Assessing the elimination of estrogenic activity in advanced wastewater treatment with a reporter gene-based bioassay

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Abstract Estrogen-like chemicals, so-called xenoestrogens, have become a topic of concern because they are potentially capable of disturbing the hormonal balance of wildlife and humans. Effluents of wastewater treatment plants (WWTPs) are presumably the major source of xenoestrogens in the aquatic environment. In this study, we investigated eight WWTPs with respect to their input, elimination efficiency, and output of estrogenic activity by means of a reporter gene-based bioassay. All WWTPs employed activated sludge treatment with nitrification/denitrification and tertiary treatment (second nitrification and/or filtration). Estradiol equivalents (EEQs) in the influents of the WWTPs were between 5.7 and 65.8 ng/L. The greatest inputs were found in plants treating pure domestic sewage and in samples collected in winter. Process waters either had no estrogenic activity or EEQs in the range of raw sewage, depending on the source of the process water. EEQs of effluents ranged from mostly below quantification limit (0.8 ng/L) to a maximum of 5.4 ng/L in secondary and 1.4 ng/L in tertiary effluents. These findings demonstrate the elimination efficiency of the activated sludge treatment and the further improvement by additional tertiary treatment. However, several concentrated effluents elicited little, but detectable estrogenic responses in the bioassay.
Keywords Activated sludge treatment; endocrine disruption; estradiol equivalents; reporter gene assay; sewage treatment; xenoestrogens

Introduction

The quality of discharges of wastewater treatment plants (WWTPs) has improved in recent years due to treatment methods introduced to decrease the pollution of surface waters with nutrients and organic pollutants. Among these organic pollutants, endocrine disrupting chemicals (EDCs) have received growing attention. Because of the specificity and susceptibility of the hormonal system, even low concentrations of EDCs remaining in treated wastewaters may affect aquatic wildlife (Purdom *et al*., 1994). The initial concentrations of estrogenic substances in the influents of WWTPs are determined by the type of wastewater, of which some are primarily domestic while others are predominated by industrial sources. The removal of estrogenic substances from wastewaters depends on the load in the influent as well as the treatment method employed. Both parameters have been investigated in recent studies, but there is still little knowledge on how elimination of these compounds can be increased by improving the treatment methods. WWTPs with primary treatment (mechanically treatment including primary settlement) remove only minor amounts of estrogenic active substances (Kirk *et al*., 2002). Biological treatment appears to be the decisive step where compounds are either degraded or sorbed into the sludge and removed in the secondary settling step (Matsui *et al*., 2000; Tanaka *et al*., 2001; Kirk *et al*., 2002). Within the biological treatment methods, the activated sludge treatment attains better elimination rates than does treatment with trickling filters, whereas advanced methods such as membrane filtration or activated carbon treatment have achieved even better results

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(Takigami *et al*., 2000; Körner *et al*., 2001). The effect of tertiary treatment steps such as sand filtration on the removal of EDCs had not yet been systematically investigated, even though many WWTPs perform advanced tertiary treatment in order to meet effluent requirements, particularly for nutrient removal. This study was conducted to assess the elimination of estrogenic activity in WWTPs employing advanced treatment methods, especially a tertiary treatment step. Since not all estrogenic substances present in wastewaters are known, a bioassay was used to measure the total estrogenic activity regardless of the single responsible substances.

Methods

The main characteristics of the investigated WWTPs, all located in Northrhine-Westfalia, Germany, are shown in Table 1. All plants used a combination of mechanical pretreatment of sewage, activated sludge treatment and at least one additional filtration step as tertiary treatment. In some plants, a second nitrification was performed before filtration. Phosphate was biologically removed or by precipitation and biological treatment included always nitrification and denitrification. In contrast to all other plants, the three plants employing simultaneous aerobic sludge stabilization had no primary settlement step before the biological treatment.

Sampling and extraction

At all WWTPs samples (0.5 L) of influent were collected after preliminary mechanical treatment but before primary settlement, except for plants A, B, and E, where samples were collected before the effluent entered activated sludge treatment. On some sampling dates, 1 L samples of secondary effluent (final settlement tank) were collected and on all sampling dates 1 L samples of tertiary effluent were taken in all WWTPs but plant A, which did not employ a final settlement because of the membrane filtration process being integrated in the biological treatment tank. All samples were taken as grab samples and, when possible, additional parallel 24-h-composite samples were collected by the WWTP staff. To determine the effect of recirculation, process waters of the sludge treatment were also collected and assessed for estrogenic activity.

The extraction procedure is described in detail in Coors *et al*. (2003). Briefly, raw sewage and process waters were filtered with glass fiber filters and the filters were Soxhlet-extracted with acetone. Aqueous samples were extracted using solid phase columns

Table 1 Characteristics of the investigated wastewater treatment plants

Plant	Inhabitant equivalents	Type of wastewater	Sludge retention time [days]	Sludge stabilisation method	2nd nitrification step	Final filtration
A	3,000	Pure municipal	~ 40	Simultaneous aerobic	No	Yes (membrane)
B	3,000	Pure municipal	~ 35	Simultaneous aerobic	No	Yes
C	8,500	Pure municipal	7–8	Anaerobic digestion	Yes	Yes
D	29,000	~ 10% wastewater from textile industry	~ 23	Anaerobic digestion	No	Yes
E	34,000	~ 10% industrial and hospital wastewater	~ 16	Simultaneous aerobic	Yes	Yes (biofilter)
F	67,000	~ 30% industrial wastewater (e.g. textile industry)	Not available	Anaerobic digestion	Yes	Yes
G	425,000	~ 20% industrial wastewater (e.g. paper industry)	~ 8	Direct incineration	No	Yes
H	458,000	~ 50% industrial wastewater (e.g. food industry)	~ 10	Anaerobic digestion	Yes	Yes

(200 mg Isolute ENV+, International Sorbent Technology, UK) and eluted with acetone. Acetone was removed and the extracts were resuspended in ethanol, filter sterilized, and reduced to 1 mL. Laboratory blanks (deionized water, Millipore) were co-extracted on all sampling dates. The efficiency of the extraction method was assessed with standard solutions of estradiol (E2), estriol (E3), and ethinylestradiol (EE2) added to Millipore water.

Bioassay and data evaluation

Human breast cancer cells stably transfected with an estrogen-responsive reporter gene coding for firefly luciferase were used to detect estrogenic activity (Pons *et al.*, 1990). Cell culture and bioassay procedures were described in detail elsewhere (Snyder *et al.*, 2001; Coors *et al.*, 2003). In brief, for the bioassay MVLN-cells were seeded into 96-well plates with 250 µL of hormone-free cellculture medium. After 24 h incubation, the cells were dosed with 2.5 µL of the extracts or dilutions thereof. Cells were incubated with extracts, standards or as blanks for 72 h, then luminescence was measured and thereafter the protein content, which was used as an index of cytotoxicity. The mean maximum response of the positive control (1,000 pM E2) was set to 100% and the relative light units (RLUs) of all other samples were expressed relative to this maximum response in the same assay (RLU in %E2max). RLUs of the estradiol standard curve were logit- and concentrations log-transformed and a linear regression was calculated for each assay. From the equation of this linear regression the estradiol equivalent (EEQ) was calculated for each extract dilution and the results were pooled to calculate the mean EEQ of each treatment step for each sampling date. Only replicates with both an RLU value between 20 and 80% of the maximum response achieved with estradiol (equivalent to the linear part of transformed dose-response curves) and a protein content of more than 75% of the negative control were included in the calculation of EEQs. The exposure concentration in the bioassay was related to the original concentration of the samples by the concentration factor (concentration factor = exposure concentration/concentration of original sample).

Results and discussion

Estradiol equivalent calculation

The concept of calculating estrogen equivalents (EEQs) by means of an indirect bioassay inherently requires that the dose-response curves of the standard (in this case 17ß-estradiol) and the sample are parallel and that both reach the same maximum response at some concentration (Villeneuve *et al.*, 2000). As can be seen from the selected dose-response curves determined for extracts of raw sewage, these assumptions were not always met (Figure 1). The decrease of the induction at greater concentration factors is, together with a decrease in protein content (data not shown), an indication of increasing cytotoxicity the more concentrated the extracts were tested. Due to cytotoxicity and due to precipitates that occurred in the extracts, no sample could be tested at concentrations sufficiently great to result in maximum reporter gene induction. The occurrence of sub-maximal induction has also been reported for other bioassays (e.g. Tanaka *et al.*, 2001). Incomplete dose-response curves and the resulting need for extrapolating beyond measured responses generally cause uncertainties in relative potencies of estrogenicity evaluated by the ratio of half-maximal responses (EC50 ratio). However, the chosen method to calculate EEQs did not require extrapolations beyond measured responses and is therefore less affected by sub-maximal dose-response curves. The dose-response curves of influent extracts were in principle parallel to that of the standard E2 (Figure 1). Only at greater concentration factors did dose-response curves became flatter than the standard curve. Therefore, EEQs calculated at greater concentration factors tended to underestimate the EEQ in the investigated wastewater. Since all dilutions with a bioassay response of at least 20% E2max on the

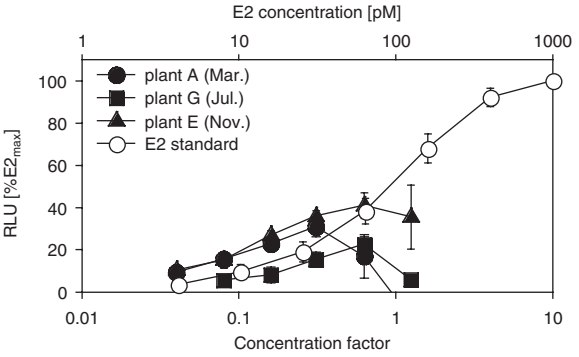

Figure 1 Dose-response curves of selected raw sewage extracts and the standard 17β-estradiol (E2) assessed with the MVLN-cell bioassay. Given are means and standard deviations of at least three independent assays as relative light units in relation to the maximal induction with E2 (RLU in %E2max)

increasing part of the respective dose-response curves were included in the calculation of the mean EEQ, the difference between EEQs calculated for respective concentration factors were greater than they would have been if the dose-response relationships for samples and standards had been strictly parallel and thus resulted in the rather large standard deviation of the mean EEQs (Table 2). However, the almost parallel dose-response curves enabled calculation of dose-equivalent concentrations of E2 for these wastewater samples.

Recovery of the extraction method

The efficiency of the extraction method was assessed by use of the synthetic estrogen ethinylestradiol (EE2) and the natural estrogens estradiol (E2) and estriol (E3). These estrogens were selected to assess the extraction and quantification method for compounds with a broad range of polarities and because of their relevance in wastewaters (Johnson and Sumpter, 2001). Results of extraction recovery experiments are shown in Figure 2. Extracts prepared from spiked deionized water induced responses from 84.4 to 96.2% and from 55.9 to 66.0% when spiked at levels equal to the induction of a 100% and 50% response, respectively. There was no difference between filtered and unfiltered samples and no estrogenic activities were observed in Soxhlet extracts of the filters, which indicates that filtration with glass fiber filters did not affect recovery of the spiked substances. Further experiments will clarify whether the recovery of estrogenic substances from wastewater samples is as good as that from pure water. Little estrogenic activity was observed in only a few Soxhlet extracts of wastewater samples, which indicates that (xeno)estrogens either did not sorb to suspended solids or could not be completely extracted with the chosen method. Neither laboratory blanks from the recovery experiments nor blanks extracted in parallel to wastewater samples showed estrogenic responses in the bioassay.

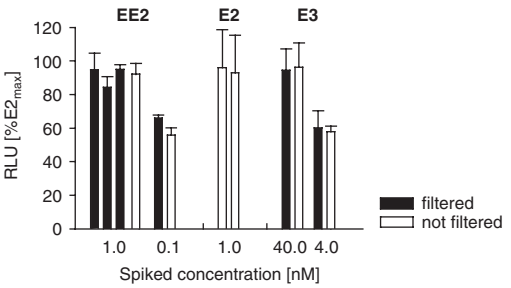

Figure 2 Recovery of ethinylestradiol (EE2), estradiol (E2), and estriol (E3) from spiked deionized water

Table 2 Mean estradiol equivalents ± SD (EEQs in ng/L) calculated from the MVLN-bioassay for influent grab and 24-h-composite samples (in brackets) of different wastewater treatment plants

	Sampling dates (2001–2003)					
Plant	**Jan./Feb.**	**Mar./Apr.**	**May/Jun.**	**Jul./Aug.**	**Sept./Oct.**	**Nov./Dec.**
A		65.8 ± 27.1		33.2 ± 14.6	36.0 ± 12.9 (36.9 ± 12.3)	
B	45.5 ± 18.8			28.9 ± 9.9		
C	14.6 ± 9.2					5.7 ± 1.6 (12.6 ± 5.1)
E			34.0 ± 9.8 (30.6 ± 12.2)	29.7 ± 9.1		59.7 ± 26.0 (25.3 ± 11.5)
G	13.1 ± 2.5	33.3 ± 17.4		17.4 ± 4.0 (14.8 ± 3.6)		
H				25.9 ± 4.9		

WWTP influents

Mean estradiol equivalents (EEQs) of raw sewage samples ranged from 5.7 to 65.8 ng/L (Table 2). These findings are consistent with published EEQs values of up to 100 ng/L in wastewater influents (e.g. Tanaka *et al.*, 2001; Kirk *et al.*, 2002). There was little difference between grab samples and respective 24-h composite samples despite the two samples taken at plants C and E in November/December. In almost all plants, the EEQ of samples taken in winter was greater than that of those taken in summer. The only exception was plant G, where the sample taken in March showed the greatest estrogenic activity. A possible explanation is the influence of rainfall, since indeed plant G was the only one which was not always sampled during dry periods. Degradation of estrogenic substances begins in the sewer system (Birkett and Lester, 2003). Because this process is influenced by temperature, higher temperatures in summer would be expected to result in enhanced degradation in the sewage system and, therefore, in the winter more estrogenic active substances pass the sewer without degradation and could have caused the measured peaks in influent estrogenicity. The greatest EEQs in influents were observed in samples from plants A and B, which treated only municipal wastewater, and plant E, which received a large proportion of municipal sewage together with wastewater from a hospital and some minor industries. This finding supports the hypothesis that mainly synthetic and natural estrogens present in domestic sewage are responsible for observed estrogenic activities (Johnson and Sumpter, 2001). However, plant C also received pure municipal sewage and had the least EEQ. Therefore, knowledge about the proportion of domestic wastewater in the influent may not be enough to predict the estrogenicity. Both paper and textile industries have been suggested as possible sources of known xenoestrogens, e.g. bisphenol A and nonylphenol (Ahel *et al.*, 2000; Fürhacker *et al.*, 2000). WWTPs receiving partly wastewaters from these industries showed in our investigation rather little estrogenic activity in the influent compared to WWTPs treating mainly municipal wastewater. Especially in plants D and F, the response of influent extracts in the bioassay was insufficient to calculate an EEQ (data are therefore not included in Table 2). These two plants with the least concentrations of EEQs in the influent were both treating a large proportion of wastewater from the textile industry. However, the low responses of these samples in the bioassay could also have been due to the greater toxicity of the extracts and/or to the presence of anti-estrogenic substances. Extracts of influents from plants D and F were toxic to the MVLN cells at concentration factors where samples from most other plants were eliciting estrogenic effects in a dose-dependent manner. The presence of anti-estrogenic substances cannot be proven, but antagonistic effects may occur in environmental samples (Körner *et al.*, 2001; Snyder *et al.*, 2001). It can be concluded that the tested wastewaters partly from industrial sources,

especially those from the textile industry, possessed little estrogenic activity or contained large amounts of toxic or antagonistic substances that may have interfered with the detection of estrogenic activity.

WWTP effluents

The estrogenic activity of WWTP effluents is shown in Figure 3 as reporter gene induction of ten-fold concentrated samples relative to the maximal induction by E2. Most samples of secondary and tertiary effluents showed little, but detectable estrogenic activity. In two plants (F and H) cytotoxicity hindered the detection of potential estrogenicity in the tertiary effluents. A tendency of greater estrogenic activity in effluents collected in winter was observed. This trend was more pronounced in secondary than in tertiary effluents. Overall, estrogenic activity was less in tertiary (median response: 8.8%E2max) than in secondary effluents (median response: 14.9%E2max). Thus, tertiary treatment was able to further reduce the estrogenicity of biologically treated wastewater. WWTPs with a 2nd nitrification step (C, E, F, and H) had a median response of 16.2% in the secondary and a median response of 9.1% in the tertiary effluent, whereas plants with only sand filtration (B, D, and G) had a median responses of 13.1 and 11.8% in secondary and tertiary effluents, respectively. Therefore, the 2nd nitrification step seemed to improve the efficiency of tertiary treatment, since the reduction in estrogenicity was greater than that achieved with only sand filtration. The greatest responses were obtained in secondary effluents of plants B and E, both operating with aerobic simultaneous sludge treatment which results in a rather long sludge retention time (SRT, Table 1). Tertiary effluents of these plants still contained measurable concentrations of estrogenic compounds. Even in plant A, which had the longest SRT and employed membrane filtration, estrogenic activity was found in the effluent. A long SRT was thought to be advantageous to the degradation of estrogenic substances, because slowly growing nitrifying bacteria, which were proposed to be involved in the degradation process of at least ethinylestradiol (Vader *et al.*, 2000), would be favored. This hypothesis was not supported by the results of our study, since no clear correlations between SRT and estrogenic output were observed. However, the ability to demonstrate a

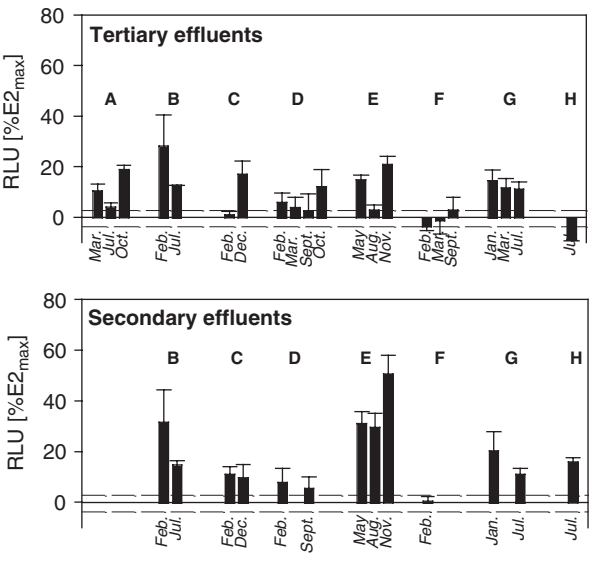

Figure 3 Estrogenic activity of tenfold concentrated effluent extracts from wastewater treatment plants A to G. Means and standard deviations of at least three independent assays are reported relative to maximal induction with estradiol (%E2max). The dotted lines indicate the threefold standard deviation of negative controls

correlation may be hampered by the fact that WWTPs with aerobic simultaneous sludge treatment and long SRTs (plants A,B, and E) were also the ones which received the greatest input of estrogenic compounds. Only a few samples resulted in an induction of more than 20%. Therefore it was seldom possible to calculate EEQs or elimination rates in order to compare the efficiency of the WWTPs. Concentrations of EEQs in effluents ranged from less than 0.8 (quantification limit) to 1.4 ng/L in tertiary and 5.4 ng/L in secondary effluents, which are both values in the lower range of published data. Other studies reported maximal EEQs up to 3.3 ng/L or even up to 15 ng/L (Körner *et al.*, 2001; Matsui *et al.*, 2000, respectively) in the effluents of WWTPs with activated sludge treatment.

The elimination rate (tertiary effluents in relation to WWTP influents) was always greater than 94% with one exception of only 82% reduction. Secondary treatment alone achieved elimination of less than 90% on two sampling dates, but more than 91% on all other dates. Since the sampling of influents and effluents did not take into account the hydraulic retention times within the plant, the calculation of the elimination rate from grab samples may be skewed by fluctuations in wastewater composition. However, the comparison of 24-h-composite samples and grab samples showed for influent as well as effluent samples little differences which allowed us to calculate the elimination rates from grab samples (Table 3). In only one plant (E) were the EEQs in the grab and composite samples differed from each other with grab samples having the greater response in the bioassay.

WWTP process waters

In all WWTPs except for plant A, process waters originating from the dewatering of anaerobic digested or undigested sludge were returned to the biological treatment step. EEQs from these process waters of different sources are reported in Table 4. The greatest amounts were found in the process water of plant D and H and minor amounts in other process waters originating from the dewatering of anaerobic digested sludge. Process waters from the dewatering of undigested sludge contained no estrogenic activity with the exception of that from plant G. This difference indicates that the presence of estrogenic substances in process waters is not simply due to desorption in the dewatering process but associated with the anaerobic degradation processes. Thus, process waters can contain estrogenic active substances and so present an additional input burden through recycling in the WWTP.

Table 3 Reporter gene induction of tenfold concentrated extracts of tertiary effluent grab and composite samples. Given are means and standard deviations in relation to the maximum induction with estradiol (%E2max)

Plant	A	A	A	E	E	F	G
Sampling month	Mar.	Jul.	Oct.	May	Nov.	Feb.	Jul.
Grab sample	10.7 ± 2.6	4.2 ± 1.7	19.0 ± 1.8	15.0 ± 2.0	21.1 ± 3.2	–3.5 ± 1.5	11.4 ± 2.8
24-h-composite sample	6.8 ± 3.3	3.6 ± 1.3	19.7 ± 3.0	8.0 ± 1.3	11.2 ± 3.7	–2.5 ± 1.2	18.0 ± 5.3

Table 4 Mean estradiol equivalents (EEQ) ± SD in the process waters of different sources

Plant	Sampling month	Process water source	EEQ (ng/L)
B	Jul.	Dewatering of anaerobic digested sludge	10.5 ± 3.2
C	Dec./Feb.	Dewatering of anaerobic digested sludge	5.2 ± 1.4/4.7 ± 2.2
D	Oct.	Dewatering of anaerobic digested sludge	55.6 ± 23.4
D	Feb.	Dewatering of undigested sludge	< 3.2
E	Aug./Nov.	Dewatering of undigested sludge	< 1.8/1.8
F	Mar.	Dewatering of anaerobic digested sludge	< 4.2
G	Jan./Mar./Jul.	Dewatering of undigested sludge	4.6 ± 1.4/28.2 ± 9.6/4.7 ± 1.7
H	Jul.	Dewatering of anaerobic digested sludge	74.3 ± 0.4

However, since the EEQs of process waters were in the range of the WWTP influents and the volumes were considerably lesser, the effects on the elimination efficiency in the treatment process were presumably negligible.

Conclusions

Estrogenic activities of influents, effluents and process waters of wastewater water treatment plants could successfully be assessed by a reporter gene-based bioassay, despite some obstructions by hindering substances. Estradiol equivalent concentrations in raw sewage were reduced within the treatment plants to non-detectable or small, but detectable, estrogenic activity. Tertiary treatment methods appeared to further improve the elimination efficiency of the activated sludge treatment. Related to the estrogenic activity in the influents, the elimination was mostly more than 92%, but whether the remaining estrogenic substances still present a hazard to aquatic wildlife needs to be investigated *in vivo*, by use of field studies.

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