MONITORING OF ESTROGENIC SUBSTANCES IN SEWAGE PLANT EFFLUENTS BY BIOLOGICAL AND CHEMICAL ANALYSIS

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Introduction

Laboratory experiments in which adult male rainbow trouts were exposed for 21 days to steroidal estrogens revealed a significant increase of estrogen-dependent plasma vitellogenin levels. The threshold levels for 17α-ethinylestradiol were below 1 ng/L [1] and for 17β-estradiol between 1 and 10 ng/L [2]. A preliminary screening of total estrogenic activity in effluents from five different municipal sewage plants in southern Germany revealed estradiol equivalent concentrations between 2.5 and 25 ng/L [3]. Therefore, induction of plasma vitellogenin levels in male fish in rivers downstream of municipal sewage plants, which has been observed in the UK [4] and the USA [5], may also happen in German streams. Since there are still open questions on the nature of the substances mainly responsible for the measured estrogenicity, we are currently performing a monitoring study on effluents of 15 different municipal sewage plants located in the state of Baden-Württemberg, Germany. The project includes the determination of total estrogenic activity by applying a miniaturized proliferation test with human MCF-7 breast cancer cells (E-screen) and the parallel analysis of various classes of estrogenic substances by GC/MS. The comparison of the results of chemical and biological analysis reveals the contribution of each substance to total estrogenicity in sewage. The results of 10 sewage plants will be presented.

Material and Methods

Sampling, extraction, and chemical analysis

Effluent samples of 10 municipal sewage plants in Baden-Württemberg, Germany, were taken between August and Nov. 1998. Each sample was collected time-proportionally with an automatical sampling device over a period of 24 hours and stored in brown glass flasks at 4 °C until solid phase extraction was carried out. The whole procedure of extraction and chemical analysis is described in fig. 1.

Biological analysis

For performance of biological analysis (E-screen assay), an extract of 1 L effluent was used, purified at 1 g silica/5% H_20 (Chromabond SiOH) and evaporated to dryness like for chemical analysis of steroidal estrogens. Each dry extract was mixed with 50 µl DMSO, diluted gradually with steroid-free cell culture medium and tested in three independent E-screen assays with MCF-7 cells, seeded into 96-well culture plates, as described in detail in [3]. Estrogen receptor(ER) agonists induce cell proliferation in human ER-positive MCF-7 breast cancer cells in relation to a hormone-free control. The proliferative effect (PE) of a sample is the ratio of the highest cell

number achieved with the sample to the cell number of the negative control. The relative estrogenic efficacy of a sample, the relative proliferative effect (RPE), is calculated by comparison of the maximum proliferation induced by a sample with that induced by 17β-estradiol (E2):

RPE = [PE-1 (sample) / PE-1 (E2)] * 100 %.

The total content of estrogenic active compounds in a sample, the 17β-estradiol equivalent concentration (EEQ), is calculated by comparison of the EC_{50} value of the sample with that of the positive control E2.

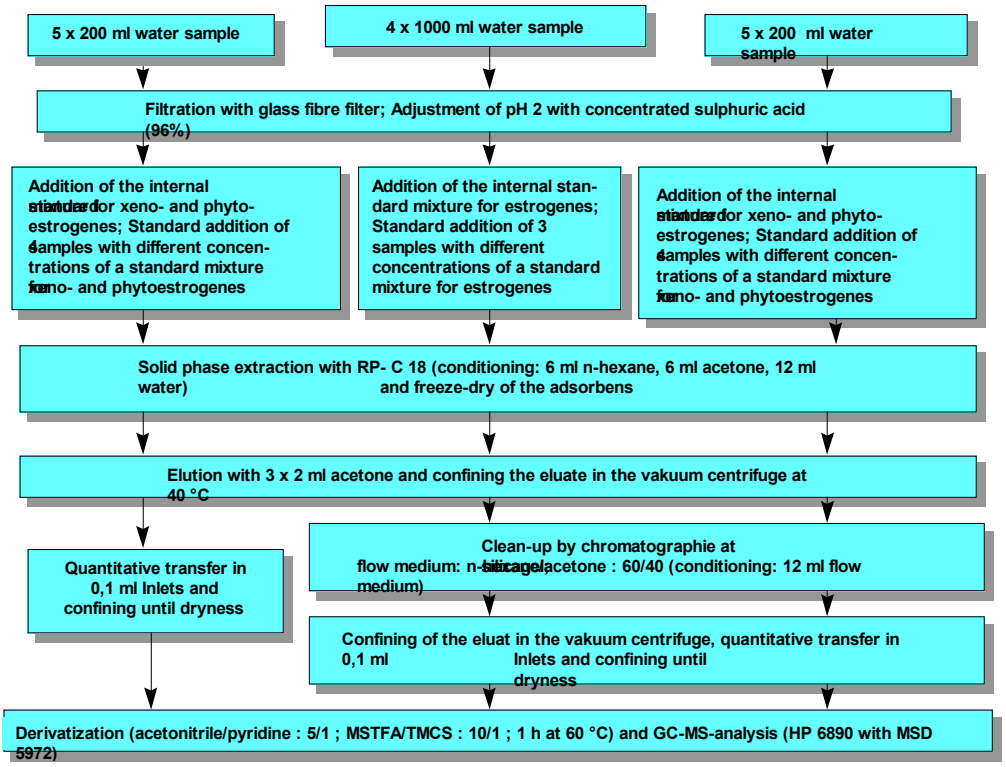


Figure 1: Procedure for the extraction and determination of steroidal estrogens and non-steroidal substances with estrogenic activity in effluents of municipal sewage plants.

Results and Discussion

The results of GC/MS analysis of effluent samples from nine sewage plants for various estrogenic chemicals, natural and synthetic estrogens, are shown in table 1. For the steroids, the limits of detection were about 1 ng/L (signal to noise ratio of 3:1) and the limits of quantification about 2 ng/L (S/R of 6:1). The natural estrogen 17β-estradiol (E2) and its major metabolite estrone were detectable in eight of nine effluent samples with median values of 2.9 and 4.3 ng/L, respectively. The synthetic estrogen 17α-ethinylestradiol (EE2) was found in similar concentrations like E2. The plastic softeners benzyl-n-butylphthalate (BBP) and di-n-butylphthalate (DBP) as well as the plastic monomer bisphenol A were present in all effluent samples in the upper ng/L range. The

median concentration of bisphenol A was 103 ng/L thereby confirming results of a recent study at one plant [6]. Although the use of nonylphenolpolyethoxylates as nonionic surfactants has been largely reduced in Germany since the eighties due to a voluntary agreement of the chemical industry, 4-nonylphenol is still ubiquitous in treated sewage in the upper ng/L range. Its metabolite 4-nonylphenoxyacetic acid (4-NP1EC), which is also estrogenic in the E-screen assay but with less potency (data not shown), was found in higher concentrations (median: 2 µg/L).

Table 1: Concentrations of estrogenic substances in sewage plant effluents in ng/L.

Plant Nr.	1	2	3	4	5	6	7	8	9	median
DBP	387	1015	531	818	585	372	257	738	206	531
BBP	119	62	123	107	284	402	450	80	n.a.	119
4-NP (sum)	360	604	331	747	955	2313	857	561	735	747
4-NP1EC	589	424	1698	2016	2475	5827	5477	2020	1986	2016
4-NP2EO	n.d.	n.d.	n.d.	n.d.	n.d.	3456	646	189	n.d.	n.d.
Bisphenol A	41	39	103	81	126	1044	52	113	136	103
17β-Estradiol	n.d.	2.1*	2.6	6.4	4.2	5.4	1.6	4.4	2.9	2.9
Estrone	2.3*	<1.9	n.d.	3.1	16.8	18.2	7.5	3.5	4.3	4.3
17α-Ethinyl-estradiol	2.4*	2.5*	1.9*	4.1	2.0*	12.2	2.7	<0.7	2.4	2.4

n.d. = not detectable n.a. = not analyzed
* values between limit of detection and limit of quantification

Table 2: Quantitative results of the E-screen test of 10 effluent samples: Estradiol equivalent concentration (EEQ) and relative proliferative effect (RPE). Each value represents mean (± SD) of three independent assays.

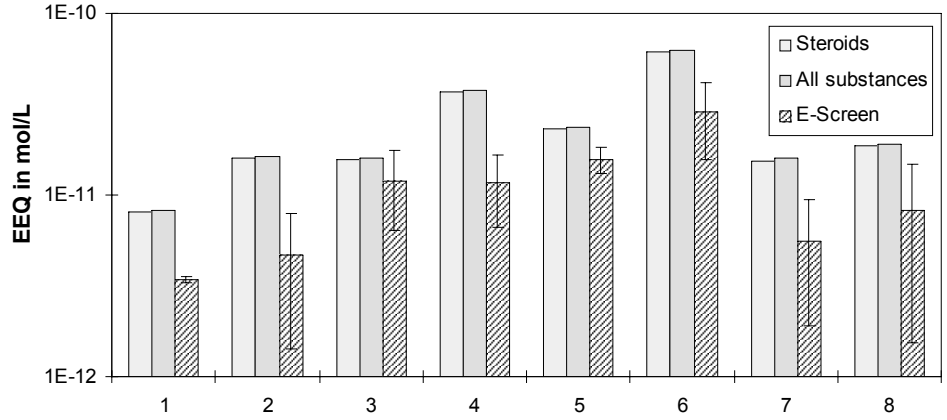
Plant Nr.	1	2	3	4	5	6	7	8	9	10	median
EEQ (ng/L)	0.94 (.03)	1.3 (0.9)	3.3 (1.5)	3.2 (1.3)	4.3 (0.7)	7.8 (3.5)	1.5 (1.0)	2.2 (1.8)	2.1*	3.3 (0.7)	2.7
RPE (in % E2)	26 (7)	31 (13)	48 (11)	54 (6)	34 (3)	67 (16)	59 (23)	47 (27)	58*	46 (10)	48

* mean of two independent E-screen assays.

Each effluent sample induced proliferation of ER-positive MCF-7 cells in a dose-dependent way. The proliferative effect relative to the positive control E2 was between 26 and 67%. The calculated estradiol equivalent concentrations (EEQ) were between 1 and 8 ng/L (median: 2.7 ng/L) and thereby lower than in a preliminary study with effluents from five municipal sewage plants which revealed EEQ levels between 2.5 and 25 ng/L [3].

For each detected substance the molar concentration was multiplied with its estrogenic potency relative to E2 determined in previous E-screen experiments (estradiol equivalency factor EEF), resulting in a estradiol equivalent concentration (EEQ) for the particular compound. The sum of EEQ values of the 3 steroidal estrogens and of all substances, respectively, was compared with the EEQ level calculated from the E-screen experiments (fig. 2). The EEQ values of chemical and biological analysis were in the same order of magnitude. For most samples, the results of GC/MS analysis were a factor of 2 to 3 higher. >97% of total estrogenic activity was caused by steroidal estrogens. Albeit their levels were much higher, the contribution of xenoestrogens to total EEQ value was less than 3% since their EEF is four to six orders of magnitude lower than that of E2.

Fig. 2: Comparison of estradiol equivalent concentrations (EEQ) of chemical and biological analysis



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