fig. 1. Sampling locations in the Rhine Meuse estuary (The Netherlands). For each location, type of location and salinity (‰) are given in parentheses. A = Eastern Scheldt 1 and 2 (remote [Rm]; 30.1; 30.7); B = Zierikzee outer harbor and Zierikzee inner harbor (Recreation [Rc], industrial [I]; 30.4); C = Lake Veerse Meer (Rc, I; 30.1); D = Haringvliet (Rm, sedimentation area [S]; 23.6); E = Bruinisse (Agricultural [A], I; 0.23); F = Dintelmond Sluices (A, I; 29.6); G = Moerdijk (I, A; 0.28); H = Nieuwe Maas (I; 0.22), I = Nieuwe Waterweg (I; 0.24); J = Rotterdam Yssel harbor and Rotterdam Second Petrol harbor (I; 16.9); K = Biesbosch Spijkerboor and Biesbosch Gat van de Vissen (S; 0.25).

using a Van Veen grab. During sampling, water salinity at the sampling location was monitored. Sediment samples were stored at 4°C until further treatment. Samples were sieved (mesh size, 2 mm), air-dried, homogenized, and stored for four months before extraction in bottles of dark brown glass. The percentage of organic carbon in sieved material was estimated by elemental carbon measurement with FlashCombustion/gas chromatography (FlaschEAtm 1112 Elemental Analyzer; ThermoQuest Italia, Milan, Italy).

Extractions

All solvents were pro analysis quality or better and were purchased from JT Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany) unless stated otherwise. A total of 10 g of dried sediment from each location was extracted with a mixture of hexane and acetone (9:1, v/v) with accelerated solvent extraction (three extraction cycles, 100°C, system pressure of 2,000 psi; ASE200; Dionex, Sunnyvale, CA, USA). The extracts were divided into two equal portions. In both portions, sulfur was removed using tetra butyl ammonium sulfite. To obtain extracts, one portion was evaporated with N_2 at 40°C. Just before dryness, 60 µl of dimethyl sulfoxide (DMSO; spectrophotometric grade, 99.9%; Acros, Geel, Belgium) were added. The other portion was used for the preparation of stable extracts. This portion was further cleaned using a multilayer sulfuric acid silica column consisting of 1 g Na_2SO_4 on top of 3 g of dried silica with 20% hexane–washed H_2SO_4 (w/w) and 3 grams of dried silica with 33% hexane–washed H_2SO_4 (w/w). The column was eluted with 40 ml of hexane:diethyl ether (97:3, v/v). The eluate was dried with N_2 at 40°C and dissolved in 60 µl of DMSO. Both total and stable extracts were tested in all bioassays except for the UMU test, in which only total extract was used.

Bioassays

DR-CALUX. The DR-CALUX-assay was performed using H4IIE Luc cells in 96-well microtiter plates (Greiner, Frick-

enhausen, Germany) according to the method developed by Murk et al. [19] with the following adaptations. Extracts were serially diluted up to 30-fold for stable extracts and 10,000-fold for total extracts. Cells were exposed for 24 h in triplicate to extracts at a maximum solvent concentration of 0.4% DMSO. Cells were harvested by lysis in 30 μl of lysis buffer (25 mM Tris, 2 mM dithiothreitol, 2mM trans-1,2-diamino-cyclohexane-N,N,N′,N′-tetraacetic acid monohydrate, 10% glycerol, and 1% Triton® X-100 [Sigma-Aldrich, Steinheim, Germany] in demineralized water, pH 7.8 overall buffer) and stored at −20°C until analysis. Microtiter plates were thawed and shaken at room temperature. To each well, 100 μl of glowmix (20 mM tricine, 1.07 mM $C_4H_2Mg_5O_{14}$, 2.6 mM $MgSO_4$, 0.1 mM ethylenediamine-N,N,N′,N′-tetraacetic acid, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.46 mM Lu-ciferine, and 0.53 mM adenosine-5′-triphosphate in demin-eralized water) were added. Luciferase activity was measured in a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min/well. For the calculation of induced re-sponses on each microtiter plate, a concentration series of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was included. For this series, a sigmoidal standard curve ($y = a1/[1 + \exp(-(x - a2)/a3]$, where y is the luciferase activity measured expressed as relative light units and x is the concentration of TCDD) was fitted using the software program SlideWrite 4.1 (Advanced Graphics Software, Encinitas, CA, USA). After correction for background signal (DMSO solvent control), lu-ciferase activities of mostly diluted extracts causing response between that of 0.9 pM and the median effective concentration (EC50) of TCDD were interpolated on this curve and ex-pressed as TCDD equivalents (TEQs) per gram sediment.

ER-CALUX. The ER-CALUX assay was performed with T47D Luc cells according to the method of Legler et al. [14]. Extracts were diluted up to 72-fold. Cells were exposed in triplicate to extracts at a maximum solvent concentration of 0.1% in 96-well microtiter plates (TC microwell 96F; Nalge Nunc International, Roskilde, Denmark). A concentration se-ries of 17β-estradiol (E_2) was included on each plate. Har-vesting, measurement of luciferase activity, and calculation of estradiol equivalents (EEQs) of samples by interpolation in standard curves were performed as described above for DR-CALUX using E_2 as positive control.

Antiestrogenicity measurements. To investigate the capacity of sediment extracts to suppress estrogen-mediated luciferase activity, T47D Luc cells were exposed to a combination of 3.5 pM E_2 (~EC50) and to sediment extracts at a total solvent concentration of 0.2%. Distinction between suppression of lu-ciferase activity caused by antiestrogenicity and suppression caused by other nonspecific effects was made by exposing cells to E_2 at a supermaximum concentration (100 pM) in combi-nation with sediment extract. Extracts were judged to be an-tiestrogenic in a estrogen receptor–mediated manner only if this combined exposure to the high E_2 concentration increased luciferase activity again. Luciferase induction after combined exposure to 3.5 pM E_2 and sediment extract was expressed as the percentage of induction after exposure to E_2 alone. Ta-moxifen, a known antiestrogen in T47D Luc cells was used at 100 nM as a positive control for antiestrogenicity.

Thyroid hormone displacement. The capacity of the sedi-ment extracts to compete with the natural thyroid hormone T_4 for binding to the thyroid hormone transport protein TTR was analyzed according to the method described by Meerts et al. [20]. After overnight incubation of 30 nM human TTR, [125]I-

labeled T_4, unlabeled T_4, and 5 μl of nonpolar stable sediment extract, radioactivity was measured in a gamma counter (1282 LKB; Wallac, Turku, Finland) with an energy window of 035 to 102 and 60% efficiency in the protein-bound [125I]T_4 con-taining eluate fraction and compared to control incubations. All measurements were performed in duplicate. A sigmoidal standard curve was fitted for a concentration series of T_4. Per-centages of T_4-TTR binding for each sample were interpolated in this curve and recalculated. Reduced binding (inhibition) compared to controls was recalculated into T_4 equivalents per gram sediment.

UMU test. The assay was performed according to the Deutches Institut für Normurg protocol 38415-3 [21] with the deviations as reported by Hamers et al. [22]. Extracts were tested in the presence as well as in the absence of induced S9 mix to assess the influence of metabolic deactivation on the total mutagenic potency of the sample. Nonpolar total extracts were tested in triplicate with 1-, 3-, 9-, and 27-fold dilutions. Responses of the extracts in the UMU test were expressed as equivalents of 4-nitroquinolin-oxid (4-NQO) or 2-aminoan-thracene (2-AA) for those concentrations yielding an induction factor of greater than 1.3. All measurements were performed in triplicate.

Vibrio fischeri assay. General toxicity of sediment extracts to *V. fischeri* bacteria was tested according to Hamers et al. [18] in an exponential dilution series (with steps of $\sqrt{10}$) of sediment extracts that had been diluted 400-fold in deminer-alized water with a final concentration of 2% (w/v) NaCl and 0.45% (v/v) DMSO. Luminescence was measured exactly 7.5 min after injection of bacteria. From the dilution series from each location, luminescence-inhibiting potency was expressed as the amount of sediment necessary to lower luminescence by 20% (EC20).

Statistics

Statistical analysis of the antiestrogenicity data using Stu-dent's *t* tests and linear regression calculations were performed with the software program SlideWrite 4.1.

RESULTS

DR- and ER-CALUX assays

Estrogenic, antiestrogenic, and dioxin-like activities were de-termined in sediment extracts using the ER- and DR-CALUX assays. Figure 2a shows examples of typical S-shaped dose–response curves of T47D Luc and H4IIE Luc cells exposed to E_2 (ER-CALUX) and 2,3,7,8-TCDD (DR-CALUX), respec-tively. Maximum induction was found at 10 pM E_2 (25-fold) and 100 pM TCDD (20.5-fold). For ER-CALUX, the average limit of detection in this study (in which 5 g of sediment was extracted and dissolved in 60 μl of DMSO) was 1.3 ± 1.0 pg EEQ/g dry weight. For our samples, we found an average var-iation coefficient of 9.7 ± 5.6 (± SE). Figure 2b shows an example of dose–response data regarding dilutions of total and stable sediment extracts from Zierikzee outer and inner harbor. This figure illustrates the method of the interpolation of lucif-erase activity by extracts in the E_z standard curve. As observed more often, the undiluted total extracts (e.g., Zierikzee outer harbor) show estrogenic potency lower than that of the eightfold-diluted extract. However, further diluting the extract (e.g., to 24-fold) leads to a quantifiable response that is then used for the EEQ calculation. For DR-CALUX, the average limit of detection was 0.5 ± 0.5 pg TEQ/g dry weight, and the average variation coefficient was 7.5 ± 4.6.
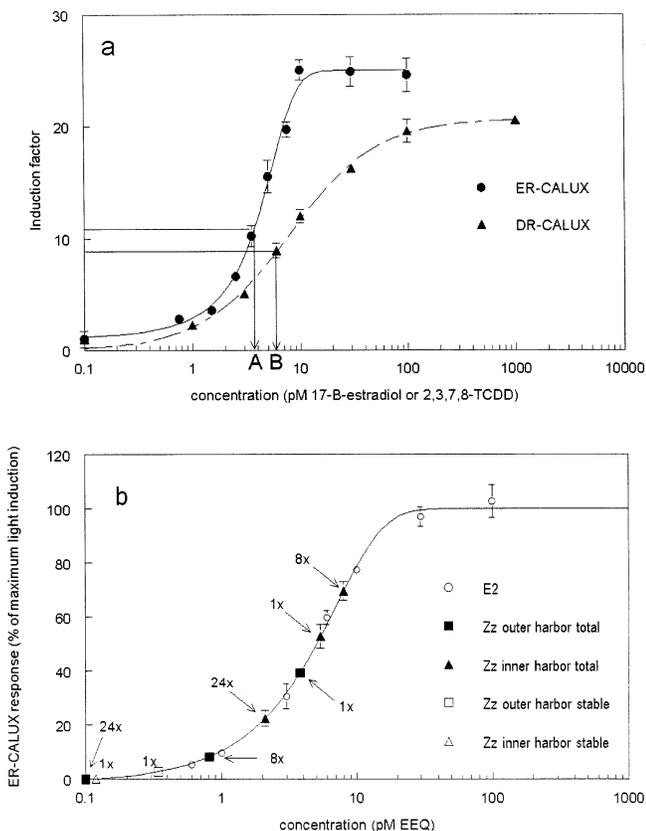
Fig. 2. (**a**) Concentration–response curves for estrogen-responsive chemical-activated luciferase gene expression (ER-CALUX) and dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX) assays after 24 h of exposure to 17β-estradiol (E$_2$) for ER-CALUX or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for DR-CALUX. The induction factor is the light production in the well expressed relative to the dimethyl sulfoxide control. Arrows indicate the calculated values of the median effective concentrations (EC50) for ER-CALUX (A; 4.2 pM E$_2$) and DR-CALUX (B; 7.9 pM TCDD). (**b**) Estrogenic potencies in the ER-CALUX assay of different dilutions of total and stable extracts of locations Zierikzee (Zz) outer and inner harbor. EEQ = estradiol equivalent.

Estrogenic activity was observed in nonpolar total extracts from all locations. Calculated values ranged from 5.0 ± 0.4 pg EEQ/g dry weight for location Easternscheldt 1 to 340.0 ± 6.9 pg EEQ/g dry weight for sediment from location Haringvliet, with a median value of 64.8 pg EEQ/g dry weight (Table 1). Nonpolar stable extracts treated with sulfuric acid did not cause any significant induction of luciferase activity in T47D Luc cells except for sediment from location Second Petrol harbor, which had some estrogenic activity above the limit of detection (3.8 ± 0.5 pg EEQ/g dry wt). Stable extracts were tested for antiestrogenic properties as well, but only slight (~30% decrease of luciferase induction) antiestrogenic activities were observed in the locations Rotterdam Yssel harbor, Dintelmond Sluices, Haringvliet, and Moerdijk (Rhine Meuse estuary) (Table 1). Haringvliet extract showed the highest antiestrogenic activity, with a reduction of 37% of the E$_2$-induced luciferase activity (Fig. 3). In the presence of 100 nM E$_2$ (dashed bars), luciferase induction was restored to uninhibited levels, indicating the involvement of the estrogen receptor in the antiestrogenicity observed. Total extracts were too crude to obtain reproducible results in antiestrogenicity measurements (data not shown).

Dioxin-like activity in H4IIE Luc cells was observed after exposure to total extracts from almost all locations. Activities found varied between 9.6 ± 0.4 pg TEQ/g dry weight (Easternscheldt 1) up to 11,305 ± 1,088 pg TEQ/g dry weight for location Zierikzee inner harbor, with a median value of 3,770 pg TEQ/g dry weight (Table 1). Unlike in the ER-CALUX assay, the stable extracts showed Ah receptor–mediated activity as well, varying between 1.6 ± 0.1 pg TEQ/g dry weight (Easternscheldt 1) and 162.5 ± 9.5 pg TEQ/g dry weight (Rotterdam Yssel harbor), with a median value of 92.3 pg TEQ/g dry weight (Table 1). Correction for the percentage of organic carbon in dried sediments (Fig. 4) resulted in a strong correlation between dioxin-like activities of total and stable extracts (r^2 = 0.77).

T$_4$-TTR binding competition assay

The in vitro T$_4$-TTR binding competition assay was performed to investigate the presence of compounds with thyroid hormone activity in sediment. Figure 5 shows the calibration curve of competition between unlabeled T$_4$ and radioactive [^{125}I]T$_4$ for binding to TTR. For this experiment, the limit of detection was 6.0 pmol T$_4$ equivalents/g dry weight. The average variation coefficient was 3.8 ± 3.4. Inhibiting potencies of the stable sediment extracts ranged from below the detection limit to 16.2 ± 0.3 pmol T$_4$ equivalents/g dry weight (Table 1). Most locations failed to inhibit T$_4$ binding to TTR with the exception of sediment extracts from locations Zierikzee outer harbor, Second Petrol harbor, Haringvliet, and Dintelmond Sluices. They showed significant ability to compete with T$_4$ for binding to TTR. Sediment extract from location Haringvliet was the most potent competitor, with the undiluted extract causing 42% displacement of T$_4$ (Fig. 5). As with antiestrogenicity measurements, total extracts were too crude to be used in this assay (data not shown).

Non–endocrine-disruption assays

The UMU test was carried out to determine genotoxic potency in total extracts of sediments and was measured directly as well as after metabolic (in)activation with S9 mix. Only total extracts were tested to determine the role of metabolites, which are not present in stable extracts because of the sulfuric acid clean up. Induction of β-galactosidase activity increased proportionally with increasing concentrations of the mutagens used as positive controls, 4-NQO and 2-AA. The results expressed as mutagenic equivalents only are given for samples with induction factors of greater than 1.3 (Table 1). Average limits of quantification were 25.7 ± 3.9 ng 4-NQO/g dry weight in the absence of S9 mix and 172.5 ± 45.0 ng 2-AA/g dry weight in its presence. Genotoxic activity of the sediment extracts was generally low, with induction factors of undiluted extracts less than 1.5. Average activities at locations showing activities higher than the limit of quantification were 44.4 ng 4-NQO/g dry weight and 269.8 ng 2-AA/g dry weight. Only for sediment extract from location Veerse Meer was activity found in the presence as well as the absence of S9 mix. Direct activity (without S9 mix) was slightly higher than activity in the presence of S9 mix.

Luminescence of the bacteria in the *V. fischeri* assay diminished with increasing concentrations of both types of sediment extracts, indicating the presence of compounds with nonspecific toxic potency in the extracts. Instead of the EC50, luminescence-inhibiting potency is expressed as the EC20 (Table 1), because the interpolated EC50 concentration often did not fall in the linear part of the dose–response curve. For total extracts, EC20 values varied between 6.1 mg dry weight/well

Table 1. Overview of endocrine-disrupting potencies, general toxicity, and genotoxicity in nonpolar total and stable extracts of 15 Dutch aquatic sediments[a]

Assay	DR-CALUX (pg TEQ/g dry wt)		ER-CALUX (pg EEQ/g dry wt)		Anti-ER	T$_4$-TTR	*Vibrio fischeri* (EC20 mg dry wt/well)[b]		UMU		OC (%)
	Total	Stable	Total	Stable	Stable (% reduction)	Stable (pmol T$_4$-eq/g dry wt)	Total	Stable	Total (−S9) (ng 4-NQO-eq/g dry wt)	Total (+S9) (ng 2-AA-eq/g dry wt)	
Easternscheldt 1[c]	9.6 ± 0.4	1.6 ± 0.1	5.0 ± 0.4	<LOD	NA[d]	<LOD	6.12	>LOD	<LOD	<LOD	0.06
Easternscheldt 2[c]	153.4 ± 7.3	4.4 ± 0.3	33.6 ± 3.0	<LOD	NA[d]	<LOD	4.71	>LOD	<LOD	<LOD	0.33
Zierikzee outer harbor	620.5 ± 75.9	31.7 ± 5.2	10.7 ± 0.3	<LOD	NA[d]	13.4 ± 0.2	0.46	0.04	27.8 ± 2.7	<LOD	2.19
Zierikzee inner harbor	11,305.1 ± 1,088.4	158.1 ± 22.0	126.3 ± 10.1	<LOD	NA[d]	<LOD	0.48	>LOD	<LOD	258.6 ± 16.5	1.53
Lake Veerse Meer	844.3 ± 57.0	19.5 ± 1.3	182.3 ± 5.0	<LOD	NA[d]	6.8 ± 0.4	0.03	21.99	80.6 ± 6.6	461.3 ± 40.6	2.96
Haringvliet	9,150.2 ± 382.7	108.1 ± 3.5	340.0 ± 6.9	<LOD	37	16.2 ± 0.3	0.16	>LOD	<LOD	<LOD	3.83
Bruinisse	6,663.8 ± 351.7	115.6 ± 9.4	124.0 ± 22.0	<LOD	NA[d]	7.8 ± 0.1	0.69	>LOD	<LOD	453.3 ± 13.9	2.79
Dintel Sluices	4,651.4 ± 529.9	92.3 ± 4.3	91.8 ± 7.0	<LOD	32	13.4 ± 0.9	0.29	>LOD	<LOD	<LOD	4.32
Moerdijk	10,986.0 ± 456.8	133.7 ± 21.0	64.8 ± 4.5	<LOD	28	6.1 ± 0.6	0.10	>LOD	<LOD	255.3 ± 36.4	4.66
Nieuwe Maas[c]	1,129.8 ± 58.4	18.3 ± 2.6	14.8 ± 0.4	<LOD	NA[d]	<LOD	2.98	>LOD	34.5 ± 0.9	<LOD	0.22
Nieuwe Waterweg[c]	881.3 ± 76.7	3.1 ± 0.1	9.8 ± 1.2	<LOD	NA[d]	<LOD	3.59	>LOD	<LOD	<LOD	0.41
Rotterdam Yssel harbor	3,659.3 ± 55.4	162.5 ± 9.5	76.3 ± 9.8	<LOD	28	6.5 ± 0.3	0.25	>LOD	<LOD	<LOD	4.21
Rotterdam Second Petrol harbor	8,826.6 ± 422.3	112.3 ± 1.3	128.3 ± 11.1	3.8 ± 0.5	NA[d]	9.2 ± 0.0	0.52	3.21	<LOD	<LOD	4.02
Biesbosch Spijkerboor	3,770.0 ± 405.6	101.3 ± 17.9	44.7 ± 4.0	<LOD	NA[d]	<LOD	0.39	>LOD	27.3 ± 2.7	<LOD	1.5
Biesbosch Gat van de vissen	4,053.4 ± 117.7	73.6 ± 2.7	40.4 ± 2.7	<LOD	NA[d]	<LOD	0.49	>LOD	51.9 ± 4.5	<LOD	1.15

a DR-CALUX = dioxin-responsive chemical-activated luciferase gene expression assay; ER-CALUX = estrogen-responsive chemical-activated luciferase gene expression assay; TTR = transthyretin; OC = organic carbon; NA = not achieved; LOD = limit of detection; EC20 = concentration causing 20% of the maximum effect; EEQ = estrogen equivalence factor; TEQ = toxic equivalence factor; UMU = UV mutagenesis gene.
b A higher value represents a lower potency.
c Sediment locations with extremely low organic carbon content (OC < 0.5%).
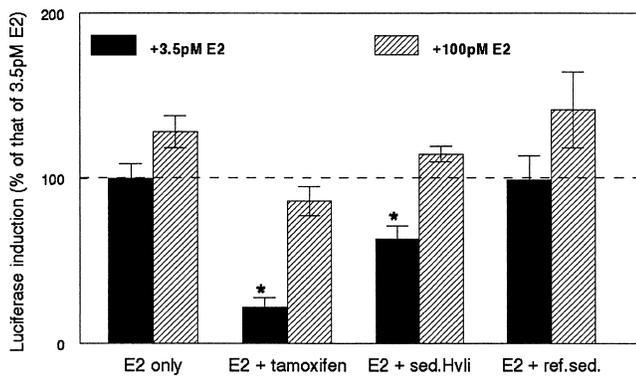d No reduction or insignificant reduction (<25% of 17β-estradiol controls observed).

Fig. 3. Antiestrogenic activity in nonpolar stable sediment extracts. Extracts were tested with coadministration of 3.5 and 100 pM estradiol (E_2). Sediment (sed.) extracts from Haringvliet (Hvli) and a reference sediment (ref. sed.; seasand) are shown. Tamoxifen (100 nM) was used as positive control for antiestrogenic activity. *Significantly different ($p < 0.05$) from E_2 alone.

Fig. 5. Concentration–response curve of thyroxine (T_4) binding to transthyretin (TTR). Arrow A indicates the calculated value of the median effect concentration (EC50) for competitive binding of radioactive T_4 to TTR (40.2 nM T_4). Stable Haringvliet sediment extract was tested in various dilutions (+). The T_4 equivalents were calculated using the undiluted extract, which was closest to the EC50 (arrow B). std = standard.

(Easternscheldt 1) and 0.1 mg dry weight/well (Moerdijk), with an average of 1.4 ± 1.9 mg dry weight/well and a median value of 0.5 mg dry weight/well (Table 1). For stable extracts, EC20 values varied between greater than the detection limit (26.5 mg dry wt/well for location Easternscheldt 2) and 0.04 mg dry weight/well (Zierikzee outer harbor), with undetectable low toxicities for 12 locations (Table 1).

DISCUSSION

In the present study, we found different potentially endocrine-disrupting activities in sediment extracts from the Dutch Delta. Estrogenic activity was found in relatively nonpolar sediment extracts. Estrogenic activity has been reported in other studies in both nonpolar extracts, such as in a nonpolar fraction of extract of PM10 air particulate matter [23] and polar fractions of extracts of sediments [9,24,25], indicating the broad range of compounds that can bind to the estrogen receptor. Estrogenic activities found in the present study are in the lower range of those found in a recently undertaken Dutch biomonitoring study concerning estrogenic compounds in the aquatic environment [9], which ranged from less than 6.8 to more than 1,000 pg EEQ/g dry weight.

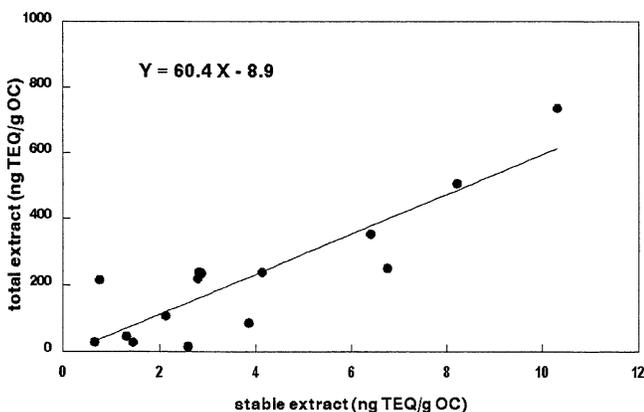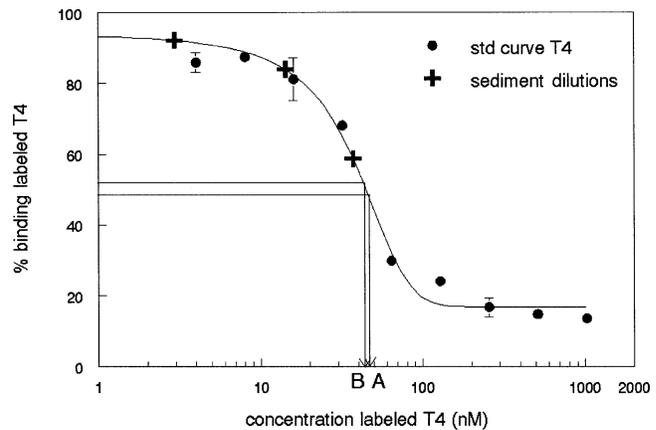Interestingly, acid silica treatment resulted in almost total

Fig. 4. Dioxin-like potencies of sediment extracts with (stable extract) and without clean up with acid silica (total extract) expressed as toxicity equivalence quotient (TEQ) values in the dioxin-responsive, chemical-activated luciferase gene expression assay. Results show a clear correlation between the dioxin-like activities in both extract types ($r^2 = 0.77$). OC = organic carbon.

loss of estrogenic activity, indicating that the estrogenic activity in our samples must have been caused by acid-labile, relatively nonpolar compounds. In contrast, in a study that measured estrogenic activity in sediment from Masan Bay, Korea, Khim et al. [25] found similar results between sulfuric acid–treated and untreated extracts of midpolarity, suggesting that the majority of the activity found in their extracts probably was accounted for by acid-treated stable compounds. Although chemical analytical determination of estrogenic compounds in sediment has not been carried out, several acid-labile (xeno)estrogens could have been responsible for the effects observed in the Dutch sediments, such as naturally occurring phytoestrogens, but also certain polyaromatic hydrocarbons [23,26,27] and some of polycyclic aromatic hydrocarbon oxymetabolites and azarenes [26].

To our knowledge, the present study is the first demonstration of antiestrogenic activity in Dutch sediment extracts. However, the antiestrogenic potency found was slight, and it occurred only in a few stable sediment extracts. It should be noted that antiestrogenicity measurements with total extracts often decreased luciferase activity in such a way that it could not be restored by addition of high E_2 concentrations (data not shown). These results indicate that clean up, such as we performed for the acid-treated stable extracts, is necessary to ensure that the signal reduction observed is caused by receptor-mediated interactions instead of other causes, such as nonspecific matrix effects or cytotoxicity. Antiestrogenic activity was found in extracts that showed estrogenic activity as well. This demonstrates the complexity in the issue of toxicity of environmental mixtures and the need for further research on the interactions of such mixtures with real organisms exposed to whole sediments. No correlation was found between antiestrogenicity and dioxin-like activity (data not shown). Apparently, although several acid-treated stable Ah-receptor agonists have been shown to be antiestrogenic (e.g., 2,3,7,8-TCDD, PCB 126, and PCB 118 [10]), in the present study other as-yet-unknown compounds possibly account for the dioxin-like and antiestrogenic effects observed.

Dioxin-like activity was observed in total as well as acid-treated stable extracts. The activity observed in total extract always was much higher (on average, 66-fold) than in acid-

treated stable extract. This suggests that most of the dioxin-like activity in untreated total extract is caused by acid-labile compounds, such as polycyclic aromatic hydrocarbons [28]. Organic compounds of natural origin with affinity for the Ah receptor could have been present in the sediments and, thus, responsible for a major part of the activity observed as well. For example, natural organohalogens, containing chlorine or bromine, have been shown to be ubiquitous in terrestrial and marine environments [29,30]. To enable comparison between dioxin-like activities in total and stable extracts, we had to express potencies measured in the total extracts as TCDD equivalents. Yet, because most of the compounds in these extracts are much more biodegradable than TCDD (data not shown), their toxicological potential cannot be equalized to that of dioxin. For this reason, other studies that do not perform comparison with stable extracts express unstable dioxin-like activities in benzo[*a*]pyrene equivalents [22,28].

Dioxin-like activity in acid-treated stable extracts found in the present study is on the same order of magnitude as those found in the Dutch national investigation concerning estrogenic compounds in the aquatic environment (7.4–71 pg TEQ/g dry wt [31]) and those found by Vondráček et al. [28] in acid-treated dichloromethane:methanol extracts from Czech river sediments. Based on biomagnification factors from sediment to fish to male otter and on effect concentrations, Smit et al. [32] derived a critical level of dioxin-like compounds in sediment of 7 ng TEQ/kg organic carbon. For the locations in the present study, this level is exceeded by an average of 540-fold. This indicates a certain risk for the development of wildlife populations at these locations. Dioxin-like activity in acid-treated stable extracts may have been caused by stable compounds, such as PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans, other polyhalogenated aromatic hydrocarbons (e.g., polychlorinated terphenyls and tetrachlorobenzyltoluenes), and polybrominated diphenyl ethers, which all are Ah-active compounds persistent enough to survive sulfuric acid treatment [33].

Normalization based on organic carbon content resulted in a strong correlation between dioxin-like potencies of total and stable extracts. A comparable constant ratio ($\sim$75) between the activities of acid-treated stable and unstable extracts has also been reported previously for freeze-dried sediment samples [34]. This might indicate that in the present study, similar substances in the total and stable extracts could be responsible for the dioxin-like activity we measured.

To our knowledge, the results of the present study demonstrate for the first time the occurrence of compounds with thyroid hormone–displacing activity in sediment extracts, as was found in stable extract from Haringvliet sediment. In fish, thyroid hormones are important regulators of physiological functions, such as energy metabolism, metamorphosis, and growth [35]. Because TTR is an important thyroid hormone transport protein in fish [36], exposure to compounds in sediment that may displace T_4 from TTR could disrupt thyroid hormone transport in target tissues and lead to effects on thyroid hormone–mediated processes. Among the chemicals known to bind to TTR in vitro and/or in vivo are metabolites of polyhalogenated aromatic hydrocarbons (PHAHs), such as hydroxylated PCBs and polybrominated biphenyls with halogen substitutions on the 3,5-positions, hydroxylated PCDDs, and pentachlorophenol and several hydroxylated brominated flame retardants (e.g., tetrabromobisphenol A and pentabromophenol) [37]. However, because most of these PHAH metab-

olites are unlikely to survive acid silica treatment, other as-yet-unknown compounds contribute to the T_4-like activity.

Non-endocrine disruption–related toxic potency in sediment extracts

Genotoxic activities observed in total sediment extracts with the UMU test were low. In some extracts, the genotoxicity in the absence of S9 mix was greater than in the presence of S9 mix. Possibly, direct genotoxic compounds present in the extracts are metabolized by the S9 mix into nongenotoxic compounds that have not yet been metabolized by the rat liver homogenate. In other cases, the highest genotoxicity was found in the presence of S9 mix, indicating the conversion of compounds into metabolites with more genotoxic capacity.

As with the DR- and ER-CALUX results, the highest toxic potency in the *V. fischeri* assay was found in total extracts, indicating the responsibility of acid-labile compounds. Total extracts were not cleaned up at all, with the exception of sulfur removal. For this reason, particles in this type of extract could have been responsible, in part, for the inhibition of luminescence measured by quenching the light produced by the bacteria. Dose–response curves of different types of sediment extracts from Rotterdam Second Petrol harbor have different luminescence-inhibiting potencies but also different slopes, suggesting the presence of both stable and labile toxic compounds with different mechanisms of action. Because *V. fischeri* bacteria respond to a huge amount of different compounds [38], the assay gives a good impression regarding the nonspecific and acute toxic activity in the extract, but as we performed an extraction covering a broad range of components, the assay does not provide an indication regarding what classes of compounds could have caused the toxicity observed.

Results from the UMU test and *V. fischeri* assay were compared with those from the assays for endocrine disruption. It can be concluded that the acute toxicity of the sediment extracts is low, as is the case for the genotoxic potential of the samples. However, the endocrine-based activities found were significant. Therefore, more attention should be directed toward possible long-term consequences of exposure to EDCs in the aquatic environment.

Suitable bioassays for endocrine disruption to direct chemical analysis with TIE

To direct chemical analysis of EDCs with a TIE approach, the bioassays selected must be sensitive, require a minimal amount of sample, be specific, and be compatible with chemical analysis as well as biologically relevant. The three selected bioassays for endocrine disruption are applicable for testing different toxic potencies of sediment extract, because the occurrence of those activities was clearly demonstrated.

Both the ER- and DR-CALUX assays, as well as the T_4-TTR binding competition assay, are sensitive enough for application in TIE analysis. The T_4-TTR binding competition assay can detect T_4 binding at the low nanogram range, whereas both CALUX assays are capable of detecting femtograms of TEQs or EEQs, respectively. In all cases, a few grams of material are sufficient to perform the assay. All three assays can be used to assess the total potential of a sample to interfere with very specific biochemical pathways known to be involved in endocrine-disrupting processes. Both CALUX assays are based on receptor-mediated mechanisms ultimately leading to transcription activation, whereas in the TTR binding competition assay, specific competition with T_4 for binding to the TTR protein is measured.

For a first screening in TIE analysis, the extract should be as crude as possible, because every step in clean up has the risk of losing relevant compounds. However, in working with crude sediment extracts, one should be aware of the possibility of measurement disturbance by matrix components. Both ER- and DR-CALUX were successfully performed in total extracts, but attempts to perform the antiestrogenicity test and the TTR binding competition assay with these extracts did not succeed, presumably because of the presence of disturbing factors in the extracts (data not shown). Therefore the potential of acid unstable compounds to cause antiestrogenicity or TTR binding competition remains as yet unknown. The demands on sample pretreatment for these assays require further research before they are compatible with a TIE analysis for acid-treated stable as well as unstable compounds.

Because of the use of intact cells, CALUX bioassays have high biological relevance (for DR-CALUX, see [39]) compared to the TTR assay, in which only the components necessary for binding the transport protein are present. In the CALUX assays, with a 24-h exposure period, influences of transmembranal transport and metabolic activation and degradation are included in the ultimate response measured, which is not the case in the cell-free T_4-TTR binding competition assay. Furthermore, DR- and ER-CALUX assays are performed in the presence of, respectively, 10% and 5% serum in the exposure medium, whereas no serum is present in the assay mixture for the TTR assay. Proteins present in the assay mixture can influence toxicity found by facilitating transmembrane transport but also by scavenging compounds from the free dissolved bioavailable fraction. These influences together could contribute to a better representation of the real-life exposure scenario. The predictive value for the in vivo situation has been demonstrated for the DR-CALUX assay by Murk et al. [33] by comparing DR-CALUX responses of pore-water extracts to their biological responses in an early life-stage test with zebra fish. The predictive value of the ER-CALUX assay has been shown by comparing in vitro ER-CALUX with measurements in a transgenic zebra fish, which was stably transfected with the same construct as the ER-CALUX cell line [40]. Good correlation between in vitro competitive binding to TTR by 4-OH-CB107 in the in vitro competitive binding assay and in vivo T_4 transport disturbance in rats was shown by Meerts [41].

Possible locations for further TIE research

Although toxicities were not correlated to the percentage of organic carbon in the sediment, locations with very low percentages of organic carbon had either no detectable or low toxicities. Often, attempts to derive sediment-quality guidelines to protect sediment-dwelling organisms use contaminant concentrations corrected for the percentage of organic carbon in the sediment sample, because organic microcontaminants will be associated with this fraction. This approach is most suitable for the comparison of locations in close connection to each other. In the present study, a few sediments sampled did have very low organic carbon content (Table 1), showing hardly any biological activity. Because most of our samples were taken at very different locations in a large area, they show a large variation in organic carbon content, and normalization for organic carbon is less appropriate. For these reasons, we expressed all activities (with the exception of those in Fig. 4) per gram dry weight.

Different potencies were found in sediment from industrial as well as remote locations. For example, in the locations Rotterdam Yssel harbor (an industrial harbor presently in use as a port of transshipment in the Rotterdam port area), Zierikzee inner harbor (a small harbor with recreation and some old industrial activity), and Moerdijk (a location in the neighborhood of a big chemical industrial plant), high dioxin-like activity was found, whereas the highest estrogenic activities were found in location Lake Veerse Meer (a remote location influenced by agriculture and recreation) and Haringvliet (a brackish location remote from industrial activity but situated in a sedimentation area for the Meuse and Rhine rivers). This shows that EDCs are widespread throughout the environment. No correlation was found between potency and salinity of water at the sampling location. The sediment extract from location Haringvliet caused high responses in all three assays for endocrine-disrupting potency but not in the assays for general toxic potency and genotoxicity. None of the other locations caused high responses in all the assays. This indicates the presence of diverse active compounds and their multiple sources and the high specificity of the effects measured, implicating the impossibility of evaluating locations using only one bioassay.

CONCLUSION

In the present study, all investigated types of endocrine-disrupting toxicities (estrogenic, antiestrogenic, dioxin-like, and T_4-TTR binding competition) were observed in sediment extracts from the Dutch delta. In ER- and DR-CALUX assays, activity found in total extracts was consistently higher than that found in stable extracts, indicating that the highest contribution to total activity was made by acid instable compounds. Thyroid hormone–like activity and antiestrogenic activity were measured in a limited number of sediment extracts. Generally, toxic potencies in sediment extracts were not mutually correlated, indicating the involvement of multiple (sources of) active compounds. Endocrine-disrupting potencies were more present than genotoxic and general toxic potencies. This implies that present testing on acute toxicity will probably fail to predict potentially adverse long-term effects.

Obviously, the selected bioassays for endocrine disruption are applicable for testing different toxic potencies in sediment extracts. Because of their high specificity, high sensitivity, low sample demands, compatibility with sample treatment for chemical analysis, and biological relevance, DR- and ER-CALUX assays are very promising and suitable tools to direct TIE studies for acid-treated stable as well as unstable EDCs. Future studies will focus on using the CALUX bioassay to direct fractionation of sediment extracts to identify (groups of) compounds responsible for endocrine-disrupting potency.

REFERENCES

1. Tyler CR, Jobling S, Sumpter JP. 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Crit Rev Toxicol* 28: 319–361.
2. Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I, Vethaak AD. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference of the European situation. *Crit Rev Toxicol* 30:71–133.
3. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
4. Gray MA, Metcalfe CD. 1997. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ Toxicol Chem* 16:1082–1086.
5. Gronen S, Denslow N, Manning S, Barnes S, Barnes D, Brouwer

M. 1999. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environ Health Perspect* 107:385–390.

6. Metcalfe TL, Metcalfe CD, Kiparissis Y, Niimi AJ, Foran CM, Benson WH. 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o,p′*-DDT in water or through maternal transfer. *Environ Toxicol Chem* 19: 1893–1900.

7. Janssen PAH. 1996. Reproduction of the flounder, *Platichthys flesus* (L.), in relation to environmental pollution. Steroids and vitellogenesis. PhD thesis. Utrecht University, Utrecht, The Netherlands.

8. Vethaak AD, Jol JG, Meijboom A, Eggens ML, ap Rheinallt T, Wester PW, van de Zande T, Bergman A, Dankers N, Ariese F, Baan RA, Everts JM, Opperhuizen A, Marquenie JM. 1996. Skin and liver diseases induced in flounder (*Platichtys flesus*) after long term exposure to contaminated sediments in large-scale mesocosms. *Environ Health Perspect* 104:1218–1229.

9. Legler J, Leonards P, Spenkelink A, Murk AJ. 2003. In vitro biomonitoring in polar extracts of solid phase matrices reveals the presence of unknown compounds with estrogenic activity. *Ecotoxicology* 12:239–250.

10. Safe S, Wang F, Porter W, Duan R, McDougal A. 1998. Ah receptor agonists as endocrine disrupters: Antiestrogenic activity and mechanisms. *Toxicol Lett* 102–103:343–347.

11. Safe SH, Foster WG, Lamb JC, Newbold RR, Van der Kraak G. 2000. Estrogenicity and Endocrine disruption. Issue Paper 16. Council for Agricultural Science and Technology, Ames, IA, USA.

12. Olsson PE, Borg B, Brunström B, Håkansson H, Klasson-Wehler E. 1998. Endocrine disrupting substances—impairment of reproduction and development. Report 4859. Swedish Environmental Protection Agency, Stockholm, Sweden.

13. Aarts JMMJG, Denison MS, Cox MA, Schalk MAC, Garrison PM, Tullis K, de Haan LHJ, Brouwer A. 1995. Species-species antagonism of Ah receptor activation by 2,2′,5,5′-tetrachloro- and 2,2′,3,3′,4,4′-hexachlorobiphenyl. *Eur J Pharmacol Environ Toxicol Pharmacol Sect* 293:463–474.

14. Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, van der Burg B. 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol Sci* 48:55–66.

15. Lans MC, Klasson-Wehler E, Willemsen M, Meussen E, Safe S, Brouwer A. 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and -dibenzofurans with human transthyretin. *Chem-Biol Interact* 88:7–21.

16. Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H. 1985. Evaluation of the new system (*umu*-test) for the detection of environmental mutagens and carcinogens. *Mutat Res* 147:219–229.

17. Bulich AA. 1979. Use of bioluminescent bacteria for determining toxicity in aquatic environments. In Marking LL, Kimerle RA, eds, *Aquatic Toxicology*. STP 667. American Society for Testing and Materials, Philadelphia, PA, pp 98–106.

18. Hamers T, Smit GD, Murk AJ, Koeman JH. 2001. Biological and chemical analysis of the toxic potency of pesticides in rainwater. *Chemosphere* 45:609–624.

19. Murk AJ, Leonards PEG, van Hattum B, Luit R, van der Weiden MEJ, Smit M. 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). *Environ Toxicol Pharmacol* 6:91–102.

20. Meerts IATM, van Zanden JJ, Luijks EAC, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman Å, Brouwer A. 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. *Toxicol Sci* 56:95–104.

21. Deutsches Institut für Normung. 1996. Bestimmung des erbgutverändernden Potentials von Wasser mit dem umu-Test (T3). Deutsche Norm, Report 38415-3. Deutsches Institut für Normung, Berlin, Germany. p 12.

22. Hamers T, van Schaardenburg MD, Felzel EC, Murk AJ, Koeman JH. 2000. The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution. *Sci Total Environ* 262:159–174.

23. Clemons JH, Allan LM, Marvin CH, Wu Z, McCarry BE, Bryant DW, Zacharewski TR. 1998. Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM10 air par-

ticulate matter using in vitro gene expression assays. *Environ Sci Technol* 32:1853–1860.

24. Legler J, Dennekamp M, Vethaak AD, Brouwer A, Koeman JH, van der Burg B, Murk AJ. 2002. Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. *Sci Total Environ* 293:69–83.

25. Khim JS, Villeneuve DL, Kannan K, Koh CH, Giesy JP. 1999. Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea. 2. In vitro gene expression assays. *Environ Sci Technol* 33:4206–4211.

26. Machala M, Ciganek M, Bláha L, Minksová K, Vondráček J. 2001. Aryl hydrocarbon receptor–mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azarenes originally identified in extracts of river sediments. *Environ Toxicol Chem* 20:2736–2743.

27. Legler J, Jonas J, Lahr J, Vethaak AD, Brouwer A, Murk AJ. 2002. Biological measurement of estrogenic activity in urine and bile conjugates with the in vitro ER-CALUX reporter gene assay. *Environ Toxicol Chem* 21:473–479.

28. Vondráček J, Machala M, Minksová K, Bláha L, Murk AJ, Kozubík, Hofmanová J, Hilscherová K, Ulrich R, Ciganek M, Neča J, Švrčková D, Holoubek I. 2001. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. *Environ Chem Toxicol* 20:1499–1506.

29. Gribble GW. 2000. The natural production of organobromine compounds. *Sci Pollut Res* 7:37–49.

30. Gribble GW. 1998. Naturally occurring organohalogen compounds. *Accounts of Chemical Research* 31:141–152.

31. Vethaak AD, Rijs GBJ, Schrap SM, Ruiter H, Gerritsen A, Lahr J. 2002. Estrogens and xenoestrogens in the aquatic environment of The Netherlands. Occurrence, potency and biological effects. RIZA/RIKZ-Report 2002.001. Dutch National Institute of Inland Water Management and Waste Water Treatment and the Dutch National Institute for Coastal and Marine Management, Den Haag, The Netherlands.

32. Smit MD, Leonards PEG, Madsen AB, van Hattum B. 1996. Bioaccumulation of PCBs in Danish otter habitats. Chapter 2. In Smit MD, Leonards PEG, Murk AJ, de Jongh AWJJ, van Hattum B, eds. *Development of Otter-Based Quality Objectives for PCBs.* Report E-96/11. Institute for Environmental Studies, Vrije Universiteit, Amsterdam, The Netherlands, pp 7–32.

33. Murk AJ, Legler J, Denison MS, Giesy JP, Van de Guchte C, Brouwer A. 1996. Chemical-activated luciferase gene expression (CALUX): A novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. *Fundam Appl Toxicol* 33: 149–160.

34. Klamer HJC, Leonards PEG, Lamoree MH, Bakker JF. 2002. Chemical and toxicological risk assessment of North Sea surface sediments. Brominated flame retardants and dioxin-type toxicity. *Organohalogen Compounds* 59:111–114.

35. Power DM, Llewellyn L, Faustino M, Nowell MA, Björnsson BT, Einarsdottir IE, Canario AVM, Sweeney GE. 2001. Thyroid hormones in growth and development of fish. *Comp Biochem Physiol C Pharmacol Toxicol* 130:447–459.

36. Schreiber G. 2002. Beyond carrier proteins. The evolutionary and integrative roles of transthyretin in thyroid hormone homeostasis. *J Endocrinol* 175:61–73.

37. Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman Å, Visser TJ. 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: Mechanisms and possible consequences for animal and human health. *Toxicol Ind Health* 14:59–84.

38. Kaiser KLE, Palabrica VS. 1991. Photobacterium phosphoreum toxicity data index. *Water Pollut Res J Can* 26:361–431.

39. Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS. 2000. Ah-receptor-based chemical screening bioassays: Application and limitations for the detection of Ah receptor agonists. *Toxicol Sci* 55:107–115.

40. Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, De Voogt P, Murk AJ, Van der Burg B. 2002. Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. *Environ Sci Technol* 36:4410–4415.

41. Meerts IATM. 2001. In vitro and in vivo interactions of organohalogens with the endocrine system—The role of metabolites and implications for human health. PhD thesis. Wageningen Agricultural University, Wageningen, The Netherlands.