Potential accumulation of estrogenic substances in biofilms and aquatic plants collected in sewage treatment plant (STP) and receiving water.

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Introduction

During the past years the estrogenic potency of natural (e.g. estrone and 17β-estradiol E2) and synthetic hormones (e.g. ethinylestradiol EE2) and xenoestrogens (e.g. pesticides, polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), dioxins (PCDDs) and furans (PCDFs), alkylphenolic compounds or bisphenol A (BPA)) has attracted increasing scientific attention[1]. Especially the occurrence and behaviour of these substances in waste water of sewage treatment plants (STPs) were often investigated. Andersen *et al*. found steroid estrogen concentrations in the effluent of a municipal STP always below the limit of quantification of 1 ng/l[2]. However, Aerni *et al*. detected E2 and EE2 concentrations up to 6 ng/l and 2 ng/l, and alkylphenols, alkylphenolmono- and diethoxylates even at µg/l concentrations in the effluent of a wastewater treatment plant with a significant industrial impact[3]. In activated and digested sewage sludge concentrations of estrone and E2 up to 37 ng/g and 49 ng/g, of the synthetic EE2 up to 17 ng/g were observed[4]. In river sediments the concentrations detected were lower with up to 2 ng/g estrone and 0,9 ng/g EE2[4].
In the meantime many studies exist about raw and treated water in STPs, but there is little knowledge about the influence of estrogenic active substances on aquatic plants so far. In this study we investigated therefore the potency of estrogenic substances to accumulate in the duckweed *Lemna minor* from STP in comparison to the estrogenicity of duckweed from a natural pond, biofilms in drain and microsieve of the STP by the *in vitro* E-Screen- and LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase)[5]. In addition, we tested the estrogenic activity of moss-like aquatic plants collected at different sites of the receiving water and analyzed the concentrations of four phenolic xenoestrogens in the effluent by GC/MS.

Methods and Materials

E-Screen-assay
The estrogenic activity of the samples was determined by an *in vitro* proliferation test with human estrogen receptor-positive MCF-7 breast cancer cells (E-Screen-assay). The medium containing charcoal-dextran stripped human serum inhibit the proliferation of these cells. Estrogens or estrogen-like substances release this inhibition and induce the cell proliferation. Details of the

procedure are given in Ref. 6. The used 96 well microtiter plates were supported by Sarstedt (Nümbrecht, Germany).

LYES-assay[5]

Following the procedure of the YES-assay (Routledge *et al*., 1996) recombinant *Saccharomyces cerevisiae* cells were incubated 48 hours at 30 °C on an orbital shaker[7]. The yeast suspension (50 ml) was centrifuged (3500 rpm, 10 min) and the supernatant replaced by fresh growth medium (10 ml). The test compounds (10 µl), dissolved in double-destilled water, and the yeast suspension (90 µl) were added to the wells. The 96 well microtiter plate was sealed with autoclave tape, vigorously shaken for 2 min and incubated for 4 hours at 36 °C. The lyticase stock solution (1 g/l) was prepared by combining lyticase (10 mg, Sigma-Aldrich, Steinheim, Germany), potassium phosphate buffer (1 M, pH 7.5, 1 ml), NaCl (5 M, 0.2 ml) and glycerol (5 ml) and completing the volume to 10 ml with double-destilled water. This stock solution was diluted 10-fold with ´Z-buffer´ (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$ and 50 mM 2-mercaptoethanol) and 40 µl of this mixture were added into each well. After an incubation time of 45 minutes at room temperature Triton X-100 (0.1 %, 35 µl) was added to each well and the plates were incubated again for 20 minutes. Subsequently CPRG solution (1 g/l, 25 µl) was added to the microplate wells and the absorbance at 550 nm was immediately measured. After an incubation time of 2 hours the absorbances at 630 nm and 550 nm were determined again. Double-destilled water was used as blank.

GC/MS analysis

The sample extracts were concentrated to 100 µl. After a clean-up step on silicagel, the samples were methylated with trimethylsulfoniumhydroxide-solution in methanol. The analysis of the mixture was performed using GC/MS-system HP 6890/HP 5973. Column: DB-5ms, 30 m length, 0.25 mm ID, 0.25 µm film thickness. Split/splitless Injector: 280 °C; initial temperature 80 °C, 7.0 °C/min 180 °C, 12 °C/min 240 °C, 20 °C/min 300 °C. Quantification: 10 point calibration using the standards n-nonylphenol, n-octylphenol and d^{16}-bisphenol A.

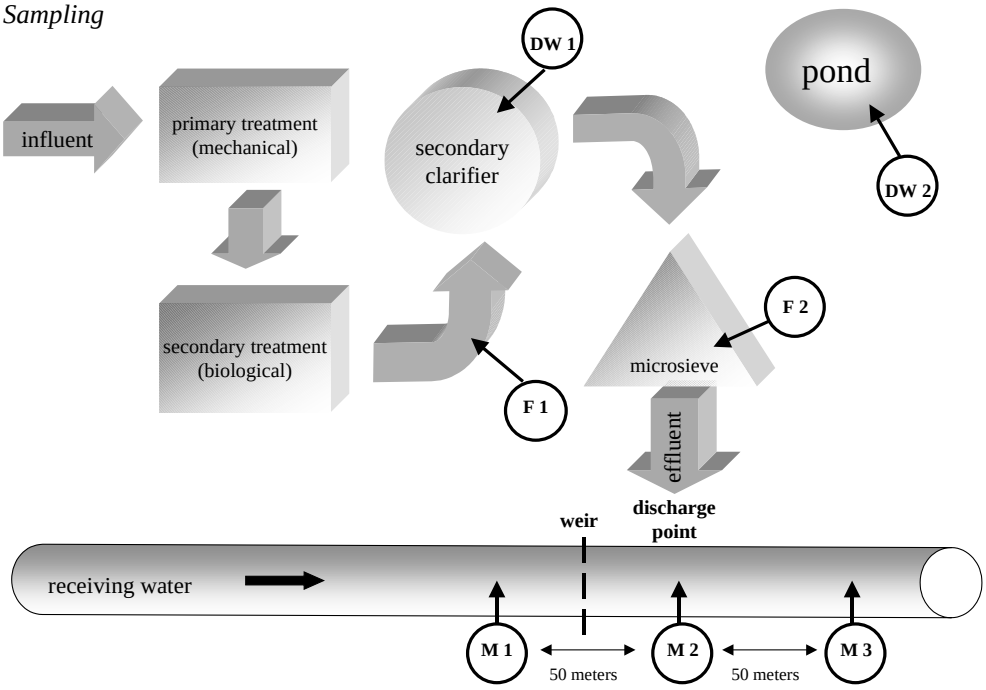

Fig. 1. Points of sampling in the sewage treatment plant (F1, biofilm taken from the wall of the drain; F2, biofilm taken from the microsieve; DW1, duckweed collected in secondary clarifier), duckweed collected in a pond (DW2) and moss collected in receiving water (M1, upstream; M3, downstream; M2, directly at the discharge point).

All samples were taken on the site of the sewage treatment plant in Stuttgart-Büsnau, Germany (Fig. 1). A sample of the duckweed *Lemna minor* (DW 1) was collected in the secondary clarifier to determine a potential estrogenic activity due to accumulated estrogenic substances from waste water. The duckweed sample DW 2 was from a natural pond, which was not influenced by waste water.

Additionally, we investigated a potential estrogenic activity of the biofilm in the waste water channel between secondary treatment and clarifier (F 1) as well as the biofilm on the surface of the microsieve at the discharge point (F 2). Finally, the estrogenicity of moss-like aquatic plants in the receiving water was investigated. For this purpose moss was sampled 50 meters upstream (M 1) and downstream (M 3) as well as directly at the discharge point (M 2). The upstream point of sampling is separated from waste water by a weir.

Sample preparation
All plant samples were freeze-dried, grinded, filled in extraction thimbles and covered with Na_2SO_4 (1 g). The samples were then extracted with methanol/diethylether/HCl (100:10:0.01, v/v/v). Diethylether (picograde) was supplied by Promochem (Wesel, Germany), methanol (pesticide grade) by Fisher Scientific (Leicestershire, UK), HCl (pro analysis) and ethanol (absolute extra pure) by Merck (Darmstadt, Germany). A part of the extracts (3 ml) was evaporated under nitrogen and took up in double-destilled water (1 ml) and ethanol (0,5 ml). The dilution series for the LYES-assay were made in double-destilled water. In the E-Screen-assay culture medium was used for the dilutions.

Results and Discussion

In the effluent of the STP, phenolic xenoestrogens were detected in concentrations up to 106 ng/l (Table 1).

Table 1. Concentrations and relative estrogenic potential of phenolic xenoestrogens in 8 days effluent samples determined by GC/MS.

	rel. molecular mass	rel. potency [#]	average value (8 days) [ng/l]	min. concentration [ng/l]	max. concentration [ng/l]
4-t-octylphenol	206.3	0.0001	79	5	172
4-nonylphenol	220.4	0.0001	95	8	251
bisphenol A	228.2	6.0E-05	106	30	257
2-hydroxybiphenyl	170.2	1.5E-06	37	4	28

[#] literature data[8]; relative to E2

The E-Screen- and LYES-assay showed very similar estradiol equivalent factors (EEF) (Table 2). Extracts of duckweed from a natural pond (DW 2) was not active in neither *in vitro*-assay in contrast to duckweed collected in the secondary clarifier (DW 1). Also the biofilm from the STP drain (F 1) and especially the biofilm on the surface of the microsiever (F 2) had a high level of estrogenic potency.
Estrogenic activity of the moss upstream the discharge point (M 1) was not detectable. However, the moss collected directly at the discharge point (M 2) showed an estrogenic level comparable to that of the biofilm in the STP drain. The estrogenicity of the moss downstream this point (M 3) was also lower.
Although only low concentrations of estrogenic active substances were determined by GC/MS in treated waste water, the collected biofilms and plants in the STP showed estrogenic potency. These results support the assumption, that estrogenic substances from waste water could accumulate in biofilms and plants in the STPs. Thus, we need to get more knowledge about the influence of estrogenic substances not only on fish and other water species but also on aquatic plants.
On the other hand, maybe we could use this ability of accumulation to eliminate estrogenic active substances in the waste water treatment. Why not use something simple like natural duckweed?

Table 2. Estrogenic activity of samples collected from aquatic plants and biofilms in a sewage treatment plant, receiving water and in natural pond determined by *in vitro* E-Screen- and LYES-assay.

Sample	LYES-assay EEF [10^{-9}]*	E-Screen-assay EEF [10^{-9}]*
DW 1 (duckweed, STP)	5.7	11.7
DW 2 (duckweed, pond)	No estrogenic activity	No estrogenic activity
F 1 (biofilm, drain)	22.0 (0.8 %)#	26.5
F 2 (biofilm, microsieve)	287.8 (4.1 %)#	238.6
M 1 (moss, upstream of discharge point)	No estrogenic activity	No estrogenic activity
M 2 (moss, at discharge point)	83.9 (24.7 %)#	13.8
M 3 (moss, downstream of discharge point)	2.3 (24.4 %)#	4.6

*EEF (estradiol equivalent factor) = EC_{50} (E2) / EC_{50} (sample), EEF (E2) = 1; #represents average value of two independent experiments with 1-fold standard deviation (%).

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References

1 Estévez-Alberola, M.-C. and Marco, M.-P. (2004) Anal. Bioanal. Chem. 378, 563.
2 Andersen, H., Siegrist, H., Halling-SØrensen, B. and Ternes, T.A. (2003) Environ. Sci. Technol. 37, 4021.
3 Aerni, H.-R., Kobler, B., Rutishauser, B.V., Wettstein, F.E., Fischer, R., Giger, W., Hungerbühler, A., Marazuela, M.D., Peter, A., Schönenberger, R., Vögeli, A.C., Suter, M.J.-F. and Eggen, R.I.L. (2004) Anal. Bioanal. Chem. 378, 688.
4 Ternes, T.A., Andersen, H., Gilberg, D. and Bonerz, M. (2002) Anal. Chem. 74, 3498.
5 Schultis, T. and Metzger, J.W., Chemosphere, submitted.
6 Körner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., Hagenmaier, H. (1999) Sci. Total Environ. 225, 33.
7 Routledge, E.J. and Sumpter, J.P. (1996) Environ. Toxicol. Chem. 15, 241.
8 Körner, W., Bolz, U., Süßmuth, W., Hiller, G., Schuller, W., Hanf, V. and Hagenmaier, H. (2000) Chemosphere 40, 1131.