Anal Bioanal Chem (2004) 378 : 688–696
DOI 10.1007/s00216-003-2276-4

ORIGINAL PAPER

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Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents

Received: 27 May 2003 / Revised: 25 August 2003 / Accepted: 4 September 2003 / Published online: 22 October 2003

Abstract Five wastewater treatment plant effluents were analyzed for known endocrine disrupters and estrogenicity. Estrogenicity was determined by using the yeast estrogen screen (YES) and by measuring the blood plasma vitellogenin (VTG) concentrations in exposed male rainbow trout (*Oncorhynchus mykiss*). While all wastewater treatment plant effluents contained measurable concentrations of estrogens and gave a positive response with the YES, only at two sites did the male fish have significantly increased VTG blood plasma concentrations after the exposure, compared to pre-exposure concentrations. Estrone (E1) concentrations ranged up to 51 ng L^{-1}, estradiol (E2) up to 6 ng L^{-1}, and ethinylestradiol (EE2) up to 2 ng L^{-1} in the 90 samples analyzed. Alkylphenols, alkylphenolmonoethoxylates and alkylphenoldiethoxylates, even though found at µg L^{-1} concentrations in effluents from wastewater treatment plants with a significant industrial content, did not contribute much to the overall estrogenicity of the samples taken due to their low relative potency. Expected estrogenicities were calculated from the chemical data for each sample by using the principle of concentration additivity and relative potencies of the various chemicals as determined with the yeast estrogen screen. Measured and calculated estradiol equivalents gave the same order of magnitude and correlated rather well (R^2=0.6).

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Keywords Endocrine disruption · Fish exposure · Yeast estrogen screen · Vitellogenin · Steroid hormones · Nonylphenol ethoxylates

Introduction

Endocrine-disrupting chemicals (EDCs) in the aquatic environment have become an increasingly important issue for scientists and regulatory bodies. As early as 1970, Tabak and Bunch were investigating the risk posed by natural urinary and especially synthetic ovulation-inhibiting hormones [1, 2], work that was later taken up by other groups [3, 4, 5, 6, 7]. The impact of the steroid hormones on the environment is highlighted by the fact that concentrations as low as 0.1 ng L^{-1} of ethinylestradiol [8] and 1–10 ng L^{-1} of estradiol [9] induce vitellogenesis in male rainbow trout. This high potency at low concentrations necessitates understanding of the fate and effects of these compounds in the aquatic environment. In the early 1990s various manmade chemicals were also identified as disrupters of the endocrine system [10, 11] and their fate and behavior became a major research topic [12]. Since then, accumulating evidence suggests that these chemicals may also affect the health and possibly the fertility of humans and wildlife [13, 14, 15].

To predict environmental concentrations of steroid hormones in river waters, Johnson and co-workers used an exposure assessment model [16, 17, 18]. They expected concentrations below 0.4 ng L^{-1} for estradiol and estrone, and 10 times less for ethinylestradiol. This expectation agrees well with values in the ng L^{-1} range reported by various groups for wastewater treatment plant effluents when the dilution of the effluent in the receiving rivers is taken into account [5, 7, 19, 20, 21, 22, 23, 24].

The very low environmental concentrations expected for natural and synthetic steroid hormones require sophisticated analytical techniques for their measurement and assessment of their effect on aquatic organisms. Biological tools are needed to measure effects, which are integrated responses to all estrogenic chemicals present, in-

cluding those that might be missed by chemical analysis. Hence, in vitro testing of environmental samples and chemicals has become an important complementary technique to classical chemical analysis [25, 26, 27, 28]. It allows targeting known and unknown chemicals and mixtures of chemicals that cause specific effects at the cellular or molecular level, and it can be used for high-throughput screening. By using the yeast estrogen screen (YES) as an in vitro test system, effects can be measured as a result of additivity in a mixture of EDCs, even if the concentration of individual EDCs is below the no-effect concentration [29]. However, in vitro tests represent an artificial system, and their responses should be validated with in vivo experiments [30]. For this reason, the induction of vitellogenin (VTG) in juvenile or male fish in vivo has become an accepted biomarker of exposure to estrogenic compounds [8, 31].

In the work presented here we have applied a combination of chemical analysis and various bioanalytical tools (in vitro and in vivo) for assessing estrogenicity in wastewater treatment plant (WWTP) effluents and their receiving waters. We have used this approach to correlate chemical data with biological effects and could show that: i) natural and synthetic steroid hormones are the major contributors to estrogenicity in WWTP effluents, ii) the measured (YES) and calculated (based on chemical analysis) estrogenicity correlated well, and iii) no false negative results were obtained in vitro, since the VTG induction in exposed male fish was matched by the response of the YES assay.

Materials and methods

Chemicals

The standards β-estradiol-17-acetate, 17-α-ethinylestradiol, 17-β-estradiol, estriol, estrone, and 2,4,6-trimethylphenol were from Sigma-Aldrich (Buchs, Switzerland). E2 and EE2 used in the YES were from Fluka (Buchs, Switzerland), 1,4-dithioerythritol (DTE, >99%) was from VWR Int. AG (Dietikon, Switzerland), 1-(trimethylsilyl)imidazole (T(M)SIM, >98%) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, ≈99%) from Fluka (Buchs, Switzerland). All solvents were puriss p.a., or HPLC-grade.

Sample collection for chemical and in vitro analysis

Three-day composite water samples were collected by using cooled (5°C) mobile sampling devices (ISCO Model 6700 Standard, IG Instrumenten-Gesellschaft, Zurich, Switzerland) fitted with acetone-rinsed 4-L glass or aluminum (France1 and France2) bottles. Sampling locations were WWTP effluents, the river upstream of the point of discharge, and Lake Lucerne at Kastanienbaum. To prevent sample degradation, samples were stabilized by adding 10 mL 36% formaldehyde solution (NPnEO analysis, n=0–2), or 10 mL methanol (steroid analysis and YES) per liter. The samples were stored at 4°C and processed within 48 h (steroid analysis and YES) or 14 d (NPnEO analysis, n=0–2), or frozen at –18°C if enrichment was delayed. An internal standard (25 ng L^{-1}) was added before the enrichment for steroid (E2-acetate) and NPnEO chemical analysis (2,4,6-trimethylphenol).

Enrichment for steroid analysis and in vitro testing

Solid-phase extraction (SPE) was performed according to the method reported by Ternes et al. [22]. Briefly, 1 L of sample was vacuum filtered through a glass fiber filter (GF/F, 90 mm, Whatman International Ltd, Kent, UK), and the pH of the filtrate was adjusted to 3 with 16% aqueous HCl. The enrichment step was carried out by using 3-mL self-packed polypropylene SPE tubes (Supelco, Buchs, Switzerland) containing a mixed solid phase (LiChrolut RP18 and LiChrolutEN; VWR Int. AG, Dietikon, Switzerland). After drying of the solid phase, analytes were eluted successively with 4×1 mL acetone. For steroid analysis, the solvent was reduced to 200 µL under a gentle stream of N_2. The extract was then further cleaned on a 6-mL SPE column containing 1 g of silica gel (Sigma-Aldrich, Buchs, Switzerland), deactivated with 1.5% H_2O (w/w). Conditioning and elution were done with hexane/acetone 6:4 (v/v). The eluate was subsequently derivatized for GC/MS analysis (see below). For the in vitro testing, the acetone was evaporated completely and the residues were then taken up in 500 µL of absolute ethanol. Blank samples were prepared from Dubendorf groundwater (chemical analysis) or deionized water (YES) and extracted and treated in the same way as the environmental samples.

GC/MS analysis of steroid hormones

The solid-phase extracts were derivatized according to the method reported by Ternes et al. [22] by using MSTFA/TSIM/DTE, 1,000:2:2 (v:v:w) and separated on a GC Fisons 8000 equipped with a PTV injector OPTIC 2 (ATAS, The Netherlands). We injected 10-µL volumes split-less onto a plug of deactivated fused silica (Restek, BGB Analytik AG, Anwil, Switzerland). The carrier gas was He (Carbagas, Zurich, Switzerland). The column was a XTI-5, 30-m, 0.25-mm ID, 0.25-µm film thickness (Restek) attached to a 2-m pre-column (Siltek, 0.53-mm ID, Restek). GC temperature program: 110°C (4 min), 17°C to 238°C, 1.7°C to 260°C, 10°C to 300°C, 300°C isothermal (7 min).

High-resolution mass spectrometry (resolution 8,000 at 5% valley) with positive electron ionization on an Autospec-Q (Micromass, Manchester, UK) was used to detect steroid hormones. The transfer lines were set to 300°C, and the source temperature to 280°C. Optimized EI+ conditions for steroid hormones resulted in an electron energy of 54 eV and a trap current of 700 µA. The detector was set to 375 V. Low-boiling PFK (Fluka, Buchs, Switzerland) was used for calibration and lock masses. Single-ion monitoring was used during data acquisition.

Recoveries determined from two independent datasets (January 2000/November 2001) for groundwater samples spiked with 30 ng L^{-1} of the standard steroid mixture prior to or after the enrichment, were 114/106% for E1, 117/100% for E2, 119/100% for EE2, and 40/25% for estriol E3 (n=8). For effluent samples, recoveries were 160/136% for E1, 122/80% for E2, 141/104% for EE2, and 166/4% for E3 (n=8). The corresponding standard uncertainties for groundwater samples were 15% for E1, 22% for E2, 25% for EE2,

Table 1 Relative estrogenic potencies (REP) determined with the yeast estrogen screen [33]

	E1	E2	E3	EE2	NP
Relative estrogenic potencies	0.38	1	2.4×10^{-3}	1.19	2.50×10^{-5}
Lower and upper 95% confidence limits	0.36–0.40	0.97–1.04	2.3×10^{-3}–2.5×10^{-3}	1.15–1.24	2.4×10^{-5}–2.7×10^{-5}

Table 2 Summary of all the fish exposures

Site	WWTP	Flow (m^3 d^{-1})	HRT[a] (h)	SRT[b] (d)	Avg T[c] (°C)	BOD[d] (mg L^{-1})	Sludge (g L^{-1})	Sampling date (weeks)	Male surviving fish (number)	Survival all fish (%)	Weight range (g)
Rontal 100%	industrial/urban population: 27,000	8,200	7	3	20.7	9.0	1.5	3–16/9/99 (2)	11	53	450–700
River Ron					19.8				11	74	
Lake Lucerne					19.8				10	64	
Glatt 100%	urban population: 88,000 approx. 20% industrial	45,000	12	4–6	17.4	4.7	2.5	18–31/10/99 (2)	12	100	286–372
					17.5			18/10–7/11/99 (3)	18	100	
River Glatt					13.0			(2)	12	100	
					12.8			(3)	16	100	
Lake Lucerne					12.4			(2)	12	100	
					12.2			(3)	16	100	
Surental 100%	industrial/urban population: 25,000 industrial: 13,000	15,000	10	14–18	16.4	1.6	3.6	5–19/10/00 (2)	9[e]	33	177–295
Surental 50%					14.9			5–26/10/00 (3)	24	89	
River Sure					13.3				14	45	
Lake Lucerne					12.6				17	89	
France1 100%	urban population: 30,000	8,100	12	10	16.3	n/a[f]	n/a	16–30/5/01 (2)	29	97	152–347
France1 50%					17.9				29	100	
River France1					19.8				30	100	
Lake Lucerne					16.6				24	96	
France2 100%	industrial/urban population: 11,000 industrial: 14,000–19,000	6,000	41	6–10	11.7	n/a	n/a	10–24/4/01 (2)	18	58	162–308
France2 50%					9.4				29	100	
River France2					7.5				29	97	
Lake Lucerne					6.6				30	100	

[a]Hydraulic retention time
[b]Sludge retention time
[c]Average temperature
[d]Biological oxygen demand
[e]High mortality in the Surental 100% effluent let us stop the experiment after two weeks
[f]Not available

and 173% for estriol E3 (n=8). For effluent samples, they were 60% for E1, 23% for E2, 42% for EE2, and 116% for E3 (n=8). The highly varying recoveries and corresponding high uncertainties for E3 can be explained by the relatively high polarity of the compound, which means that recoveries are affected strongly by the activity of the silica column. Only slight changes in the activity can lead to large losses. Limits of quantitation at signal-to-noise ratios of 10:1 were strongly matrix-dependent and had to be determined separately for each experiment.

Enrichment and analysis of alkylphenolethoxylates

The analytical procedure for determining nonylphenol (NP), nonylphenolmonoethoxylate (NP1EO), and nonylphenoldiethoxylate (NP2EO) in environmental samples is based on the work published by Ahel et al. [32]. We enriched 100-mL samples by liquid–liquid extraction with 3×2 mL hexane. The hexane phase was dried with anhydrous sodium sulfate, and the volume reduced to 500 µL under a gentle stream of N_2. The analysis was performed by using normal-phase HPLC on an HP1090 Series II liquid chromatograph (HPLC) equipped with a photodiode array detector (HP 1090) and a programmable fluorescence detector (HP 1046) from Agilent Technologies AG (Basel, Switzerland).

In vitro analysis (YES)

The yeast cells were stably transfected with the human estrogen receptor gene and an expression plasmid containing estrogen-responsive elements (ERE) which control the β-galactosidase-encoding reporter gene *lacZ*. The translated β-galactosidase is secreted into the medium, and its activity is determined by measuring the absorbance at 540 nm. Culture and exposure of the yeast cells were performed as described by Routledge and Sumpter [25]. Inhibition of yeast cell growth was regarded as an acute toxic effect of a tested sample or compound; this was observed as a reduction of absorbance at 620 nm, compared to reference wells. Dilution series of E1, E2, EE2, E3, NP, and the WWTP effluent extracts were prepared in ethanol. Ten µL of each dilution of the standard compounds and 20 µL of the dilutions of the effluent samples were added to 96-well microtiter plates. The ethanol was evaporated and the yeast cells were added in growth medium. At least four replicate wells per dilution were used.

Calculated and measured estradiol equivalents (E2-EQ)

Estrogenic activity (expressed as estradiol equivalency E2-EQ calc in ng L^{-1}) calculated from steroid and nonylphenolethoxylate chemical data was based on potencies relative to E2 as determined with the YES (Table 1). Associated uncertainties were calculated based on the uncertainties of the chemical determination and relative potencies by following the rules of error propagation [33].

Estradiol equivalency of samples measured with the YES was determined by interpolation from E2 standard curves [33]. Standard deviations of replicate measurements with the YES are shown.

Fish exposure experiments

Five exposure experiments were performed with fish at three Swiss and two French WWTPs. For reasons of confidentiality, the French WWTPs will be called France1 and France2. The WWTPs chosen for this study received either mostly urban (Glatt, France1) or a mixture of urban and industrial influent (Rontal, Surental, France2; see Table 2). The five plants had activated sludge treatment and were operating under nitrifying conditions. The actual population in the catchment area ranged from 11,000 to 88,000 people.

Before the start of the exposure, mostly male adult rainbow trout (*Oncorhynchus mykiss*) purchased from a hatchery (Teddy Waser, Andelfingen, Switzerland) were allowed to acclimate in the laboratory for two weeks. During this time they were fed *ad libitum* with commercially available pellets (Hokovit, Silver Cup, Hofmann AG, Butzberg, Switzerland). The fish were anesthetized in a solution containing MS222 (100 mg L^{-1} 3-aminobenzoic acid ethyl ester, Redmont, USA) and individually marked with numbered jaw tags [34] for future identification. Body length and weight were measured and blood samples were collected by using lithium-heparinized monovettes (Sarstedt, Numbrecht, Germany).

After acclimation, the fish were exposed to 100% WWTP effluent, or 50% WWTP effluent mixed with upstream river water (Surental, France1, and France2 only), or upstream river water (0% WWTP effluent), or Lake Lucerne water (laboratory control) in black 400-L polyethylene (HD) tanks. The accuracy of the 50% mixing was verified by comparing the expected with the actual measured temperature (R^2 values for the linear regressions were 0.95, 0.99, and 0.85 for Surental, France1, and 2, respectively). During each experiment the fish were kept in the dark without feeding. Each treatment was done in three replicates using three tanks with an average of 10 fish. When an experiment ended, the fish were killed with a blow to the head and processed immediately. Length and total weight were measured, blood samples collected, and the sex determined. The fish were then dissected and the liver and gonads weighed. During the exposures the following standard parameters were measured: temperature, oxygen concentration, pH, conductivity, ammonium, nitrate, nitrite, and phosphate.

Determination of VTG blood plasma concentrations

Blood was collected from the caudal vein of each fish in every treatment. After centrifugation at 2,000 g and 4°C, blood serum was collected in Eppendorf tubes, snap-frozen in liquid nitrogen and stored at –80°C until further analysis. VTG levels were analyzed in the blood samples from the fish exposed at all five sites by using a new homologous, polyclonal anti-rainbow trout vitellogenin antibody in a newly established sandwich ELISA protocol. For the production of polyclonal anti-VTG antibody, New Zealand white rabbits (M. Moerter, Uerschhausen, Switzerland) were immunized by subcutaneous injections of 30 µg rainbow trout VTG (Biosense, Norway) in complete Freunds adjuvant (day 0) followed by 2 booster injections of 20 µg VTG each in incomplete Freunds adjuvant on days 28 and 56 (both from Sigma-Aldrich, Buchs, Switzerland). Test bleedings were done on days 42 and 70 by puncture of the *arteria auricularis*. After 12 weeks, the animals were terminally bled by heart puncture under narcosis.

ELISA protocol

Plates were coated with 3,000×diluted primary monoclonal antibodies BN-5 (Biosense, Norway) in blocking buffer (PBS, 0.05% Tween 20, 2% milk powder) and washed with washing buffer (PBS, 0.05% Tween 20). The plates were subsequently incubated with 100 µL of 750-fold-diluted rainbow trout plasma for 2 h, washed with washing buffer, and further incubated overnight at 4°C with polyclonal anti-VTG antibodies, then diluted 1:7,500 in blocking buffer. After rinsing with washing buffer, the plates were incubated with goat anti-rabbit horseradish peroxidase (diluted 1:3,000 in blocking buffer) for 2 h at room temperature and rinsed with washing buffer. Horseradish peroxidase activity was quantified using chemiluminescence substrate according to the manufacturer's instructions (Promega, Mannheim, Germany).

Results and discussion

Chemical analysis

Chemical data for steroid hormones and nonylphenolethoxylates acquired during all the exposure experiments, together with the estradiol equivalents (E2-EQ) determined with YES, are shown in Table 3. Maximal values obtained

Table 3 Chemical data for nonylphenol (NP), nonylphenolmonoethoxylate (NP1EO), nonylphenoldiethoxylate (NP2EO), estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol, estrogenicity measured with the YES (E2-EQ) and calculated (E2-EQ calc)

Site (number of samples)	Date	NP (µg L^{-1})	NP1EO (µg L^{-1})	NP2EO (µg L^{-1})	E1 (ng L^{-1})	E2 (ng L^{-1})	E3 (ng L^{-1})	EE2 (ng L^{-1})	E2-EQ (ng L^{-1})	E2-EQ calc (ng L^{-1})[a]
Glatt Exposure 100% Effluent (*n*=7)	18/10–8/11/1999	0.17–0.28	0.31–0.65	0.41–0.85	4.9–18.8	<LOQ–1.0	3.3–11.0	<LOQ	2.4–5.5	1.9–7.2
River Glatt (*n*=7)		<LOQ	<LOQ	<LOQ	<LOQ–1.6	<LOQ	<LOQ–1.4	<LOQ	<LOQ–0.3	<LOQ–0.6
Lake Lucerne (*n*=7)		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Rontal Exposure100% Effluent (*n*=4)	3–15/9/1999	0.89–1.61	0.90–2.58	1.08–3.61	4.0–(50.5)	<LOQ–6.4	2.3–17.5	<LOQ-2.8	0.4–(53.0)	2.1–(29.0)
River Ron (*n*=4)		<LOQ	<LOQ	<LOQ	0.5–0.7	<LOQ–1.1	<LOQ	<LOQ	<LOQ	0.3–1.3
Lake Lucerne (*n*=4)		<LOQ	<LOQ	<LOQ	0.2–0.4	<LOQ–0.6	<LOQ–0.5	<LOQ	<LOQ	0.1–0.8
Surental Exposure100% Effluent (*n*=5)	5–19/10/2000	0.11–0.19	0.10–0.16	<LOQ-0.22	2.0–5.9	(0.8–3.2)	<LOQ	<LOQ	0.5–2.2	(1.6–4.9)
50% Effluent (*n*=7)	5–25/10/2000	0.09–0.19	0.08–0.17	<LOQ-0.17	1.6–3.7	(1.0–3.8)	<LOQ	<LOQ	0.2–1.6	(1.6–4.8)
River Sure (*n*=7)	5–25/10/2000	<LOQ–0.17	<LOQ–0.27	<LOQ–0.11	<LOQ	(0.8–3.8)	<LOQ	<LOQ	<LOQ	(0.8–3.8)
Lake Lucerne (*n*=7)	5–25/10/2000	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ-1.1	<LOQ	<LOQ	<LOQ	<LOQ–1.1
France1 Exposure100% Effluent (*n*=5)	16–30/5/2001	0.66–1.27	0.49–1.03	0.43–0.84	<LOQ–10.2	1.3–4.0	<LOQ	<LOQ	<LOQ–2.7	1.9–6.4
50% Effluent (*n*=5)		0.39–0.88	0.36–0.71	0.24–0.58	1.2–7.1	<LOQ–4.3	<LOQ–3.1	<LOQ	1.0–3.6	0.5-5.5
River France1 (*n*=5)		<LOQ–0.49	0.12–0.38	<LOQ–0.19	1.2–2.7	<LOQ	<LOQ–7.1	<LOQ	1.0–1.9	0.5–1.1
Lake Lucerne (*n*=2)	22+29/5/2001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ, 2.0	<LOQ	<LOQ	<LOQ
France2 Exposure100% Effluent (*n*=4)	11–24/4/2001	0.49–1.74	1.10–2.52	0.81-3.97	1.9–6.5	3.2–9.8	<LOQ	<LOQ	2.1–5.3	3.9–12.2
50% Effluent (*n*=4)		0.20–0.88	0.48–1.09	0.41–2.04	<LOQ–3.2	<LOQ–3.2	<LOQ	<LOQ	2.0–2.9	<LOQ–4.1
River France2 (*n*=4)	11–15/4/2001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ–0.5	<LOQ
Lake Lucerne (*n*=2)	18+23/4/2001	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a]Values below LOQ were considered to be zero for E2-EQ calc

in the 90 samples analyzed were 50.5 ng L^{-1} estrone (Rontal 100% effluent), 9.8 ng L^{-1} estradiol (France2 100% effluent), 17.5 ng L^{-1} estriol (Rontal 100% effluent), and 2.8 ng L^{-1} ethinylestradiol (Rontal 100% effluent) and a measured estrogenicity of 53.0 ng L^{-1} (YES E2-EQ, Rontal 100% effluent). The 50.5 ng L^{-1} maximal estrone concentration determined in the Rontal effluent is well matched by the measured estrogenicity as determined with the YES (53.0 ng L^{-1}) and two other in vitro tests ([33], data not shown), but this value clearly deviates from the rest, which did not exceed 5.5 ng L^{-1} for measured estrogenicity (YES E2-EQ; Glatt 100% effluent). Since this was a singular event, it was not included in the data set used for the correlation analysis, for reasons of clarity.

The E2 values determined during the Surental exposure (100% effluent: 0.8–3.2 ng L^{-1}, 50% effluent: 1.0–3.8 ng L^{-1}, and river: 0.8–3.8 ng L^{-1}) were not considered for further analysis, because except for the Lake Lucerne concentrations (<limit of quantitation (LOQ) –1.1 ng L^{-1}), all were similar, indicating a contamination of unknown origin. On the other hand, E1 concentrations (100% effluent: 2.0–5.9 ng L^{-1}, 50% effluent: 1.6–3.7 ng L^{-1}, and river: <LOQ) and the E2-EQ obtained with the YES (100% effluent: 0.5–2.2 ng L^{-1}, 50% effluent: 0.2–1.6 ng L^{-1}, and river: <LOQ) decreased, clearly reflecting the expected dilution.

LOQs at the ng L^{-1} level are influenced greatly by the matrix of the sample. Hence, LOQs must be determined independently for each environmental sample. In 100% WWTP effluent LOQs ranged from 0.7 to 1.0 ng L^{-1} for E1, 0.5 to 0.9 ng L^{-1} for E2, 1.0 to 1.5 ng L^{-1} for E3, 0.7 to 1.0 ng L^{-1} for EE2. LOQs in Lake Lucerne water were 0.1 ng L^{-1} for E1, 0.3 ng L^{-1} for E2, 0.3 ng L^{-1} for E3, and 0.2 ng L^{-1} for EE2. The LOQs for the alkylphenols were 0.09 µg L^{-1} for NP, and 0.08 µg L^{-1} for NP1/2EO in all matrices.

In light of the ongoing discussion about effects of mixtures of causative agents at individual concentrations below effect levels [29], there is clearly a need to further lower the LOQs for the determination of steroidal estrogens. Affinity techniques and the use of immunosorbents have been shown to be promising selective enrichment or clean-up tools [24, 35, 36].

Maximal concentrations in all 90 samples analyzed were up to 1.74 µg L^{-1} for NP, up to 2.58 µg L^{-1} for NP1EO, and up to 3.97 µg L^{-1} for NP2EO.

Effluent concentrations similar to those reported here were found throughout Europe and correspond well to predictions made by Johnson et al. [17, 37]. Generally, the highest concentrations are found for E1, followed by E3 and E2. These relative concentrations agree with the relative amounts found in the urine of pre-menopausal women. Post-menopausal women excrete more E2 than E3, but the absolute hormone concentrations are lower [38].

However, chemical data on steroidal estrogens have to be considered with the corresponding uncertainties in mind. Standard uncertainties for effluent samples ranged between 23% for E2 and 116% for E3. Even if this seems rather high, it clearly reflects the problems encountered in ultra-trace analysis in complex sample matrices. Albert and Horwitz analyzed almost 10,000 intercalibration exercises and derived the following relationship between the relative standard deviation (RSD; %) and sample concentration (c; mass ratio in g g^{-1}): RSD=2^[1–0.5log$c$] [39]. Thus, at a concentration of 1 ng L^{-1}, the expected relative standard deviation is 130%, without accounting for the physical and chemical properties of the analytes. Estriol, for example, is the most polar of the steroid metabolites investigated and for this reason recoveries for E3 depend strongly on the activity of the silica used in the clean-up step. Furthermore, E2-acetate proposed by Ternes et al. [22] is not the ideal internal standard for this polar compound, but since estrogenic activity was also measured with the YES, we preferred not to use deuterated standards. This explains the large uncertainty associated with the chemical data for E3, which however still is well within the values predicted by Albert and Horwitz [39]. Since the relative estrogenic potency and the measured concentrations are relatively low (Tables 1 and 3), E3 does not greatly affect the total calculated estrogenicity.

Calculated estrogenicity versus estrogenicity measured with the YES

The relative potencies determined with the yeast estrogen screen cover a range of five orders of magnitude, with the natural and synthetic steroid hormones being the most potent (Table 1). The relative estrogenic potency of E3 is comparable with previously published results using a yeast-based estrogenic assay [40], but is much lower when compared to cases in which binding affinities with the ER have been determined [41, 42]. The estrogenic activity is calculated based on the concept of concentration additivity, which applies since all investigated EDCs target the same receptor [29, 43, 44]. The contribution of nonylphenol-ethoxylates to the total estrogenicity of the effluents measured is only minor. For instance, even the highest NP concentration measured (1,740 ng L^{-1}, France2), corresponds to an estrogenicity of 0.04 ng L^{-1} E2, which only accounts for 0.8% of the total estrogenic activity of 4.9 ng L^{-1} measured in this sample with the YES. Similarly insignificant contributions are obtained for NP1EO and NP2EO.

Data acquired during the five exposure experiments (with the exception of the Surental dilution and the singular event in Rontal mentioned above) has been used to analyze the correlation between measured estrogenicity (YES) and calculated E2 equivalents. Figure 1 lists E2-EQs calculated (E2-EQ calc) based on the chemical data as a function of the measured estrogenicity (YES) for each sample. The error bars reflect the standard error of the measured estrogenicity (replicate measurements) and E2-EQ calculated for each sample. The error bars for the calculated E2-EQs incorporate the uncertainties of the chemical measurements (up to 116% for E3) and the relative estrogenic potencies. Since a traditional least-squares estimation would be biased due to these uncertainties, we calculated the geometric mean functional relationship [45].

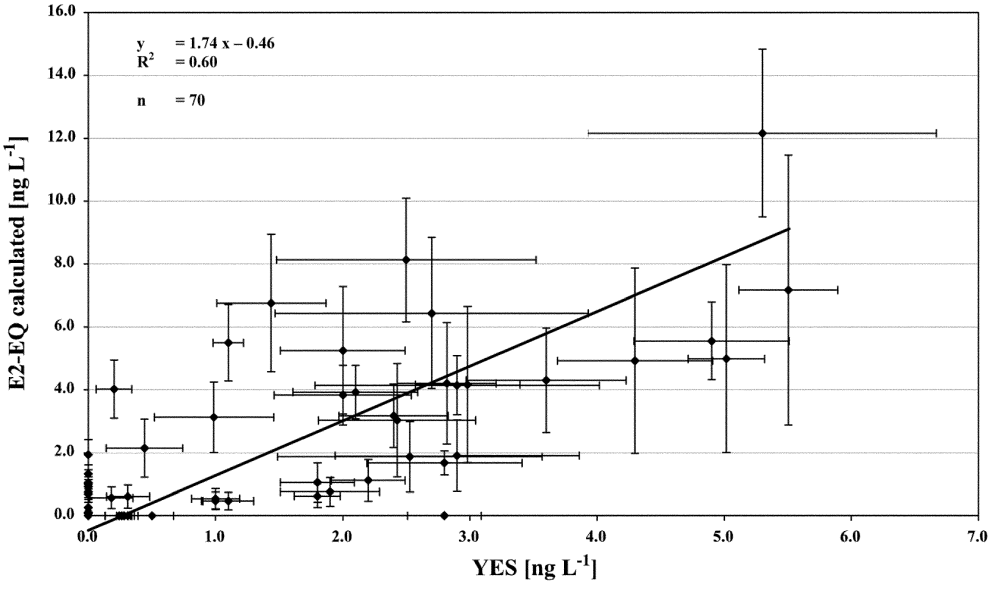

Fig. 1 Estrogenicity determined with the yeast estrogen screen (YES) versus calculated estradiol equivalents, based on chemical data (E2-EQ calculated)

A positive correlation (R^2=0.60) was observed between these two parameters, indicating that the natural and synthetic steroid hormones represent the major contributors to the measured estrogenicity. However, alkylphenols and their ethoxylates and other xenoestrogens might have to be monitored in special cases for instance when the WWTP influent has a high industrial component. The graph also shows that the calculation tends to overestimate the estrogenicity. However, due to the measurement uncertainties involved, this should by no means be over interpreted.

Fish exposure experiments

Most exposed individuals (>95%) were male except for the WWTP Rontal experiment (51% male). Individual VTG data from before the experiment was not available for the Rontal exposure due to loss of tags. The fish were exposed for a period of two weeks for all treatment experiments, except for a three-week exposure at Surental, and one of the two Glatt experiments (Table 2). The three exposures in Switzerland were done in the fall (first week of September to first week of November), while the French exposures were undertaken in spring (end of April and end of May).

Survival of the fish in the experiments conducted at the WWTPs Glatt and France1 were high (>95%). Rainbow trout used for the exposure at the Rontal WWTP had a fungal skin infection (*Saprolegnia*) and had higher mortality in all treatments. Fish survival during the Surental effluent treatment was low (33%) for unknown reasons, while the observed increased mortality in the river-water treatment was caused by an accidental oversaturation with oxygen. The high mortality found for the effluent-treated trout at the WWTP France2 was caused by very high ammonia concentrations of up to 26 mg L^{-1} on exposure day 7. This concentration was much higher than the reported acute toxic concentration of 200 µg L^{-1}, for non-ionized ammonia (NH_3) [46, 47]. To avoid total loss in the effluent and mixed water treatment groups, the treatments were interrupted for 12 h and run with river water to allow the trout to recover from the ammonia peak.

HSI (hepatosomatic index) and GSI (gonadosomatic index) were calculated for each single fish. However, in none of the experiments were changes in HSI and GSI significant between treatment and control.

In vitro versus VTG concentrations in blood plasma

Significant estrogenic activities were measured in vitro with the YES in all WWTP effluents analyzed. The measured values obtained with the YES correlated well with the estradiol equivalents calculated based on the chemical data, and most importantly did not give false negative results (Fig. 1). This clearly supports the idea of using YES as an in vitro tool for a first screening of environmental samples. YES is an easy, relatively fast, and robust in vitro assay that has recently been shown to give results similar to those of other in vitro tests, based either on the use of primary fish hepatocytes or fish cell lines [33]. Potential hazards can thus be determined in a sensitive way and relatively fast. In the case of a positive in vitro response, a further level of biological effect integration can be envisaged by using in vivo effect analysis, which also takes into account toxicokinetic and toxicodynamic effects. The importance of this validation step has recently been examined by Legler et al. [48]. They showed that effects seen in vitro do not necessarily mean observable in vivo effects.

In the work presented here we observed a clearly induced vitellogenesis in male rainbow trout exposed to sewage effluents from the WWTPs Glatt and Rontal. This is visualized by an increased number of fish having higher

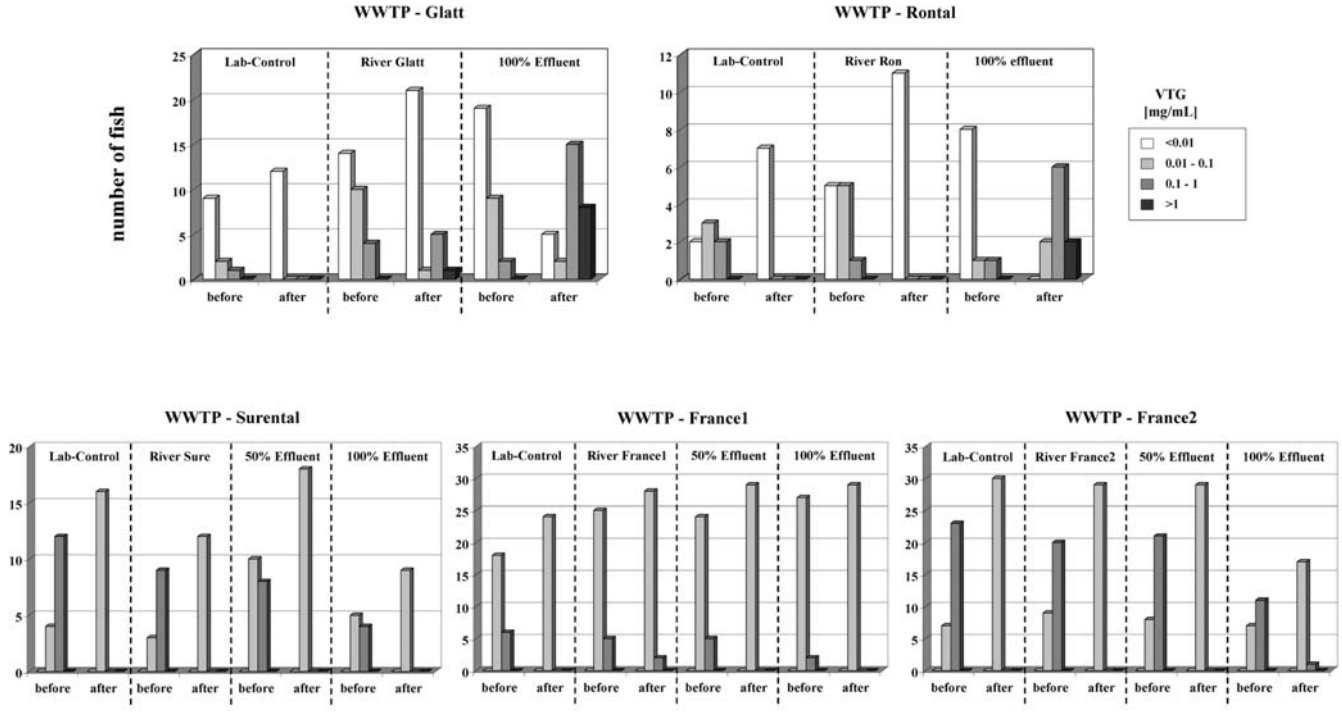

Fig. 2 Blood plasma VTG concentrations determined in male rainbow trout before and after exposure to dilutions of WWTP effluents (100% Effluent, 50% Effluent), to River and to Lake Lucerne water

concentrations of VTG in Fig. 2. These results indicate estrogenic activity in both effluents. Interestingly, no significant increase of VTG blood plasma concentrations were found at the Surental, France1, and France2 sites (see Fig. 2). We do not understand the different responses in vitro versus in vivo. The hERα is used in YES, while rtERα is present in the in vivo test. Differential binding for the two estrogen receptors for steroid hormones and for xenoestrogens has been described controversially [41, 49, 50, 51]. This differential binding can, however, not explain why no in vivo responses have been observed. Owing to the annual cycle of reproductive hormones in both females and males [52] and as a consequence the inducibility of VTG, the seasonally different exposure schemes (autumn in exposures in Switzerland; spring in exposures in France), could partly have contributed to the different response patterns. The negative in vivo response in estrogenic effluents could be due either to the presence of anti-estrogens in the effluents of these treatment plants, or hepatotoxicity-induced reduction of VTG synthesis, or diet. However, anti-estrogenicity could have been detected with the YES assay. The observed reduction of VTG levels during exposure (Surental, France1, and France2) is most likely caused by estrogenic compounds in the pellets used for feeding at the hatchery before the experiment. Indeed, estrogenicity was measured with the YES (0.12 ng g^{-1} dry weight) in an aqueous extract of the feeding pellets, which very possibly accounts for the elevated VTG levels at the onset of the experiments. No steroid hormones were detected either in aqueous, or hexane extracts of the feeding pellets (A.C. Vögeli, unpublished results). We have no explanation for the different in vivo response in the various effluents, but the results clearly illustrate the difficulty of predicting a propagation of effects onto a higher level of biological organization. A positive effect seen in vitro should hence clearly be defined as a potential hazard, rather than a proof for a biological effect in higher organisms.

Conclusion

In this work we have assessed the estrogenic activity of wastewater treatment plant effluents and combined chemical analysis and biological effect analysis, in vitro as well as in vivo. We showed that natural and synthetic steroid hormones are the major contributors to estrogenicity in the effluents tested. We can, however, not exclude that xenoestrogens might have a significant contribution in other effluents. The calculated (based on chemical analysis and using the principle of concentration additivity) and measured (YES) estrogenicity correlated well (R^2=0.6). No false negative results were obtained in vitro thereby supporting the use of YES as an in vitro tool for first screening purposes. Effects seen in vitro must not necessarily mean that in vivo effects will occur. The fact that three out of five estrogenic effluents did not induce vitellogenesis clearly shows that prediction of effects in vivo from in vitro data is and will be extremely difficult. It also shows that we are still very far away from understanding and predicting endocrine disruption, particularly if the effects of complex environmental mixtures are being studied.

Acknowledgements This work was part of the European "Community Programme of Research on Environmental Hormones and Endocrine Disruptors" (COMPREHEND), supported by the Swiss

Federal Office for Education and Science (BBW), grant Nr. 98.0090. The authors would like to thank John Sumpter (Brunel University, Uxbridge, UK) who kindly provided the recombinant yeast estrogen screen, Patricia Holm (EAWAG) and Helmut Segner (Centre for Fish and Wildlife Health, University of Berne, Switzerland), as well as all COMPREHEND consortium partners for fruitful and lively discussions. Karl Fent is acknowledged for his contributions in the initial phase of the project. Mark Borsuk and Beate Escher (EAWAG) are greatly acknowledged for their help with the statistical treatment of the data shown in Fig. 1, as are Michel Gibert and David Benanou (Vivendi Water, Paris, France) and all WWTP personnel for their on-site help during the exposures at the five WWTPs. Our special thanks go to Alan Pickering (Windermere, UK) for his outstanding coordination of COMPREHEND, and Alexander Zehnder (EAWAG) for his continuous support during the whole project.

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