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Behavior and occurrence of estrogens in municipal sewage treatment plants — I. Investigations in Germany, Canada and Brazil

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Abstract

The developed method enables the quantification of estrogens in sewage samples down to 1 ng/l and in river water down to 0.5 ng/l. Mean recoveries of the analytes in ground water after SPE extraction, clean-up and derivatization generally exceeded 75%. The determined R.S.D. varied from 0 to 14% at a spiking level of 0.05 μg/l. Even in the raw influent and the final effluent from municipal STPs the mean recoveries of estrogens were mostly above 70%. Using this method the behavior and occurrence of natural estrogens and synthetic contraceptives in municipal sewage treatment plants (STP) were investigated in German and Canadian facilities. In the sewage of a German municipal STP close to Frankfurt/Main 17β-estradiol and estrone were determined, with mean concentrations of 0.015 μg/l and 0.027 μg/l, respectively. In two investigated municipal STPs, 17β-estradiol and 16α-hydroxyestrone were eliminated with a higher efficiency than 17α-ethinylestradiol and estrone. In Canadian and German STP discharges estrone, 17β-estradiol, 17α-ethinylestradiol and 16α-hydroxyestrone were frequently detected within the lower ng/l-range. A maximum concentration was found for estrone with 70 ng/l. In 15 investigated German rivers and streams only estrone was present with a maximum concentration of 1.6 ng/l.

Keywords: Estrogens; Contraceptives; Sewage treatment plants; Rivers; GC/MS/MS

1. Introduction

Many effects observed in the aquatic environment concerning the reproductive system, for instance the feminisation of male fish within sewage treatment plant (STP) effluents, are attributed to the presence of endocrine disrupters (Purdom et al., 1994; Sumpter and Jobling, 1995). The individual compounds which are responsible for these harmful effects are currently unknown, whereas many substances like nonylphenols, phthalic esters, PCBs, dioxins, phytoestrogens and human

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estrogens are suspected to influence the hormonal system (Roembke et al., 1996). Recently, it has been hypothesized that the statistically derived decrease in sperm counts over the last decades, increasing incidents of testicular cancer and other disorders regarding male infertility may be caused by the intake of estrogens via food or drinking water (Sharpe and Skakkebaek, 1993). However, there is a lack of data about the exposure of the aquatic environment as well as food and drinking water to estrogens, although the first results had already been published in 1970 (Tabak and Bunch, 1970).

Several drugs are used in medicine to influence the endocrine (hormonal) system. One well known example are the contraceptives utilized as ingredients of birth control pills. In general, municipal sewage and therefore excreted human pharmaceuticals and natural hormones have to pass through an STP prior to entering rivers or streams. In the literature little information is available dealing with the behavior of natural and synthetic estrogens in STPs (Norpoth et al., 1973; Tabak et al., 1981; Shore et al., 1993).

A potential contamination of soil and ground water may be caused by the application of digested sludge from municipal STP on agricultural fields. Additionally, transport of hormones via bank filtration from contaminated surface water into ground water, as well as the infiltration of waste waters directly from leakage in drains is possible. However, the principal pathway should lead to the municipal STP and after incomplete removal release into the receiving waters. In order to evaluate the potential risk of the endocrine disrupting, the occurrence of individual compounds needs to be documented. In vitro test systems like competitive ligand binding assays (e.g. yeast cell assay) or cell proliferation assays (e.g. E-screen assay) exhibited at least two orders of magnitude higher estrogenic activity for 17β-estradiol than for other potential endocrine disrupters like nonylphenols or PCBs (Jobling and Sumpter, 1993; Soto et al., 1994, 1995; Jobling et al., 1995; Arnold et al., 1996a,b; Zacharewski, 1997). Therefore, a precise quantitation of natural estrogens and contraceptives in the STP effluents is essential for a risk assessment regarding endocrine disrupting effects in the aquatic environment.

2. Materials and methods

2.1. Analytical methods

2.1.1. Solid phase extraction

The solid phase materials 0.10 g Lichrolut®-EN and 0.25 g RP-C_{18} (both from Merck Darmstadt, Germany) were successively filled into a glass cartridge and were then conditioned by flushing with 3×2 ml hexane, followed by 1×2 ml acetone and 3×2 ml methanol. The cartridges were then washed with 5×2 ml of water adjusted to pH 3. One litre of the sample was glass fibre filtered (< 1 μm) and spiked with 17β-estradiol-17-acetate from Sigma (Deisenhofen, Germany) as surrogate standard. After adjusting to pH 3 the native samples were sucked through the packed glass cartridges at a flow rate of ~ 20 ml/min. Subsequently, the solid phase was dried completely by a nitrogen stream for 1 h and the analytes were eluted four times with 1 ml of acetone. The acetone extracts were evaporated to 200 μl by a gentle nitrogen stream.

2.1.2. Silica gel clean-up

One gram of silica gel (silica gel 60, 70-230 mesh, from Merck deactivated with 1.5% water) was stirred in 4 ml of hexane and the slurry was manually filled into 6-ml glass cartridges. Before use, the silica gel column was rinsed by flushing with 10 ml hexane/acetone (65:35, v/v). The preconcentrated extracts were quantitatively transferred to the prepared 1 g silica gel column. Finally, the analytes were eluted using 6 ml of hexane/acetone (65:35, v/v) and the sample extracts were evaporated to dryness.

2.1.3. Derivatization

For the detection by GC/MS/MS, the extracts were derivatized by adding 50 μl of the derivatization mixture (*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA))/trimethylsilylimidazole (TMSI)/dithioerytrol (DTE), (1000:2:2;

v/v/w). MSTFA and TMSI were purchased from Sigma and DTE from Merck. After a reaction time of 0.5 h at 60°C the solution was evaporated to dryness by a gentle nitrogen stream and the residue was dissolved in 200 μl of hexane. Finally, 250 μg of mirex (from Promochem Wesel, Germany) was added to the final extract as internal standard.

2.1.4. GC and MS/MS detection — operating conditions

Separation and detection of the analytes was achieved using a GC/MS/MS system: Varian GC 3400 coupled to a Varian Saturn 4 mass spectrometer.

The gas chromatograph was equipped with a PTV-injector, which was coupled to a retention gap (1 m × 0.32 mm) and a capillary column (Restek Bad Soden, Germany; XTI-5/30 m × 0.25 mm × 0.25 μm). The head pressure was 60 kPa helium. The retention times, the selected ion masses and the collision induced dissociation (CID) resonant amplitudes are listed in Table 1.

GC injection parameters: 4 μl splitless; 50°C; 100°C/min — 300°C, 300°C isothermal 10 min. GC temperatures: 50°C isothermal 3.5 min, 20°C/min — 240°C, 2°C/min — 290°C, 290°C isothermal 10 min.

MS parameters: temperature of transfer line (direct interface): 280°C, ion source: EI mode, electron energy: 70 eV, ion trap temperature: 250°C, collision induced dissociation (CID) RF: 120 V, window for MS/MS: 3 m/z.

2.1.5. Determination of recoveries

One litre of a ground water which is not appreciably influenced by man-made organic compounds, as well as raw and treated sewage were spiked with 50 ng/l of the individual estrogens. The recoveries were determined in relation to a non-enriched standard solution, which was only derivatized. For evaluation, mirex a non-enriched internal standard, was used. SPE extraction, clean-up, derivatization and detection by GC/MS/MS were performed as described above.

2.1.6. Calibration, detection limits and blank samples

The calibration was performed over the whole procedure after spiking ground water with the standard mixture in order to adjust seven different calibration concentrations. One litre of ground water was extracted by SPE for analysing estrogens in native samples. Always seven point calibrations were used ranging from 0.5 ng/l to 150 ng/l. Detection limits allowing for the quantitation of analytes in native samples were set as the lowest or the second lowest calibration point of the linear correlation. As blank samples ground water spiked with the surrogate standard 17β-estradiol-17-acetate were included in every series of analysis.

2.2. Sampling of corresponding influents and effluents of STP

In a sampling period from 23 November to 30 November 1997 over a period of 6 days composite

Table 1
Precursor, product ions and retention times used in GC/MS/MS-detection

Substances	Retention time (min)	Precursor ion (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	CID resonant amplitude (V)
Mirex	20.34	272	–	–	–
Estrone	21.31	342	257	244	0.31
17β-Estradiol	22.03	416	326	285	0.37
Mestranol	23.06	367	349	193	0.37
17β-Estradiol-17-acetate	23.54	386	326	297	0.37
17α-Ethinylestradiol	24.28	425	231	193	0.31
16α-Hydroxyestrone	24.38	286	244	230	0.37
17β-Estradiol-17-valerate	30.31	428	326	297	0.41

samples of a German municipal STP were taken daily from the raw influent and the corresponding final effluent. Sampling was carried out by a flow proportional automatic sampler, whereby the composite samples of the final effluent were taken time related to the influent. The municipal STP near Frankfurt/Main is connected to about 312 000 population equivalents. It consists of three commonly used main treatment steps: preliminary clarification followed by an aerator tank with the addition of Fe(II)chloride for phosphate elimination and finally an end point clarification. The average flow rate at the sampling period was 41 200 m^3/day. All cooled water samples were analyzed within 2 days in order to keep microbial degradation to a minimum.

In another sampling period (16–20 June 1997; 23 June 1997) random samples from a Brazilian municipal STP, located in Penha/Rio de Janeiro, were taken over a period of 6 days at 08:00, 12:00, 18:00 h from the raw influent as well as from the effluents of the aerator tank and a parallel operating trickling filter, so-called 'biological filter'. All six daily samples from each site were combined. The municipal STP is connected to about 624 000 population equivalents. The sewage treatment plant consists of a preliminary clarification followed by an aerator tank or alternatively by the biological filter and finally an end point clarification. The average daily flow rate was 120 096 m^3; 71% was passing the aerator tank and 29% the trickling filter. All water samples were cooled immediately and were extracted using SPE within 5 days. The clean-up procedure and the detection by GC/MS/MS were conducted in Germany.

2.3. Sampling of STP effluents and rivers

Effluents of 16 municipal German STPs and 10 Canadian STPs, treating mainly household discharges, were sampled between 20 and 27 November 1997 and 3 and 13 November 1997, respectively. All STP consisted of three commonly used main treatment steps: preliminary and final clarification and an aerator tank. Additionally, the German STPs are equipped with phosphate elimination using $Fe(III)Cl_3$ or $Fe(II)Cl_2$, while the 10 Canadian STPs (all located in Ontario) used aluminum sulfate and a final disinfection step. Five Canadian STPs applied low-pressure and one plant medium-pressure mercury arcs. Further disinfection methods were chlorination by sodium hypochlorine (one plant) and chlorine (two plants). One Canadian STP used alternatively either low pressure UV-radiation or sodium hypochlorine. Daily composite samples were taken both from the German and Canadian STPs. Samples were cooled immediately and were extracted within 2 days.

Random samples were taken from the German rivers Lahn (Limburg), Main (Kostheim), Rhine (Wiesbaden), Nidda (Höchst), Elbe (Hamburg) as well as from nine streams mostly located in the Hessian Ried area (close to Frankfurt/Main). From the stream Schwarzbach (Trebur), a daily composite sample was investigated. Sampling was carried out between March 30, and April 1, 1998.

3. Results and discussion

3.1. Recoveries and detection limits of the analytical method

Mean recoveries of the analytes in ground water after SPE extraction, clean-up and derivatization generally exceeded 75% at a spiking level of 0.05 μg/l (Table 2). Only 16α-hydroxyestrone had a lower recovery (41%) relative to the other estrogens. The sufficient precision and reproducibility of the applied analytical method for these estrogens is indicated by a relative standard deviation varying from 0% to 14%.

Even for the case of the raw influent and the final effluent from municipal STPs, the method allows mean recoveries of estrogens frequently above 70%. The lower recoveries observed for 16α-hydroxyestrone and 17β-estradiol-17-valerate were presumably the result of two factors, incomplete adsorption by the used SPE-material and loss by the silica gel clean-up procedure. For instance, 17β-estradiol-17-valerate had only an 80% recovery rate after SPE-extraction, whereas the other analytes were quantitatively extracted above 93% at a spiking level of 0.05 μg/l (16α-hydroxyestrone was not included in this investigation).

Table 2
Recovery of various estrogens in % (all spiked with 50 ng/l; ground water: $n = 3$; raw sewage: $n = 2$; STP effluent: $n = 2$)

Spiked in	Mean value[b] ground water (%)	R.S.D.[a] (%)	Mean value raw sewage (%)	Mean value STP effluent (%)
Estrone	90	2	86	82
17β-Estradiol	77	14	84	76
Mestranol	90	1	77	74
17α-Ethinylestradiol	85	0	88	76
17β-Estradiol-17-valerate	75	13	58	56
16α-Hydroxyestrone	41	9	56	52
17β-Estradiol-17-acetate	88	3	–	–

[a] Relative standard deviation.
[b] Evaluation using mirex as internal standard.

The developed method allows the quantification of the estrogens in sewage samples down to 1 ng/l and in river water down to 0.5 μg/l. This high sensitivity was possible due to the effective clean-up step and the MS/MS-detection. The silica gel clean-up removes most of the co-extracted impurities like humic acids. The improved selectivity of MS/MS-detection was favorable for identification and quantification. The baseline could be enormously reduced and hence the signal to noise ratio increased. Especially the improved confirmation using GC/MS/MS exhibits an important advantage of this powerful novel technique. For instance, in Fig. 1 the EI-spectra of 17α-ethinylestradiol and an unknown impurity are shown. Both compounds exhibited exactly the same retention time and both EI spectra showed the m/z values of 440 (molecular weight of silylated 17α-ethinylestradiol) and 425 ($M_W - CH_3$), however, with a different ratio. Using MS/MS-detection of the target ion m/z 425, a confirmation with regard to identification and quantification of 17α-ethinylestradiol can be carried out. Due to the fact that the MS/MS-spectra of the contraceptive and the unknown impurity are different, a precise quantitation is possible using the product ions m/z 193 and m/z 231 of the precursor ion 425 m/z. For the unknown compound the excitation energy used was not appropriate to decompose the target ion m/z 425 completely and the product ions m/z 193 and m/z 231 were not formed. Using single MS detection the probability to determine excessive concentrations of 17α-ethinylestradiol is relative high. It cannot be excluded that this was the reason for the higher concentrations of 17α-ethinylestradiol in German STP discharges detected by Stumpf et al. (1996).

3.2. Elimination of estrogens after passage through a German and Brazilian municipal STP

In the raw sewage of the Brazilian STP of Penha/Rio de Janeiro the natural estrogens 17β-estradiol and estrone were detected with average concentrations of 0.021 μg/l and 0.040 μg/l, respectively, yielding a load up to 5 g/day (Fig. 2). The observed removal rates ranged from 64% for 17α-ethinylestradiol in the effluent of the 'biological filter' to above 99.9% for 17β-estradiol in the effluent of the aerator tank. Estrone and 17α-ethinylestradiol were comparably eliminated up to 83% and 78%, respectively. The activated sludge treatment step obviously removed the estrogens with a higher level of efficiency than the biological filter (trickling filter).

In the German municipal STP close to Frankfurt/Main the raw sewage was contaminated by 17β-estradiol and estrone with average concentrations of 0.015 μg/l and 0.027 μg/l, respectively, yielding loads of up to 1 g/day (Fig. 3). The evaluated removal rates were much lower than those obtained in the Brazilian STP. For instance, the loads of estrone and 17α-ethinylestradiol were not appreciably reduced while passing through the German STP. Considering the standard deviation no elimination rate could be evaluated. However, 16α-hydroxyestrone and 17β-estradiol were

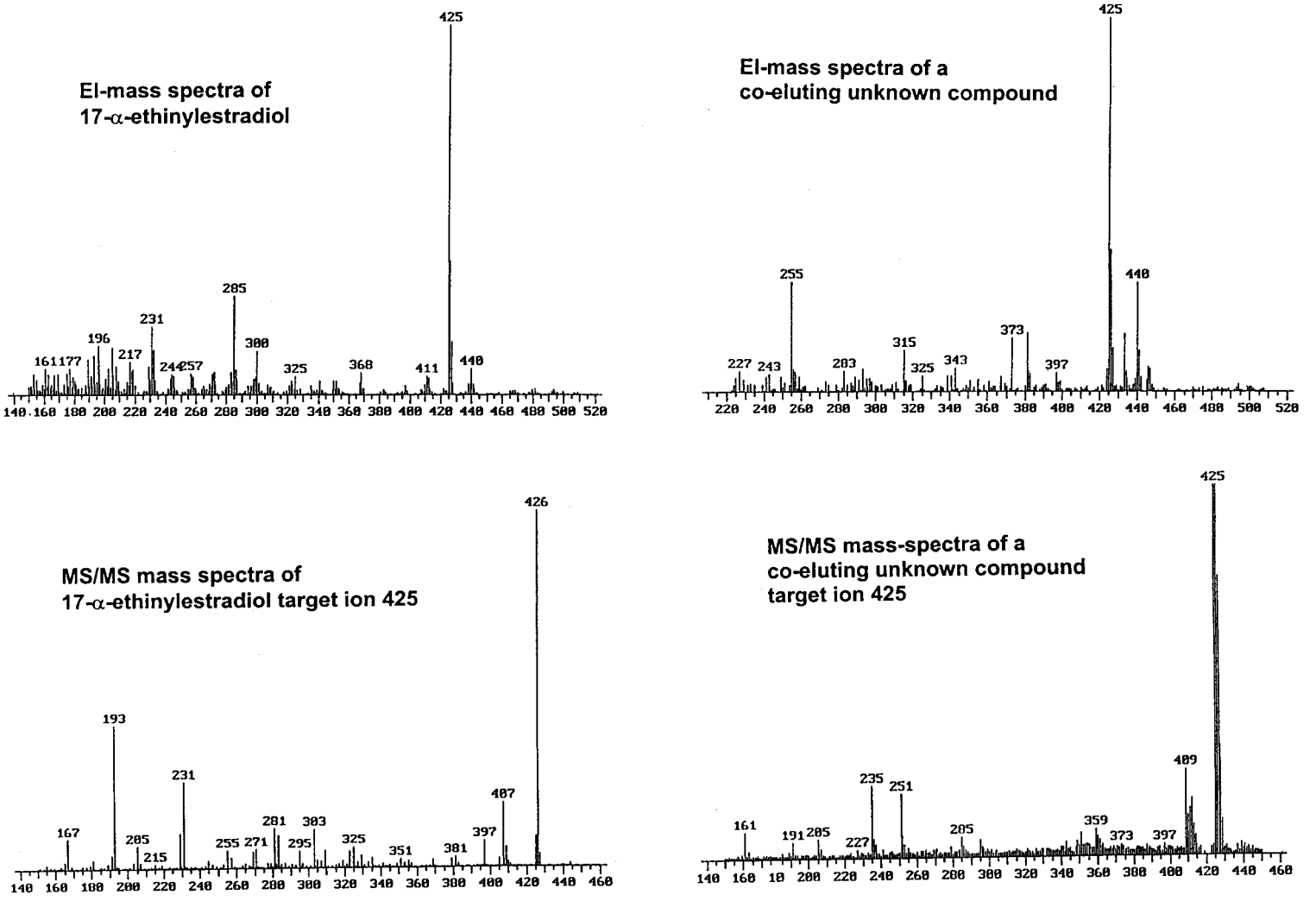

Fig. 1. MS- and MS/MS-spectra of 17α-ethinylestradiol and an unknown co-extracted impurity.

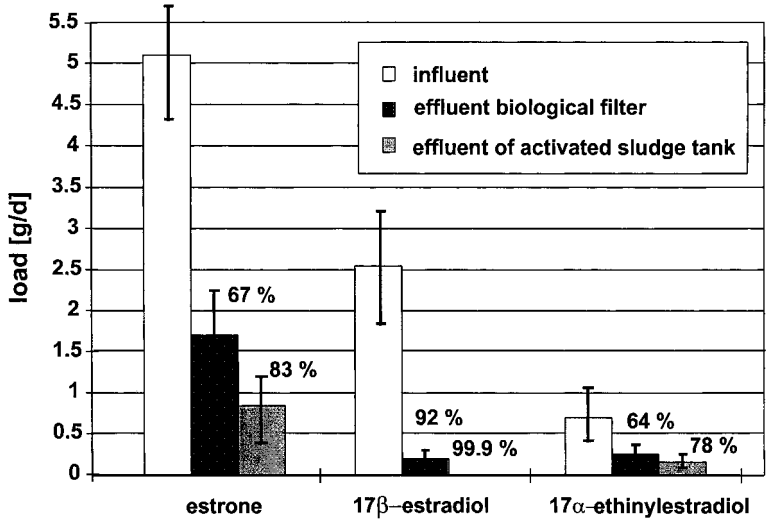

Fig. 2. Elimination in % and loads of estrogens during passage through a municipal sewage treatment plant located in Penha, RJ, Brazil over 6 days. Sampling periods: 16–20 June 1997; 23 June 1997.

removed, with reductions in concentrations of 68% and about 64%, respectively. The differences between the absolute removal rates of the German and Brazilian STP might be caused by the low temperatures in the German sampling period with −2°C on average compared to above 20°C in Rio de Janeiro. Due to the fact that the efficiency of an STP for the elimination of drugs is influenced by several parameters such as microbial activity or rain events (Ternes, 1998), only a long term study can reveal whether the differences are as a result of increased temperatures or whether other contributory factors were responsible. However, 17β-estradiol and 16α-hydroxyestrone were

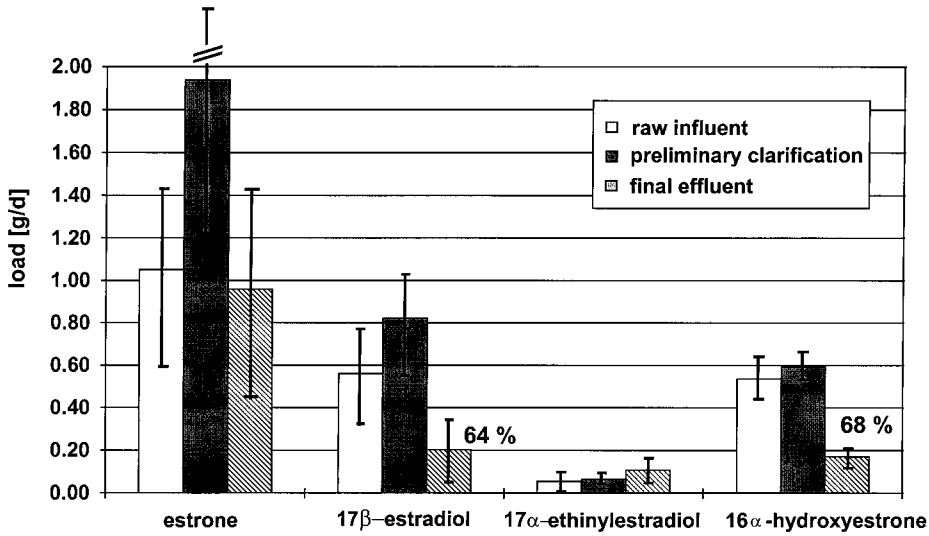

Fig. 3. Elimination in % and loads of estrogens during passage through a municipal sewage treatment plant located near Frankfurt/Main over 6 days. Sampling periods: 23–30 November 1997.

basically eliminated with a higher efficiency than 17α-ethinylestradiol and estrone. After preliminary clarification, which generally removes only the solid materials of the sewage, the loads of estrone and 17β-estradiol in the German STP is elevated relative to the inflow (Fig. 3), although the error bars overlap. It appears likely that depending on the processing time conjugates like glucuronides, which are the principal excreted metabolites, are cleaved in the STP. Therefore, in order to explore the behavior of estrogens in the municipal STP, batch experiments using activated sludge from a real STP were carried out in Ternes et al. (1999).

3.3. Examination of STP discharges and rivers

In STP discharges mainly the natural estrogens estrone, 17β-estradiol, 16α-hydroxyestrone as well as the synthetically altered contraceptive 17α-ethinylestradiol could be measured in the lower ng/l-range. Mestranol was only present in two samples and 17β-estradiol-17-valerate could not be detected at all (Table 3).

In German STPs predominantly estrone was determined with a maximum concentration of 0.070 μg/l and a median value of 0.009 μg/l. Additionally, median values could be evaluated for 17α-ethinylestradiol and 16α-hydroxyestrone, both with 0.001 μg/l (detection limit). In comparison, the Canadian STP discharges were contaminated in the same ng/l-range by these natural and synthetic estrogens. Median concentrations of 17β-estradiol and 17α-ethinylestradiol were higher and of estrone were lower in Canadian effluents compared to those determined in the German STP effluents. However, in order to assure that these differences are significant, further comparable investigations are essential. Especially the concentrations of 17α-ethinylestradiol in the Canadian STP discharges were relatively high (median: 0.009 ng/l). Due to the small number of samples, further screening programs are necessary to substantiate these results. The contamination of STP discharges are within the concentration ranges found by Aherne and Briggs (1989), Desbrow et al. (1998) and Routledge et al. (1998). In the UK, Desbrow et al. (1998) reported that natural estrogens like 17β-estradiol or estrone were dominant in STP effluents compared

Table 3
Concentrations of estrogens in STP effluents

Conc. substances (μg/l)	LOD[a]	German STP effluents				
		Samples	$n >$ LOD	Median (μg/l)	90-percentile (μg/l)	Maximum (μg/l)
Estrogens						
17β-Estradiol-17-valerate	0.004	16	0	n.d.	n.d.	n.d.
Estrone	0.001	16	14	0.009	0.022	0.070
16α-Hydroxyestrone	0.001	15	11	0.001	0.004	0.005
17β-Estradiol	0.001	16	8	n.d.	0.002	0.003
17α-Ethinylestradiol	0.001	16	9	0.001	0.004	0.015
Mestranol	0.001	16	3	n.d.	0.001	0.004
Conc. substances (μg/l)	LOD[a]	Canadian STP effluents				
		Samples	$n >$ LOD	Median (μg/l)	90-percentile (μg/l)	Maximum (μg/l)
Estrogens						
17β-Estradiol-17-valerate	0.004	10	0	n.d.	n.d.	n.d.
Estrone	0.001	10	8	0.003	0.010	0.048
16α-Hydroxyestrone	0.001	0	–	–	–	–
17β-Estradiol	0.001	10	8	0.006	0.014	0.064
17α-Ethinylestradiol	0.001	10	9	0.009	0.029	0.042
Mestranol	0.001	10	0	n.d.	n.d.	n.d.

[a] Limit of detection in μg/l (quantification limit).
n.d., not detectable (below LOD).

to the synthetic 17α-ethinylestradiol and mestranol. Differences arise with regard to the results reported by Tabak et al. (1981), who determined much higher concentrations of 17α-ethinylestradiol yielding up to the μg/l-range in STP effluents. It cannot be determined whether changes in prescriptions of the contraceptive 17α-ethinylestradiol in the USA over the last two decades or other factors were responsible for the enormous differences reported. However, in 1997/98 the contamination of German, Canadian and Britain STP discharges by 17α-ethinylestradiol are between one and two orders of magnitude lower than those reported by Tabak et al. (1981). Whether the concentrations of estrogens determined in the lower ng/l-range are sufficiently high to cause environmental endocrine effects has to be explored in further investigations.

Estrone was the only estrogen detected in rivers and streams investigated in this study. In three of 15 German rivers estrone was detected between 0.7 and 1.6 ng/l (Table 4). Other investigated natural estrogens and contraceptives were not detected, although the detection limit was as low as 0.5 ng/l. This was presumably caused by the low concentrations in the municipal STP effluents and the dilution in rivers and streams.

4. Conclusions

The natural estrogens 17β-estradiol, estrone and the metabolite 16α-hydroxyestrone as well as the contraceptive 17α-ethinylestradiol were frequently detected in STP discharges, due to their incomplete removal during passage through the STP. However, the concentrations were mainly in the lower ng/l-range, so that the loads which enter the receiving waters are relatively small. The detection of 17β-estradiol and 16α-hydroxyestrone in STP discharges was unexpected, due to their behavior in contact with activated sludge (see Ternes et al., 1999). Therefore, it is a topic for further investigation, whether the insufficient STP processing time, a late cleavage of glucuronides or other reasons are responsible for the contamination. One possible approach to isolate and identify the main endocrine disruptors is based on the application of the TIE (Toxicity Identification and Evaluation) as described by Desbrow et al. (1998). Using TIE the authors identified natural and synthetic estrogens in British municipal STP discharges. Therefore, estrogenic effects observed downstream to STP discharges might be caused by the detected levels of natural estrogens and 17α-ethinylestradiol. However, the relation of concentrations and estrogenic effects of these estrogens has to be compared to those of other potential endocrine disrupting compounds. Whether the extremely small concentrations of estrone up to 1.6 ng/l detected in German rivers might cause endocrine effects is questionable, because fish and other aquatic animals exhibit naturally low concentration of estrone and 17β-estradiol. In any case, further investigations are essential to enlighten these aspects confidently.

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Table 4
Concentrations of estrogens in 15 German rivers and streams

	LOD[a] (μg/l)	Rivers	n > LOD	Median (μg/l)	90-percentile (μg/l)	Maximum (μg/l)
Estrogens						
17β-Estradiol-17-valerate	0.0020	15	0	n.d.	n.d.	n.d.
Estrone	0.0005	15	3	n.d.	1.0	1.6
17β-Estradiol	0.0005	15	0	n.d.	n.d.	n.d.
17α-Ethinylestradiol	0.0005	15	0	n.d.	n.d.	n.d.
Mestranol	0.0005	15	0	n.d.	n.d.	n.d.

[a] Limit of detection in μg/l (quantification limit).
n.d., not detectable (below LOD).

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