Input/Output Balance of Estrogenic Active Compounds in a Major Municipal Sewage Plant in Germany

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Introduction

In the last few years, up to about 40 non-steroidal chemicals have been identified to mimic the effects of the natural estrogen 17ß-estradiol [1-4]. Recently, the crystal structure of the human estrogen receptor (ER) was determined [5]. As the gap of the ligand binding domain is much larger than 17ß-estradiol requires there is space for a variety of other molecules to interact with the ER. Thus, the recognition of estrogenic properties largely depends on empirical testing. The wide structural variety of the phyto- and xenoestrogens presently known, reflecting the structure of the human ER, sets a limit to single compound chemical analysis of environmental samples regarding time and labour. Therefore, sensitive and specific *in vitro* biotests are required for the screening of extracts and fractions for their content of compounds with receptor-mediated estrogenic activities regardless of their chemical structure (functional analysis).

Many of the known xenoestrogens as well as natural and synthetic estrogens are expected to end up in the aquatic environment via sewage. Recently, it was demonstrated in the UK [6, 7] and the USA [8] that male fish held in treated sewage effluents or in rivers below sewage plants showed a pronounced increase of estrogen dependent plasma vitellogenin levels. Since 25 years it is known that the synthetic estrogens ethinylestradiol and mestranol used in oral contraceptiva are not completely biodegradated in sewage plants [9]. Several studies proved the occurrence and persistence of 4-nonylphenol [10-12] and Bisphenol A [13] in sewage plants and surface water. These findings underpin the general need for analytical monitoring data of substances with estrogenic activity in sewage effluents and rivers.

In the present study we determined levels of estrogenic active substances in parallel in the raw and treated sewage of a major municipal sewage plant South Germany, to evaluate the persistence of these compounds during modern waste water treatment and to determine the magnitude of their release into rivers. For this purpose we measured quantitatively the total content of estrogenic activity in raw and treated sewage with ER positive human MCF-7 breast cancer cells (E-screen assay). Second, we used a recently developed GC/MS method [14] to analyze the concentrations of nine phenolic xenoestrogens. The comparison of the data of chemical and biological analysis

indicated the contribution of phenolic chemicals to total estrogenic activity in sewage.

Materials and Methods

Sampling: On March 11, 1998, samples of sewage influent and effluent were taken automatically every 2 h over a period of 24 h in the municipal sewage plant Steinhäule, Neu-Ulm, a major plant in South Germany with a capacity of 350,000 people equivalents. About 60 % of the wastewater comes from households, the rest from industries, hospitals, and research institutes. According to the average residence time of the sewage in the plant, the effluent sample was taken 8 h later than the influent sample.

Extraction: Solid phase extraction (SPE) of 1 L sample was performed on 200 mg of the polystyrene copolymer ENV+ (ICT, Bad Homburg, Germany) covered with silanized glass wool to prevent the blockade of the column by suspendend matter. Prior to extraction, the pH of each sample was adjusted to 2 - 3. After washing and drying of the extraction column, elution was performed with 5 ml acetone. The extract was concentrated to 0.5 ml with a stream of nitrogen.

GC/MS analysis: A 10 % aliquot (50 µl) was methylated with 50 µl of a 0.1 M solution of phenyl-trimethylammoniumhydroxide in methanol. After addition of biphenyl as internal standard for quantification HRGC/LRMS analysis of nine phenolic xenoestrogens was performed on a 15 m DB-XLB fused silica capillary column with 0.25 mm inner diameter and 0.25 µm film thickness (J&W Scientific Products, Köln, Germany). Details are described elsewhere [14].

E-screen assay: 50 µl of dimethylsulfoxide were added to the remaining extract as a keeper. Then the acetone was evaporated. The extracts were redissolved in 4.95 ml of steroid- and phenolred-free DME cell culture medium (experimental medium) and filtered sterile. MCF-7 cells seeded into 24 well plates were incubated for five days with 1:10 to 1:10,000 dilutions of the stocks testing each dilution in four wells. The samples were tested in three independent experiments. Cell number was determined photometrically by fixation of the cells and staining with sulforhodamine B. The cell number relative to the hormone-free negative control is the basic endpoint of the assay and was used for calculating the EC_{50} values. Total concentration of estrogenic active compounds in the extracts was assessed by comparing the EC_{50} of the sample with that of the positive control 17ß-estradiol (E2). Details on the performance of the E-screen assay are described elsewhere [4].

Results and Discussion

The proliferative effect PE is the ratio of the highest cell number achieved with the sample or the positive control 17ß-estradiol (E2), respectively, and the cell number of the negative control (Figure 1). The relative proliferative effect (RPE) compares the maximal proliferation induced by a sample with that induced by E2: RPE = [PE-1 (sample) / PE-1 (E2)] * 100 %

Influent as well as effluent sample showed a clear dose-dependent estrogenic activity in the E-screen assay. The RPE was 75 ± 18 % for the raw sewage and 30 ± 0.5 % (n=3) for the treated sewage indicating that the concentrations of full ER agonists and/or strong partial ER agonists were lower in the effluent. The proliferative effect of both samples was completely inhibited by co-incubation with 5 nM of the ER receptor antagonist ICI 182,780 (data not shown).

Expressed in 17ß-estradiol equivalent concentrations (EE), total estrogenic activity was 39.8 ng E2/L in the influent and 4.5 ng E2/L in the effluent (figure 1) resulting in an overall elimination rate of estrogenic compounds of 89 %. The EE in the effluent was within the range of 1 - 21 ng E2/L we recently found in four other sewage plant effluents in Germany. For ethinylestradiol it is known that levels in the lower ng/L range induce estrogenic effects in male rainbow trouts [6].

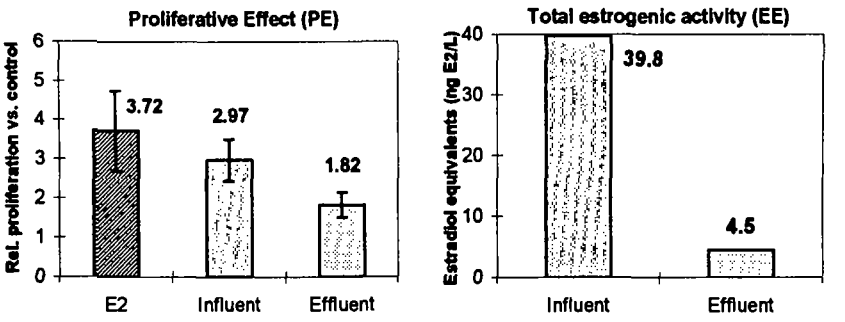

Figure 1: Estrogenic activity in the influent and effluent of a major municipal sewage plant in human MCF-7 breast cancer cells. The PE values represent means (± SD) of three independent experiments, values for total estrogenic activity (EE) are the mean of two independent experiments.

Table 1: Levels of phenolic xenoestrogens in the influent and effluent of a major municipal sewage plant.

Compound	influent (µg/L)	effluent (µg/L)	reduction (%)
4-t-octylphenol	0.195	0.290	+ 48.4
techn. 4-nonylphenol, peak 1*	0.531	0.426	19.8
techn. 4-nonylphenol, peak 2*	0.469	0.339	27.8
Bisphenol A	0.556	0.155	72.1
3-t-butyl-4-OH-anisole	n.d.	n.d.	
2-t-butyl-4-methylphenol	n.d.	n.d.	
4-OH-biphenyl	n.d.	n.d.	
2-OH-biphenyl	1.89	0.015	99.2
4-Cl-3-methylphenol	0.167	n.d.	100
4-Cl-2-methylphenol	n.d.	n.d.	

* two major peaks n.d. = not detectable

The GC/MS method recently developed and validated for quantitative analysis of nine phenolic compounds with known estrogenic activity [14] was successfully applied to treated as well as raw sewage. The limits of determination were between 10 and 20 ng/L. In the sewage influent 4-t-octylphenol (4-OP), 4-nonylphenol (4-NP), Bisphenol-A (BPA), 4-chloro-3-methylphenol, and 2-hydroxybiphenyl were found in the upper ng/L range while for other phenols were not detectable (table 1). In the effluent the levels of the two major isomers of 4-NP were only slightly lower and 4-OP was even present in higher concentrations. BPA levels were reduced by 72 %. 4-chloro-3-methylphenol and 2-hydroxybiphenyl were eliminated quantitatively.

Using the estrogenic potencies of the phenolic xenoestrogens in the E-screen assay relative to 17ß-estradiol (E2) we calculated E2-equivalent concentrations (EE) for the phenols present in raw and treated sewage (Table 2). The results were compared with the total estrogenic activity determined by the E-screen assay (Figure 1). The detected levels of phenolic xenoestrogens only contributed to 0.5 % of total estrogenicity in the raw sewage and to 3.3 % in the effluent.

As the levels of phenols only explain a small part of total estrogenicity in the effluent further analytical studies are necessary to identify the most important estrogenic substances. Regarding their fate in the aquatic environment, concentrations of alkylphenols and Bisphenol A in the upper ng/L range in sewage effluent cannot be neglected as these compounds are known to adsorb readily to sediments. The Bisphenol A derivative 4,4'-bisphenol-(2,2'-methylethylideneoxiran-)-

dimethylmethane was recently detected as a major toxic compound in the sediment from a River Elbe tributary downstream a chemical production plant [15].

Table 2: Comparison of chemical analysis of phenolic xenoestrogens and determination of total content of estrogenic active compounds with theE-screen assay for raw and treated sewage.

Compound	Molar mass	Rel. potency (E2=1)	Influent Concentration ng/L	mol/L	E2-equ. (EE) mol/L	Effluent Concentration ng/L	mol/L	E2-equ. (EE) mol/L
4-t-OP	206.3	0.0001	195	9.5E-10	9.5E-14	290	1.4E-09	1.4E-13
4-t-NP	220.4	0.0001	1000	4.5E-09	4.8E-13	765	3.5E-09	3.7E-13
Bisphenol-A	228.2	6.0E-05	556	2.4E-09	1.4E-13	155	6.8E-10	4.0E-14
2-OH-Biphenyl	170.2	1.5E-06	1890	1.1E-08	1.7E-14	15	8.8E-11	1.3E-16
Sum			3641	1.9E-08	**7.4E-13**	1225	5.6E-09	**5.5E-13**
E-screen					1.5E-10			1.7E-11
% of E-screen					**0.51**			**3.3**

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