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RESEARCH ARTICLE

The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by in vitro bioassays and chemical analysis

Stefanie Grund · Eric Higley · René Schönenberger · Marc J-F. Suter · John P. Giesy · Thomas Braunbeck · Markus Hecker · Henner Hollert

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Abstract
Introduction The present study was part of a comprehensive weight-of-evidence approach with the goal of identifying potential causes for the declines in fish populations, which have been observed during the past decades in the Upper Danube River.
Methods The specific goal was the investigation of the endocrine disrupting potential of sediment extracts from different sites along the Danube River. Parallel to the identification and quantification of target estrogens, two in vitro bioassays were employed to assess the estrogenic potential (yeast estrogen screen, YES) of the sediment samples and to evaluate their effects on the production of testosterone (T) and E2 (H295R Steroidogenesis Assay). Using a potency balance approach, the contribution of the measured compounds (Chem-EEQs) to the total endocrine

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S. Grund · T. Braunbeck · H. Hollert
Aquatic Ecology and Toxicology Section, Department of Zoology, University of Heidelberg,
Im Neuenheimer Feld 230,
69120 Heidelberg, Germany

E. Higley · J. P. Giesy · M. Hecker
Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan,
Saskatoon, SK, Canada

R. Schönenberger · M. J.-F. Suter
Eawag, Swiss Federal Institute of Aquatic Science and Technology,
Ueberlandstrasse 133, PO Box 611, 8600 Duebendorf,
Switzerland

J. P. Giesy
Department of Zoology, Michigan State University,
East Lansing, MI, USA

J. P. Giesy
Centre for Coastal Pollution and Conservation and Department of Biology and Chemistry, City University of Hong Kong,
Kowloon,
Hong Kong, SAR, China

J. P. Giesy
State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University,
Nanjing 210093, People's Republic of China

J. P. Giesy
State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University,
Xiamen, People's Republic of China

M. Hecker
ENTRIX, Inc,
Saskatoon, SK, Canada

H. Hollert
Department of Ecosystem Analysis, RWTH Aachen University, Institute for Environmental Research (Biology V),
Worringerweg 1,
52074 Aachen, Germany

S. Grund (✉)
Department of Zoology, Aquatic Ecology and Toxicology Section, University of Heidelberg,
Im Neuenheimer Feld 230,
69120 Heidelberg, Germany
e-mail: steffi.grund@web.de

activity measured by the YES (YES-EEQs) was calculated.
Results and discussion Of the nine sediment extracts tested five extracts exhibited significant estrogenic activities in the YES, which suggested the presence of ER agonists in these samples. The xenoestrogens nonylphenol (NP) and bisphenol A (BPA) and the natural estrogen estrone (E1) were detected while concentrations of 17β-estradiol (E2) and ethinylestradiol (EE2) were less than their respective limits of quantification in all sediment extracts. A comparison of the measured YES-EEQs and the calculated Chem-EEQs revealed that as much as 6% of estrogenic activity in extracts of most sediments could be explained by two xeno- and one natural estrogen. Exposure of H295R cells to sediment extracts from four different locations in the Danube River resulted in significantly increased concentrations of E2, but only slight inhibition of T synthesis. Furthermore, application of the H295R Steroidogenesis Assay provided evidence for endocrine disrupting potencies in sediment samples from the Upper Danube River, some of which were not detectable with the YES. In conclusion, differential endocrine activities were associated with several sediments from the Upper Danube River. Further investigations will have to show whether the observed activities are of biological relevance with regard to declines in fish populations in the Upper Danube River.

Keywords Endocrine disruptors · Sediment · Yeast estrogen screen · H295R Steroidogenesis Assay · Testosterone · Estradiol

1 Introduction

Over the last decade several studies have documented reproductive impairment of wild fish populations around the world (Sumpter and Johnson 2005). There is increasing evidence that local populations of both estuarine and freshwater fish may be exposed to endocrine-disrupting chemicals (EDCs) at concentrations sufficient to cause disruption of their reproductive physiology (reviewed by Jobling and Tyler (2003), Segner (2005)). Fish from a variety of contaminated ecosystems have been described as showing injury that was likely to be related to alterations in endocrine function, alterations in sexual development or altered fertility, fecundity, and reproductive behavior (Gross-Sorokin et al. 2006; Hecker et al. 2002; Jobling and Tyler 2003; Jobling et al. 2006; Toft and Guillette 2005; Vos et al. 2000). Endocrine disruption has a multitude of mechanisms and actions, and the effects may be attributed to various classes of chemicals. Therefore, identification of the chemicals responsible for these reproductive alterations is difficult and demands the development of specific procedures to analyze these compounds in complex environmental mixtures.

To date, much research has been focused on the effects of xenobiotics mediated via binding to the estrogen (ER) or androgen receptor (AR) or the cross-talk of these receptors with the arylhydrocarbon receptor (AhR; Villeneuve et al. (1998), Wilson et al. (2002; 2004)). However, several studies have demonstrated that some xenobiotics exert their effects on the endocrine system without acting as direct hormone mimics but via other mechanisms such as disrupting production of steroid hormones or steroidogenic enzymes (Hecker et al. 2006; Kime 1995; Rainey et al. 1993; Sanderson 2006; Staels et al. 1993). Thus, the screening for EDCs in complex contaminated matrices and their effects on endocrine systems on the basis of bio-analytical tools that only detect one mode of action (e.g., ER mediated effects) might not be sufficient to provide an objective assessment of a given exposure. Instead, a combination of in vitro assays with different endpoints and bio-analytical methods is recommended (Giesy et al. 2002).

The present communication is part of a comprehensive weight-of-evidence approach using multiple lines of evidence (Chapman and Hollert 2006) to identify potential factors that might be responsible for the decline in fish populations in the Upper Danube River reported during the last decade (Keiter et al. 2006; Keiter et al. 2008). Whereas there are still uncertainties regarding the direct contribution of sediment-related toxicants to these fish declines, previous investigations of sediments from this area by meta analyses and toxicity assays clearly revealed acute and specific toxic potential associated with certain local sediments (Grund et al. 2010; Keiter et al. 2006; Keiter et al. 2008; Otte et al. 2008; Seitz et al. 2008). Sediments are known to accumulate and retain many pollutants released by human activities, and are well known to have the potential to negatively affect aquatic organism (reviewed by Karlsson et al. (2008)). Thus, impacts on local fish populations due to the previously reported toxic potentials in certain areas of the Danube River cannot be excluded.

The present study focused on a detailed characterization of sediment samples from the Danube River in an attempt to determine their endocrine disrupting potential. Parallel to the instrumental identification and quantification of target estrogens (NP, BPA, E1, EE2, and E2) two in vitro bioassays were utilized to assess the potential interaction of sediment-associated contaminants with the ER and the production of the sex steroids T and E2.

The potential of sediment extracts to interact with the ER was assessed by use of the in vitro recombinant yeast estrogen screen (YES, Routledge and Sumpter (1996)), assisted by enzymatic digestion with lyticase (Schultis and Metzger 2004). By using the potency-balance approach, total estrogen equivalents determined by the YES assay were compared with the sum of the potencies of the individual compounds identified by chemical analysis in

order to estimate the degree as to which analyzed substances account for the biological effectiveness of environmental extracts. This strategy has been widely used for dioxin-like compounds (e.g., (Brack et al. 2005; Safe 1990; Van den Berg et al. 1998) and has also been adopted for estrogenic compounds (Giesy et al. 2002; Hilscherova et al. 2000; Hollert et al. 2005; Korner et al. 2000).

The potential of sediment extracts to interact with steroidogenic processes was assessed using the H295R Steroidogenesis Assay for investigating the effects on the production of the steroid hormones E2 and T (Hecker et al. 2006, 2007). The human H295R adrenocarcinoma cell line has been shown to be useful as an in vitro model to screen for xenobiotic effects on steroidogenic pathways and processes (Gracia et al. 2006, 2007, 2008; Hecker et al. 2006; Hecker and Giesy 2008; Hilscherova et al. 2004; Oskarsson et al. 2006; Sanderson 2006; Xu et al. 2006; Zhang et al. 2005). To the best of our knowledge, the present communication is the first application of the H295R Steroidogenesis Assay for screening the effects of contaminated sediment extracts on the production of steroid hormones.

The combination of chemical analyses and in vitro bioassays was used to evaluate the endocrine disrupting potential of sediment extracts from different sites along the Upper Danube River as an additional line of evidence in a weight-of-evidence framework towards determining the possible causes for the declines observed in certain fish populations. Finally, the present study was intended to validate the H295R cell line as an in vitro assay to screen for the alterations of hormone production caused by sediment extracts.

2 Materials and methods

2.1 Sediment samples

In 2006, near-surface bottom sediment samples (i.e. the first 10 cm) were collected at seven locations along the Upper Danube River as well as at two tributary streams just upstream of their confluence with the Danube River, by means of a van Veen-gripper or a stainless steel shovel. Sampling sites were chosen in accordance with suspected sediment contamination gradients (Grund et al. 2010; Keiter et al. 2008; Seitz et al. 2008), and/or because of their exposure to sewage treatment plant effluents (Fig. 1). Several sediment sub-samples (n=7–10) from each location were pooled, homogenized, freeze-dried and sieved (mesh size 1.25 mm) immediately after return to the laboratory. Dried and sieved sediments (20 g) were Soxhlet-extracted for 14 h with 400 mL of a 1:1 (v/v) mixture of acetone (Ac; p.a.; AppliChem, Darmstadt, Germany) and hexane (Hx; p.a.; Merck, Darmstadt, Germany) according to Hollert et al. (2005). A second set of sediment samples were subjected to an additional extraction with methanol (MeOH; p.a.; AppliChem), and both Ac/Hx,-extracted and MeOH-extracted samples were analyzed by LC-MS/MS. Elemental sulfur was removed by copper treatment. Finally, the solvent was changed to dimethyl sulfoxide (DMSO; Fluka, Buchs, Switzerland) for H295R bioassays or ethanol (p.a.; Merck) for the YES assay, and samples were stored at −20°C until testing. To investigate possible biological and chemical interference due to the solvents, empty extraction thimbles (with fiberglass) were subjected to extraction and assessed in all experiments as a process control.

2.2 Yeast estrogen screen

Samples were tested for receptor-mediated estrogenic activity using the in vitro recombinant YES transactivation assay assisted by enzymatic digestion with lyticase (L-YES; Schmitt et al. (2008), Schultis and Metzger (2004), Wagner and Oehlmann (2005)), a further development of the YES presented by Routledge and Sumpter (1996). The L-YES assay has been found to be a very good alternative to existing estrogenic in vitro assays since it has good sensitivity, is inexpensive and much faster than other assays such as the conventional YES assay, E-Screen assay and receptor binding-assay (Schultis and Metzger 2004).

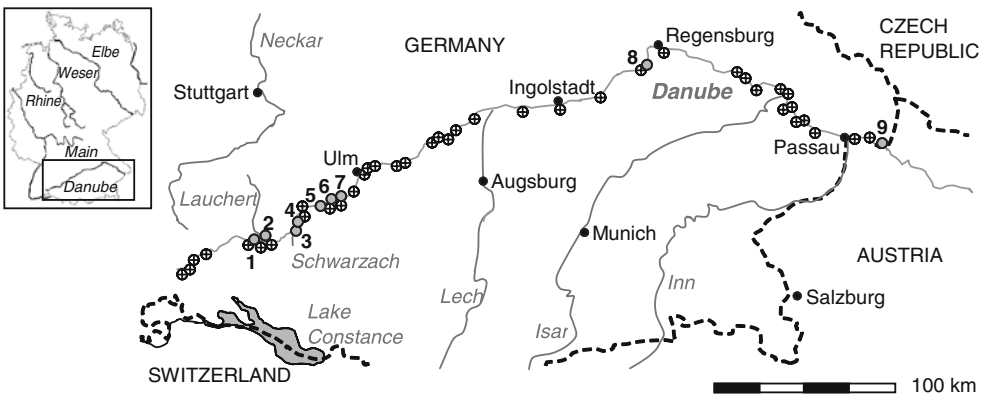

Fig. 1 Sampling sites along the Upper Danube River: *1* Sigmaringen, *2* Lauchert (tributary), *3* Riedlingen, *4* Schwarzach (tributary), *5* Rottenacker, *6* Ehingen, *7* Oepfingen, *8* Jochenstein, *9* Bad Abbach). ● Sampling sites. ⊕ Sewage treatment plants (>10,000 residents according to LFW (2005))

All experiments were conducted in 96-well cell culture plates (TPP; Renner, Trasadingen, Switzerland) and repeated three times for each sample. Ethanol (p.a.; Merck) was used as solvent at a final concentration of 0.1% *v/v*. On each plate 12 concentrations of each sediment extract were tested in quadruplicate, as well as blanks (assay medium without cells; each with 12 replicate wells) and three rows of solvent controls (SCs). Parallel to each experiment one plate including a blank, a solvent control and a full concentration range of the positive control 17β-estradiol (E2, 1 pM–1 nM; each with eight replicate wells) was run. After 24 h of incubation with the samples, the absorbance was measured at 595 nm. Then 100 μL of the lyticase stock solution containing chlorophenolred-ß-D-galactopyranoside were added to each well (for details see Wagner and Oehlmann (2005)), and absorbance was measured in five to seven intervals of 15 min at 540 nm. Absorbance was corrected for blank and cell number. The time point at which the concentration response relationship of E2 had the EC_{50}, the least absorption in the negative control and the greatest regression coefficient (r^2) was chosen as the optimum measurement. Visual observations for abnormal growth patterns or cell death as signs for cytotoxicity were made after the 24 h exposure period before measurements. Furthermore, first measurement at 595 nm gives information about the cell number in each well and, therefore, by comparison between the different concentrations, also about cytotoxicity. This data were used to check for cytotoxic effects as well.

2.3 Chemical analyses

Chemical analysis was performed based on a method published by Vermeirssen et al. (2005) using a HP Series 1100 high-performance liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an online vacuum degasser (DG4, Henggeler Analytic Instruments, Riehen, Switzerland), a binary high-pressure gradient pump, an auto-sampler kept at 10°C, a heated column compartment (23°C), and a UV detector monitoring 230 nm. The HPLC was coupled to an API 4000 triple quadrapole tandem mass spectrometer (Applied Biosystems, Rotkreuz, Switzerland), using electrospray ionization in the negative ion mode.

Preliminarily, a clean-up step was made for all sediment extracts using silica gel columns. The extracts were transferred onto the silica gel columns (1.00±0.01 g silica gel in 5 ml bottles) and the analytes were eluted by rinsing with 7.1 ml hexane:acetone (60:40). Separation of the target compounds was achieved on a C18 column (Waters X Terra, 3.5 μm; 2.1 mm×10 cm (Waters, Bad-Dättwil, Switzerland), combined with a C18 pre-column (Waters X Terra MS C18, 3.5 μm, 2.1×10 mm). 10 μl were injected onto the system. Eluent A was 10% acetonirile (ACN) in water, eluent B 90% ACN. A six-step gradient was performed with a flow rate of 0.25 mL/min. 100% Eluent A was kept for 2 min, decreased to 0% in 19 min, kept for 3 min at 0%; then initial conditions were re-established in 1 min and the column was re-equilibrated for 10 min. The total time per analysis was 35 min. The ionization efficiency, and hence the overall sensitivity, could be improved by post-column addition (micro-HPLC-pump, Bischoff GmbH, Germany) of a 2.5% ammonia solution at a flow rate of 30 μL/min. For the target analysis, the ions monitored were 269 to 145 for estrone, 271 to 145 for 17β-estradiol, 295 to 145 for 17α-ethinylestradiol, 227 to 212 for BPA and 219 to 133 for NP. For the limit of quantification (LOQ), a minimal signal-to-noise ratio of 10:1 was requested. The LOQs were highly matrix dependent. In sediment extracts LOQs averaged 0.4 ng/mL sediment extract for E1, 2.5 ng/mL for E2, 10.5 ng/mL for EE2, 28.0 ng/mL for NP and 13.5 ng/mL for BPA.

The isotope labeled steroids estrone-2,4,16,16-d_4 (C/D/N Isotopes Quebec, Canada), estradiol-3,4-^{13}C2 (Cambridge Isotope Laboratories (CIL), USA), 17α-ethinylestradiol-2,4,16,16-d_4 (CIL), *p-n*-nonylphenol ^{13}C6 (CIL), bisphenol A-d_{16} (Supelco, USA) were chosen as internal standards. Bisphenol A, estrone, 17β-estradiol, and 17α-ethinylestradiol were obtained from Sigma-Aldrich (Buchs, Switzerland). The 4-nonylphenol isomer mixture was obtained from Acros Organics (Belgium). The water used was Nanopure®-grade, all other solvents and reagents were HPLC-grade and provided by Merck (Darmstadt, Germany).

2.4 H295R steroidogenesis assay

2.4.1 Cell culture

NCI-H295R cells (ATCC, Manassas, VA, USA; Cat# CRL-2128) were cultured in medium supplemented with Nu-Serum (BD Bioscience; 355100) at 37°C under a 5% CO_2 atmosphere as described previously (Hilscherova et al. 2004). Briefly, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium with Ham's F-12 Nutrient mixture (DMEM/F12; Sigma D-2906; Sigma, St. Louis, MO, USA) supplemented with 1.2 g/L Na_2CO_3, 5 mL/L of ITS+Premix (BD Bioscience; 354352), and 12.5 mL/L of BD Nu-Serum (BD Bioscience) unless specified differently.

2.4.2 Preliminary tests (results not shown)

To ensure that modulations in hormone synthesis were not a result of cytotoxic effects, viability of the cells was assessed with the MTT bioassay (Mosman 1983) before initiation of exposure experiments. Only non-cytotoxic doses (>80%

viable cells per well) were evaluated regarding their potential to affect steroidogenesis. For hormone analyses, preliminary tests with sediment extracts from all sampling sites (at concentrations of 0.5, 2 and 5 mg SEQ/mL) and a process control (PrCo) were conducted. Sediment samples that caused an induction of E2 greater or equal to twofold relative to the SCs were chosen for further investigations.

2.4.3 Exposure experiments

Hormone analyses were performed according to the optimized H295R Steroidogenesis Assay protocol described previously (Hecker et al. 2007) with slight modifications. Briefly, all experiments were conducted in 24-well cell culture plates (TPP). One milliliter of cell suspension, at a concentration of approx. 300,000 cells/mL, was added to each well and the cells were allowed to attach for 24 h. After the attachment period, the medium was changed and the cells were exposed to the extracts for 48 h in the same 24-well plates. Dimethyl sulfoxide (DMSO) was used as a carrier solvent at a final concentration of 0.1% *v/v*. Five different concentrations of each sediment extract as well as a DMSO solvent control and a blank control were run in triplicate on each plate. Parallel to each experiment a quality control (QC) plate with a known inducer (forskolin, 1 μM) and inhibitor (prochloraz, 0.3 μM) was run as a performance control (Hecker et al. 2007). After the exposure period, the medium was removed for extraction and cell viability was assessed using the MTT bioassay to evaluate potential differences in the number of viable cells among wells (Mosman 1983).

2.4.4 Hormone analyses

Prior to hormone measurement medium was extracted twice with diethyl ether (Sigma, Deisenhofen, Germany). Total medium T and E2 concentrations were determined using commercially available enzyme-linked immunoassays (EIA-ELISA-Kits; Cayman Chemical Company, Ann Arbor, MI, USA; Testosterone: Cat# 582701, 17β-estradiol: Cat# 582251). Extracts of culture medium were diluted 1:2 and 1:5 for E2, and 1:50 and 1:100 for T, respectively. Each dilution was measured in duplicate. For relative increase/decrease evaluations, data were normalized to the mean SC value, and results were expressed as fold-change (FC) relative to the SC (FC=1 for the SC). Three independent experiments were conducted using cell passages between 5 and 7. All samples were analyzed for possible interferences with the antibody-based hormone detection assay prior to exposure of H295R cells to control for potential changes in hormone production as a function of components in the extracts directly interacting with the ELISA.

2.5 Statistical analyses

Statistical analyses of H295R Steroidogenesis Assay and YES data were conducted using SigmaStat 3.5 (SYSTAT Software Inc., Point Richmond, CA, USA). All data obtained with the H295R and the YES assays were expressed as mean±standard error of the mean (SEM). For evaluation of relative increases/decreases, results were normalized to the mean SC value for each assay, and results were expressed as fold-change relative to the SC (SC=1).

Prior to analysis, all data were tested for normality using the Kolmogorov–Smirnov test. When parametric assumptions were met, one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to determine which treatments differed significantly from the SCs. In cases where the data or transformed data did not conform to parametric assumptions a non-parametric Kruskall–Wallis test followed by Dunn's post-hoc test was used. Differences were considered significant at $p<0.05$.

For comparison and ranking of the endocrine disrupting effects of sediment extracts in both in vitro assays utilized in this study, the lowest observed effect concentration (LOEC) was determined for each sample. In addition, estrogenic potencies as measured by the YES were ranked by calculating E2 equivalent concentrations (YES-EEQ) for each sample. For this purpose, normalized data (relative to SC) were plotted as a function of the logarithm of concentrations, and effective concentration values (EC) were calculated by interpolation from E2 standard curves. Typically, only non-cytotoxic ranges of concentration-response curves were selected for calculation of EEQs. Due to increasing concomitant toxic activity in some sediment extracts, however, the concentration-response curves failed to reach the E2-EC_{20} level for some samples. Thus, YES-EEQs were calculated using EC_{10} values (Eq. 1).

$$\mathrm{YES-EEQ[ng/g]} = \mathrm{E2-EC_{10}[ng/L]/Sediment\ sample-EC_{10}[g/L]} \quad (1)$$

For comparison of the results of the YES assay and chemical analysis, and to estimate the contribution of target analytes to the overall estrogenicity of the original sediment samples, analytically derived E2 equivalents (Chem-EEQs) were determined. This was done by multiplying concentration data (c) obtained by LC–MS/MS analysis with relative estrogenic potencies (REPs) determined in former studies with the modified YES assay (Schultis and Metzger 2004) and YES (Beck et al. 2006) assays (Table 1; Eq. 2).

$$\Sigma\ \mathrm{Chem-EEQ}(i) = \Sigma\ \mathrm{REP}(i) \times \mathrm{c}(i) \quad (2)$$

Chem-EEQs were calculated only for the estrogen concentrations measured in Ac/Hx-extracted samples, since

Table 1 Total concentrations of target estrogens in sediment extracts from the upper Danube River measured by LC-MS/MS (-ESI) and their relative estrogenic potencies (REPs) determined with the yeast estrogen screen (YES) in previous studies

[ng/g SEQ]	NP		BPA		E1		EE2		E2
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a/b*
Sigmaringen	6.5	4.1	15	11	0.12	0.053	<LOQ	<LOQ	<LOQ
Lauchert	138	84	1.2	1.2	0.14	0.051	<LOQ	<LOQ	<LOQ
Riedlingen	<LOQ	2.3	6.0	0.78	0.053	<LOQ	<LOQ	<LOQ	<LOQ
Schwarzach	<LOQ	<LOQ	11	7.1	0.15	0.037	<LOQ	<LOQ	<LOQ
Rottenacker	150	270	7.0	6.8	0.097	0.19	<LOQ	<LOQ	<LOQ
Ehingen	801	881	22	6.2	0.019	0.037	<LOQ	<LOQ	<LOQ
Oepfingen	<LOQ	1.4	6.2	2.6	0.098	0.076	<LOQ	<LOQ	<LOQ
Jochenstein	<LOQ	5.9	6.5	2.7	0.12	0.15	<LOQ	0.8	<LOQ
Bad Abbach	1364	1011	8.6	12	0.24	0.095	<LOQ	<LOQ	<LOQ
PrCo	26	22	1.8	1.6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
REPs	0.000011*		0.00012*		0.13*		0.73*		1*

SEQ sediment equivalent, *a* sample extracted with acetone: hexane (Ac/Hx, 1:1 *v/v*). *b* sample extracted with methanol (MeOH), *<LOQ* less than limit of quantification, *PrCo* process control, *REP* relative estrogenic potency according to *Schultis and Metzger (2004) and **Beck et al. (2006), respectively

the extracts used in the in vitro assays were also extracted with Ac/Hx.

3 Results

3.1 Characterization of ER agonists in Danube sediments by LC-MS/MS

The xenoestrogens NP and BPA as well as the natural estrogen E1 were frequently detected in sediment samples collected at the sampling sites along the Upper Danube River (Table 1). Greatest concentrations of NP and BPA were observed in sediments from the sites at Bad Abbach, Ehingen, and Rottenacker. Furthermore, relatively great concentrations of BPA were also measured at Sigmaringen and Schwarzach. Low concentrations of NP and BPA were also detected in the process control, which might be caused by contamination of the filters used for Soxhlet extraction. The greatest concentration of E1 was measured in the sediment extracts collected from the site at Bad Abbach. The exposure scenarios were typically dominated by NP, which was measured in sediment extracts at up to 160- and 40,000-fold greater concentrations, if compared to BPA and E1, respectively. Concentrations of the synthetic estrogen EE2 and the natural estrogen E2 were less than the limits of quantification with the exception of EE2 concentrations (0.80 ng/g SEQ) in the methanol-extracted sample from the location at Jochenstein.

Generally, distinct differences in the concentration of the analyzed target compounds were observed between the methanol- and acetone:hexane-extracted sediment samples. The differences suggest acetone:hexane to be a more effective solvent for extraction of BPA and E1 in sediment samples while no such trend could be observed for NP.

3.2 Yeast estrogen screen

3.2.1 Sediment extracts ER-agonist potencies

Generally, yeast colonies incubated with the majority of sediment extracts exhibited either abnormal growth patterns or cell death after exposure to elevated sediment extract equivalents (results not shown). This suggested that one or more compounds in the extracts exerted acutely cytotoxic effects on the yeast cells and effectively inhibited growth of the yeast. For evaluation of the YES data, cytotoxic doses were excluded. Furthermore, in s006Fme of the SC wells directly adjacent to exposure wells a slightly increased estrogenic effect was observed, which was probably caused by cross-contamination from adjacent samples containing wells (data not shown). Consequently, only the SC wells of the upper row (not located next to any exposure wells) were used for data analysis.

Of the nine sediment samples tested, five extracts caused a significant estrogenic response in the YES assay. Sediment extracts collected from the sites at Bad Abbach, Ehingen, Riedlingen, Jochenstein, and Sigmaringen induced a concentration-dependent response with fold-changes being significantly greater than the SCs at sediment concentrations ≥6.25 mg SEQ/mL (Sigmaringen; SEQ= sediment equivalent), 12.5 mg SEQ/mL (Bad Abbach), 25.0 mg SEQ/mL (Riedlingen) and 50.0 mg SEQ/mL (Ehingen and Jochenstein; Fig. 2). Conversely, no significant activities were observed in sediment extracts from the sites at Lauchert, Oepfingen, Rottenacker, Schwarzach (data not shown), as well as in the process control (Fig. 2). With a maximum induction of 2.7-fold, E2 proved to be an effective positive control for producing strong dose-dependent estrogenicity. Generally, the estrogenic responses in yeast cells after exposure to various sediment extracts as well as to E2 were characterized by a relatively

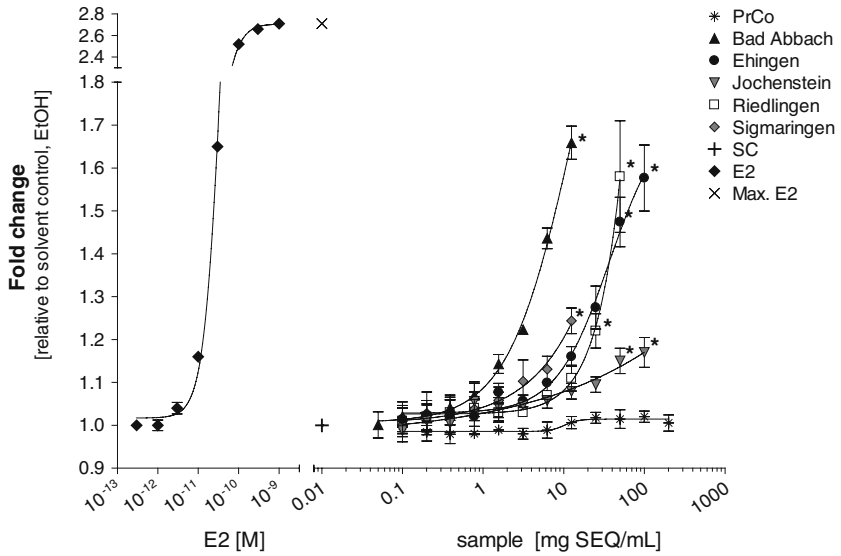

Fig. 2 Estrogenic activities of sediment extracts from the Upper Danube River (*right panel*) compared to the dose–response curve of 17β-estradiol (*E2*, *left*) measured by the yeast estrogen screen (YES). Only the results for sediment extracts that showed significant estrogenic activities as well as for the process control (PrCo) are shown. Cytotoxic doses were excluded. The estrogen equivalency is expressed as fold change compared to solvent control (SC=1). Data are given as means±SEM from three independent exposures. Significant differences between treatment concentrations and SCs are indicated by asterisks (*$p<0.05$). *SEQ* sediment equivalent

small variation among experiments (coefficients of variation <10%).

Maximum fold change (1.7) and maximum YES-EEQ (1.3±0.20 ng EEQ/g SEQ) as well as a low LOEC (12.5 mg SEQ/mL) were determined for the extract from the site at Bad Abbach (Figs. 2 and 3). A LOEC of 6.25 mg SEQ/mL, and a YES-EEQ of 0.89±0.16 ng EEQ/g SEQ were observed in the sediment extracts from the site at Sigmaringen. Samples collected at Ehingen and Riedlingen also revealed relatively great fold-changes (1.6) but LOECs (50 and 25 mg SEQ/mL) and YES-EEQs (0.25±0.02 and 0.18±0.03 ng EEQ/g SEQ) were less than those reported for extracts from Bad Abbach and Sigmaringen. The least estrogenicity that was significantly different from the

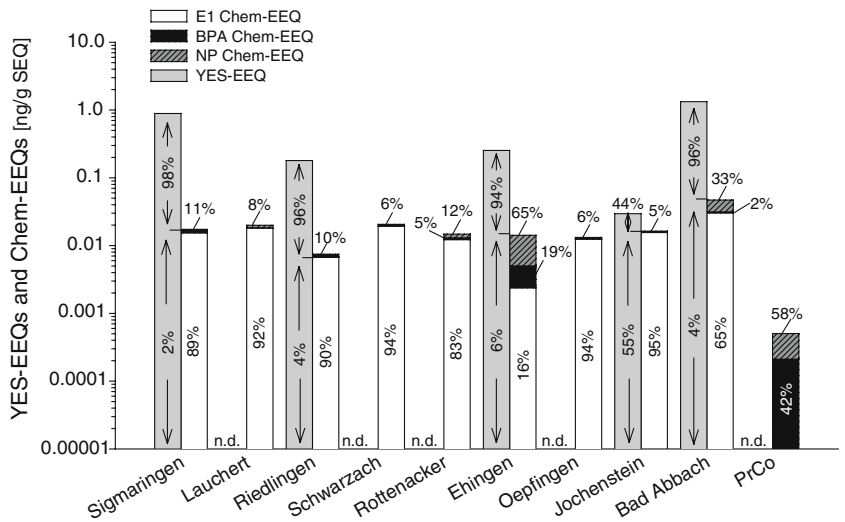

Fig. 3 Comparison of 17β-estradiol equivalent concentrations measured in the yeast estrogen screen (*YES-EEQ; gray bars*), the Chem-EEQs derived by means of chemical analyses (*stacked bars*) as well as the known and unknown portion (*in percent*) of the overall activities regarding the YES-EEQs and the Chem-EEQs in sediment extracts from the Upper Danube River. White, diagonally striped and black bars represent the contribution (%) of nonylphenol (*NP*), bisphenol-A (*BPA*) and estrone (*E1*), respectively, to the calculated overall estrogenicity. Chem-EEQs of target analytes were calculated by multiplying the concentration of analyzed estrogens (extracted with acetone:hexane (1:1, *v/v*)) with the corresponding relative estrogenic potencies taken from literature (cf. Table 1). *n.d.* YES-EEQ was not detectable

controls was observed for the sediment sample from the site at Jochenstein (YES-EEQ=0.03±0.004 ng EEQ/g SEQ; fold change=1.2; LOEC=50 mg SEQ/mL).

3.2.2 Comparison of calculated E2-equivalents

By comparison of the Chem-EEQs for each target compound to the sum of the Chem-EEQs it became evident that, with the exception of the site at Ehingen, the natural estrogen E1 was determined to be the major contributor to the overall estrogenicity calculated from chemical data (Fig. 3). Measured concentrations of E1 accounted for more than 60% of the sum of Chem-EEQs in almost all samples. While present at greater concentrations, the xenoestrogens NP and BPA accounted for less than 12% of the sum of Chem-EEQs in all samples except for the PrCo (NP=58% and BPA=42%) and the sediment extracts from the site at Ehingen (NP=65% and BPA=19%) and Bad Abbach (NP=33%) due to their considerably lesser estrogenic potencies.

3.2.3 Comparison of measured and calculated E2-equivalents

A comparison of the measured YES-EEQs and the calculated Chem-EEQs revealed that as much as 6% of estrogenic activity in extracts of most sediments could be explained by the analyzed target estrogens (Fig. 3). The percentage contribution of the calculated Chem-EEQs to the overall estrogen equivalents measured with the YES assay was between 2% and 6% except for the sediment extract from the site at Jochenstein, for which the analyzed target estrogens could explain 55% of the measured estrogenic potential. About 94% of the measured overall activities have to be attributed to unknown components in almost all sediment extracts.

3.3 H295R steroidogenesis assay

Based on the results of preliminary tests with sediment extracts from all sampling sites, the samples collected at the sites at Riedlingen, Rottenacker, Oepfingen, and Bad Abbach were further assessed for their potentials to interfere with hormone production in H295R cells. No significant adverse effects on cell viability were observed for any of the sediment extracts tested except for the sample from location at Oepfingen. A significant decrease in cell viability (67% viable cells) was determined after exposure to the greatest concentration (8 mg SEQ/mL medium) of this sediment extract.

Exposure of H295R cells to five concentrations of sediment extracts from four different locations at the Danube River resulted in significant changes in the production of E2 and slight alterations in T synthesis (Fig. 4).

E2 concentrations increased in a concentration-dependent manner, with concentrations being significantly greater than the SCs at sediment concentrations greater or equal to 5.0 mg SEQ/mL at the site at Rottenacker and greater or equal to 3.0 mg SEQ/mL at all other sites, respectively. Maximum induction for all sediments occurred at the greatest concentration tested with fold-changes between 2.6 (Riedlingen) and 4.2 (Oepfingen). Furthermore, variation in E2 production among the experiments was below 20% (coefficient of variation) for responses of all samples except for the effects of the Oepfingen extract at sediment concentrations of 3.0 and 5.0 mg SEQ/mL (CV=

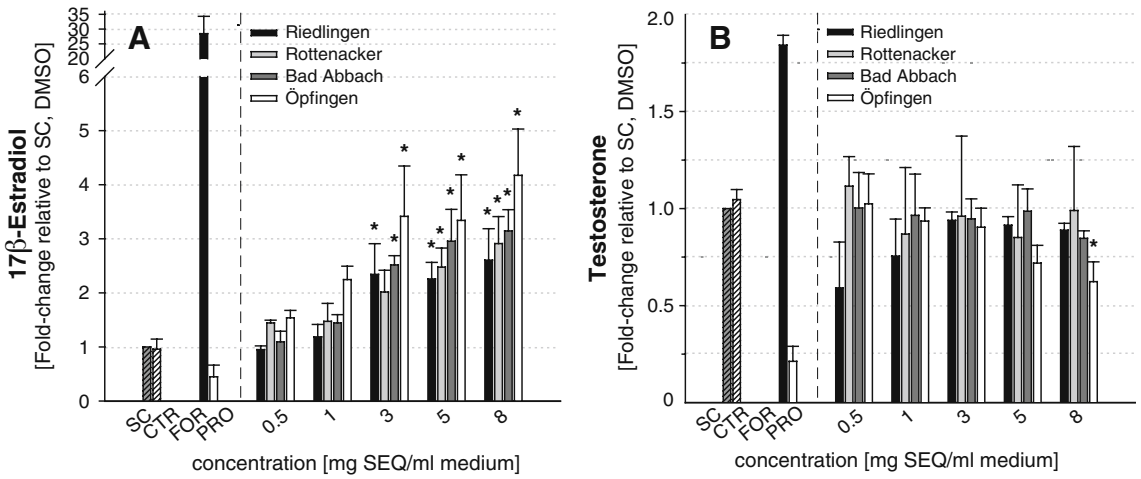

Fig. 4 Changes in 17β-estradiol (**a**) and testosterone (**b**) production by H295R cells after exposure to sediment extracts of the Upper Danube River expressed as relative changes compared to the solvent control (*SC*). Data are given as means of three independent experiments±SEM. *CTR* Blank. *FOR* forskolin [1 μM]. *PRO* prochloraz [0.3 μM]. *SEQ* sediment equivalent. *$p<0.05$. Significant effects at the greatest concentration of the Öpfingen sediment extract (8 mg SEQ/mL) have to be regarded with care as significant decrease in cell viability was determined at this concentration

27% and 25%) and the Riedlingen extract at 3.0 mg SEQ/mL (CV=24%).

No marked effects on T concentration were observed after exposure to the sediment extracts from Riedlingen, Bad Abbach, and Rottenacker. However, increasing concentrations of the sample collected at Oepfingen resulted in a dose-dependent decrease of T production by H295R cells. The changes in T production were significant at the greatest concentration of 8.0 mg SEQ/mL only, though at which a significant decrease in cell viability was determined. There were no significant differences in T and E2 concentrations between the blank (non-treated cells) and SCs. Furthermore, none of the sediment extracts tested did directly interfere with the hormone detection assays (results not shown).

4 Discussion

4.1 Receptor-mediated estrogenic activity measured by the YES assay

The results of the YES bioassay confirm the presence of ER agonists in five out of nine sediment samples investigated along the Upper Danube River. The results for the samples that caused no significant effects in the YES assay suggest cytotoxic effects at sediment concentrations greater than 6.25 mg SEQ/mL that might mask potential ER agonist potencies of these extracts. Furthermore, the presence of anti-estrogenic compounds needs to be considered as possible reason for the lack of response in some of the extracts (Legler et al. 2002). The masking effects of antagonistic compounds being present in complex chemical mixture has been demonstrated and discussed (Weiss et al. 2009) and the presence of anti-estrogenic compounds, e.g., polycyclic aromatic hydrocarbons (PAHs) are very likely in river sediment extracts (Santodonato 1997). Thus, the estrogenic potentials of the sediments from the sites at Lauchert, Schwarzach, Rottenacker, and Oepfingen could not be assessed, and further analysis, such as the utilization of fractionation techniques, are recommended to separate cytotoxic and/or antagonistic effects from potential endocrine disrupting activities in these samples (Brack et al. 2005; Luebcke-von Varel et al. 2008).

The E2 equivalents (YES-EEQs) of the samples that revealed a positive receptor-mediated response were between 0.03 and 1.3 ng EEQ/g SEQ. These values are comparable to YES-EEQs reported by other studies including sediments collected from United Kingdom rivers with 0.20–13 ng EEQ/g SEQ (Thomas et al. 2004) and estuaries with 0.021–0.03 ng EEQ/g SEQ (Peck et al. 2004) as well as to E2-equivalents measured during the analysis of sediments from Dutch inland and estuarine waters using the ER-mediated chemically activated luciferase gene expression assay (ER-CALUX), which ranged from 0.10 to 1.2 ng EEQ/g SEQ (Legler et al. 2003). However, sediment YES-EEQs reported in our study are much greater than those reported in another study including sediments from Dutch inland and estuarine waters with 0.005 and 0.34 ng EEQ/g SEQ (Houtman et al. 2006).

4.2 Characterization of ER agonists in sediments by combination of LC-MS/MS and YES

The chemical analyses revealed the presence of elevated concentrations of the xenoestrogens NP and BPA and the natural estrogens E1 in Danube River sediments. In addition, EE2 was detected in the sediment sample from the site at Jochenstein, but only in the sample extracted with MeOH. Conversely, concentrations of the natural estrogen E2 were less than the LOQ in all sediments. Total concentrations of NP, BPA, and E1 were comparable to concentrations of these estrogens found in river sediments of more industrialized regions in the United Kingdom (Peck et al. 2004), Italy (Vigano et al. 2006; Vigano et al. 2008), Germany (Heemken et al. 2001), US and Canada (Bennie 1999) as well as Korea (Khim et al. 1999).

Comparison of data from chemical analyses with the in vitro results of the YES showed that these three compounds contributed to as much as 6% to the overall estrogenic activity measured with the YES for most of the sediment samples. An exception was the extract the sediment collected at Jochenstein. In this sediment, the analyzed target estrogens could explain 55% of measured estrogenic potential whereby E1 contributed to 53% to the measured effect in the YES. Conversely, the calculated Chem-EEQs of the sediment samples from the sites at Lauchert, Schwarzach, Rottenacker, and Oepfingen would suggest an estrogenic potential in these samples, but no significant ER agonist activity could be determined with the YES. These discrepancies support the speculation that cytotoxic and/or anti-estrogenic contaminants are present in these samples, which might mask potential estrogenic effects. In general, the calculation of the contribution chemically analyzed compounds to the full potency and the confirmation step within effect-directed analysis and are today one of the largest challenges due to the complexity of environmental samples (Brack et al. 2007). In this study no fractionation to simplify the matrix was performed and hence are all the amount of known and unknown compounds contributing (or masking) the endocrine disrupting potency.

Overall, the majority of the in vitro bioassay responses remains uncharacterized and seems to be related to other compounds not analyzed in this study which may be associated with Danube River sediments. The inability to

account for the biological effectiveness may also be explained by the low sensitivity of this analytical approach, which suggest that estrogens with high endocrine potencies (e.g., EE2 and E2) could be present in the sediments at concentrations less than the LOQ but in quantities potentially contributing to the positive response in the biosassay (Hollert et al. 2005). Assuming that the estrogens E2 and EE2 were present in the extracts at concentrations close to their detection limits, a maximum of 42% and 59% of the biological effectiveness of the more polluted samples from the sites at Bad Abbach and Sigmaringen, respectively, could be explained. It is, however, important to note that the Chem-EEQs of the less contaminated samples from the sites at Riedlingen, Ehingen, and Jochenstein would also increase to a multiple of the YES-EEQs, and the real biological effectiveness of the YES-EEQs would be overestimated by the calculated Chem-TEQs. Furthermore, the discrepancies between chemical and biological analyses might also be explained by additive or even synergistic effects of the estrogenic compounds as well as matrix interference (Giesy et al. 2002; Gomes et al. 2004). Previous studies reported some difficulties associated with LC–MS/MS analysis for detecting estrogens in complex sample matrices (Aerni et al. 2004; Hirobe et al. 2006; Mauricio et al. 2006). In fact, the presence of co-extracted substances in extracts of complex matrices such as sediments can produce signal suppression in LC-MS/MS analysis with electron spray ionization, as has been described for E1, E2, and EE2 (Gomes et al. 2004; Ingrand et al. 2003; Schluesener and Bester 2005). In conclusion, the results corroborates the view that even the use of most comprehensive chemical analyses only explains a limited fraction of the biologically effective endocrine disrupting potential. Thus, bioassays are definitely indispensable for the evaluation of the endocrine disrupting potential of environmental samples (Ankley et al. 1998; Islinger et al. 1999; Matthiessen and Sumpter 1998).

4.3 Non-receptor-mediated endocrine disrupting activities applied by the H295R steroidogenesis assay

Our study revealed significant up-regulations of E2 production after exposure to the sediment extracts of sediments from four sampling sites as well as slight down-regulations of T concentration in H295R cells for one extract. Whereas significant effects (decrease) in T hormone production were observed only for the greatest concentration of the Oepfingen extract at which also a significant decrease in cell viability was determined, significant concentration-dependent increases in E2 hormone production were observed for all four samples investigated. Thus, E2 production was generally more sensitive to chemical disturbance than that of T, which may be a result of greater basal production of T than E2 in H295R cells (Hecker et al. 2006). Furthermore, E2 is the final product of the steroidogenesis pathway, whereas T only represents an intermediate product. Thus, it is possible that changes in T can be better compensated by the cells than those in E2, e.g., after exposure to chemicals that might interact with steroidogenesis at the level of aromatase.

To the best of our knowledge, this study is the first to focus on the modulation of steroid hormones in H295R cells by sediment extracts. Effects of extracts from coastal marine areas and from two major waste water treatment plants in the vicinity of the city of Hong Kong on the production of E2 and T using the H295R cell bioassay were investigated by Gracia et al. (2008). Even though these areas where characterized by elevated contamination as the result of public waste filling, marine disposal, sewage discharges, and intense aquaculture, only one of the 22 marine samples had a statistically significant effect on the production of E2. Furthermore, none of the extracts significantly affected T production. The maximum effect was a 3.6-fold induction of E2, which is comparable to the maximum effect determined in our study for the Oepfingen extract (4.1-fold induction). Up-regulation of E2 production with similar fold changes in H295R cells have been reported in literature for a binary mixture of ethinylestradiol and trenbolone (3.77-fold; Gracia et al. (2007)) and to a polybrominated diphenyl ether (3.3-fold; He et al. (2008)). Greater E2 fold-inductions were only found for the model chemical forskolin (FOR; 7- to 21-fold; Gracia et al. (2006), Hecker et al. (2006), Watanabe and Nakajin (2004)), for the herbicide prometon (sixfold; Villeneuve et al. (2007)), and for the binary mixture of forskolin and ketoconazole (15-fold; Gracia et al. (2006)). Consequently, our results indicate the presence of compounds in sediments from the sites Riedlingen, Rottenacker, Oepfingen, and Bad Abbach, which are able to strongly interfere with steroidogenic pathways and cause comparatively great changes in steroid hormone production. Furthermore, potential endocrine disrupting activities might be masked by cytotoxic and/or antagonistic effects that occurred at greater sediment concentrations at the site at Oepfingen, and thus, it may be speculated that higher concentrations of the sediment extracts would result in even greater effects on hormone synthesis. For verification of this issue, further investigations such as the utilization of fractionation techniques are required to separate cytotoxic effects from potential endocrine disrupting activities (Brack et al. 2005; Brack et al. 2007; Hollert et al. 2005).

A possible explanation for the measured up-regulation of E2 production might be a stimulation of the expression of steroidogenic genes, especially of the CYP19 gene, which regulates the production of the aromatase enzyme respon-

sible for converting T to E2. Previous studies suggest a relationship between increased E2 production and accompanying up-regulation in the expression of CYP19 and/or aromatase activity in H295R cells by forskolin (Gracia et al. 2006; Hecker et al. 2006; Watanabe and Nakajin 2004), and PCB 126 (Li 2007). For the effects of the Oepfingen extract, this speculation is supported by the fact that increasing E2 concentrations were accompanied by a slight decrease of T production. However, given the complex regulatory mechanisms controlling steroidogenesis, there are multiple points along the steroid synthesis pathway at which steroid production can be affected. For example, alternative steroidogenic pathways such as the conversion of estrone (E1) to E2 via 17β-hydroxysteroid dehydrogenase (Poutanen et al. 1995) may also have contributed to the overall production of E2. However, such scenarios remain speculative until the samples are characterized by further analysis, e.g., via measurement of effects on expression of steroidogenic genes and enzymes or combinations of effect-directed analysis and gene expression alterations.

Nevertheless, the H295R Steroidogenesis Assay proved to be a useful tool for determining endocrine disrupting potencies in sediment samples from the Upper Danube River, some of which were not detectable with the YES. Whereas the sediment extracts from Riedlingen and Bad Abbach showed significant effects in both the YES and the H295R assay, the YES alone would have indicated no endocrine disrupting potential in the samples from the sites at Rottenacker and Oepfingen, which showed significant effects in the H295R assay. Thus, our results clearly emphasize the utility of the combination of in vitro bioassays based on both receptor- and non-receptor mediated mechanisms for a comprehensive evaluation of the endocrine disrupting potentials of complex environmental samples.

4.4 Proposals of potential identity, sources and environmental effects of endocrine disrupters in Danube river sediments

In addition to the estrogens analyzed in the present study, a large number of environmental chemicals have been shown to act as EDCs via different modes of action. In a previous study within the weight-of-evidence approach for investigation of the fish decline in the Upper Danube River, PAHs, polychlorinated dibenzodioxins, and dibenzofurans as well as polychlorinated biphenyls were identified in sediment samples taken at comparable locations along the Danube River (Keiter et al. 2008). These compounds have repeatedly been shown to affect steroidogenic processes in H295R cells (Blaha et al. 2006; Li and Wang 2005; Li 2007; Sanderson et al. 2001; Villeneuve et al. 2007; Xu et al. 2006), as well as to interact with the ER (Clemons repeatedlyet al. 1998; Garcia-Reyero et al. 2005; Giesy et al. 2002; Ulrich and Stahlmann 2004). Thus, the presence of many other estrogenic compounds at high or low concentrations, with strong or weak potencies are all contributing to the response determined with the H295R Steroidogenesis Assay and the YES in the present study. More examples of compounds present and with estrogenic potency are, e.g., PBDEs and phthalates, (Legler et al. 2002; Meerts et al. 2001; Weiss et al. 2009).

Xenoestrogens as well as natural and synthetic estrogens enter the aquatic environment not only through the effluents of sewage treatment plants, but also through multiple other point and diffuse sources. Furthermore, they can be transported in a few hours over tens of kilometers (Holthaus repeatedlyet al. 2002; Kjaer et al. 2007; Peck et al. 2004; Wang et al. 2003), making an estimation of potential sources difficult. For example, Keiter et al. (2006) investigated sewage treatment effluent extracts from similar sites along the Danube River with the YES. Interestingly, reported E2-EQs (0.45–2.3 ng/L) were up to 586-fold lower than the estrogenic activity (YES-EEQs) measured in the sediments investigated in the present study. This discrepancy indicates the accumulation of EDCs in sediments, which is in consistent with several studies which have shown that surface sediments are frequently a sink for EDCs in riverine environments (Blaha et al. 2006; Labadie et al. 2007; Liu et al. 2004; Peck et al. 2004; Petrovic et al. 2002; Vigano et al. 2006; Williams et al. 2003). Furthermore, recent studies have shown that not only sediments, but also periphyton and invertebrates can accumulate natural estrogens and their mimics (Maenpaa and Kukkonen 2006; Peck et al. 2007; Takahashi et al. 2003; Vigano repeatedlyet al. 2006). These organisms can variably contribute to the diet, and thus, the exposure of fish and other species (Pedersen et al. 2003; Pickford et al. 2003; Stewart et al. 2001). Therefore, the accumulation of EDCs in Danube sediments suggest that bottom-dwelling organisms as well as fish populations might be simultaneously exposed to elevated concentrations of EDCs (Sumpter and Johnson 2005).

Relatively few studies have investigated the endocrine disrupting potentials of river sediments and their effects on aquatic organisms. While the results of the in vitro assays applied in this study do not allow direct extrapolation to physiological in vivo responses, the results of the YES assay documented the presence of estrogenic compounds with the ability to bind to the ER-receptor in Danube sediments. Detrimental reproductive impacts in fish, which have been linked to the presence of ER agonists included feminization, induction of intersex, modulation of immune function and hormone levels as well as reduced gamete production and fertilization capability (Filby et al. 2007;

Gercken and Sordyl 2002; Jobling et al. 1998; Kidd et al. 2007; Liney et al. 2006; Sumpter and Johnson 2005; Vigano et al. 2008). The YES-EEQs measured in sediments collected from two United Kingdom rivers (0.021 and 0.03 ng EEQ/g SEQ), where male wild roach (*Rutilus rutilus*) populations exhibited a high proportion of intersex pathologies and increased plasma vitellogenin (VTG) concentrations (Peck et al. 2004) are considerably lower (up to 60-fold) in comparison to the YES-EEQs determined for sediment samples from the Danube River in the present study (0.03 and 1.3 ng EEQ/g SEQ). Intersex has also been observed for wild barbel (*Barbus plebejus*) in the River Po in Italy (Vigano et al. 2001). The estrogenic activities, expressed as YES-EE2 equivalents, measured in sediments from a tributary to the River Po ranged from 1.9 to 15.6 ng EE2EQ/g SEQ (Vigano et al. 2008). Comparing EE2 with E2 equivalents is not misleading, because the two estrogens basically have similar potency in the YES (Beck et al. 2006; Murk et al. 2002). Much lower estrogenic activities than those measured in our study were reported for sediment samples from Dutch inland and estuarine waters, where wild male bream (*Abramis brama*) have shown evidence of hermaphroditism, as well as increased levels of plasma VTG (Vethaak et al. 2005). Estrogenic activities measured in sediments from these locations using the ER-CALUX assay ranged from 0.005 to 0.34 ng/g SEQ (Houtman et al. 2006), and from 0.10 to 1.2 ng/g SEQ (Legler et al. 2003), respectively. Likewise, European flounder (*Platichthys flesus*) fed on mussels held in the River Tees, a United Kingdom river with high estrogenic potential (13 ng EEQ/g SEQ), showed a tenfold increase in plasma VTG (Matthiessen et al. 2002).

Furthermore, in fish, evidence comes from different types of data that not only the treatment with exogenous estrogen, but also alterations in the activity of steroidogenic enzymes and subsequent effects on endogenous estrogens can affect sex differentiation (reviewed by Piferrer and Guiguen (2008)). Given the importance of steroid sex hormones regulating reproduction in vertebrates and numerous other processes that are related to development and growth (Norris 1997), changes in hormone production represent a biologically relevant endpoint. Thus, the determined potential to alter steroid hormone production in H295R cells in sediments from the Danube River might result in disruptions that could adversely affect development, growth, and/or reproduction in fish.

5 Conclusions

The results of our study indicate that combination of the YES and H295R Steroidogenesis Assays and chemical analyses is a powerful tool to determine differential endocrine disrupting potentials as well as the nature of some of the responsible contaminants in sediment extracts. Both in vitro assays clearly demonstrated comparatively elevated endocrine disrupting potentials in selected sediments from the Upper Danube River and subsequently confirm an accumulation of EDCs in sediments, which can affect the endocrine system via receptor-mediated as well as non-receptor-mediated mechanisms. Therefore, the sediments at some sites along the Danube River might pose a potential risk to benthic and developing fish as well as to aquatic biota. However, the relevance of determined endocrine disrupting potencies in sediments for the aquatic fauna, and in a broader sense for the decline of fish populations observed is currently unknown und further studies will have to focus on: (1) investigations of potential signs of endocrine disruption in wild fish from the Upper Danube River (e.g., plasma vitellogenin (VTG) synthesis, plasma sex steroid levels, histopathological alterations in gonads); (2) chemical identification of EDCs in sediments via bioassay-directed fractionation; (3) determination of specific mechanism(s) of action of responsible EDCs, and (4) establishment of cause–effect relationships.

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