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Behaviour and occurrence of estrogens in municipal sewage treatment plants — II. Aerobic batch experiments with activated sludge

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Abstract

Aerobic batch experiments containing a diluted slurry of activated sludge from a real sewage treatment plant (STP) near Frankfurt/Main were undertaken, in order to investigate the persistence of natural estrogens and contraceptives under aerobic conditions. The batch experiments showed that while in contact with activated sludge the natural estrogen 17β-estradiol was oxidized to estrone, which was further eliminated in the batch experiments in an approximate linear time dependence. Further degradation products of estrone were not observed. 16α-hydroxyestrone was rapidly eliminated, again without detection of further degradation products. The contraceptive 17α-ethinylestradiol was principally persistent under the selected aerobic conditions, whereas mestranol was rapidly eliminated and small portions of 17α-ethinylestradiol were formed by demethylation. Additionally, two glucuronides of 17β-estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were cleaved in contact with the diluted activated sludge solution and thus 17β-estradiol was released. The glucuronidase activity of the activated sludge was further confirmed by the cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUF-β-glucuronide) in a solution of a activated sludge slurry and Milli-Q-water (1:100, v/v). The turnover rate obtained was approximately steady state, with a turnover rate of 0.1 μmol/l for the released MUF. Hence, it is very likely that the glucuronic acid moiety of 17β-estradiol glucuronides and other estrogen glucuronides become cleaved in a real municipal STP, so that the concentrations of the free estrogens increase.

Keywords: Estrogens; Contraceptives; Glucuronides; Sewage treatment plants; Batch experiments; Activated sludge

1. Introduction

Natural hormones and contraceptives influence the hormonal system and belong therefore to potential endocrine disrupters in sewage treatment plants (STPs), as recently assumed by Desbrow et al. (1998) and Routledge et al. (1998). The daily production rate of natural estrogens by humans is in the microgram range, ranging up to approximately 400 μg for 17β-estradiol for women (Lauritzen, 1988; Römbke et al., 1996). In humans and mammalians estrogens undergo various transformations mainly in the liver. Frequently, they are oxidized, hydroxylated, deoxidated and

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methylated prior to the final conjugation with glucuronic acid or sulphate (Lauritzen, 1988; Mutschler, 1996). For instance, 17β-estradiol is rapidly oxidized to estrone which can be further converted to estriol, the major excretion product. Many other polar metabolites like 16-hydroxy-estrone, 16-ketoestrone or 16-epiestriol are formed and can be present in urine and faeces. However, the estrogens are principally excreted as inactive polar conjugates. 17β-estradiol was predominantly found as 17β-estradiol-3-glucuronide, estrone as estrone-3-sulphate and estriol as estriol-16-glucuronide. The daily excretion rates of natural estrogens for women was observed to be in the lower microgram range with a maximum of 64 μg/day for estriol (Römbke et al., 1996). Estrone is excreted at a rate between 3 and 20 μg/day and 17β-estradiol between 0.5 and 5 μg/day.

The prodrug mestranol is converted after administering into 17α-ethinylestradiol by demethylation. The contraceptive 17α-ethinyl-estradiol is mainly eliminated as conjugates, whereas other metabolic transformations occur, but are of minor relevance (Ranny, 1977; Taubert and Kuhl, 1995). The question arises, whether these endocrinically inactive conjugates of natural estrogens and contraceptives are cleaved in the raw sewage and in the STP, so that the released active estrogens cause elevated environmental concentrations. Due to the presence in the STP of high population densities of microorganisms, such as *Escherichia coli*, which exhibit glucuronidase and sulphatase activity, a separation of the glucuronic acid and sulfate moieties is reasonable. This hypothesis is supported by the detection of several unconjugated estrogens like 17β-estradiol, estrone, 16α-hydroxyestrone and 17α-ethinylestradiol in municipal STP discharges (Tabak and Bunch, 1970; Tabak et al., 1981; Aherne and Briggs, 1989; Shore et al., 1993; Stumpf et al., 1996; Desbrow et al., 1998; Ternes et al., 1999). However, to our knowledge a proof of the experimental cleavage of estrogen glucuronides and sulphates is unavailable. Additionally, there is a lack of information about the behaviour of natural estrogens and contraceptives within the STP process.

The aim of this work was to study the transformation of natural estrogens, contraceptives and estradiol glucuronides in contact with real activated sludge using aerobic batch experiments.

2. Materials and methods

2.1. Analytical methods

2.1.1. Solid phase extraction

The solid phase extraction of the 100 ml fractions obtained from the 1-l batch experiments (spiked with 17β-estradiol, 17β-estradiol-17-glucuronide or 17α-ethinylestradiol, all from Sigma, Deisenhofen, Germany) were carried out as described for the 1-l native water samples in Ternes et al. (1999). The only difference was the reduced sample volume of 100 ml used. 17β-estradiol-17-acetate was spiked as a surrogate standard, before the samples were sucked through the filled glass cartridges.

2.1.2. Silica gel clean-up, derivatisation, GC/MS/MS detection

Silica gel clean-up, Derivatisation, GC/MS/MS detection were performed as described in Ternes et al. (1999).

2.1.3. Calibration, detection limits and blank samples

The calibration was performed over the whole procedure after spiking ground water from the highland Taunus with the standard mixture in order to adjust six different calibration concentrations. The sample volume and the enrichment procedure of the calibration points were selected according to the samples which were analysed. For instance, 100 ml of ground water was extracted by SPE for analyzing estrogens in 1-l batch experiments (1 ng/ml) and 1 ml of the ground water was evaporated to dryness for the determination of the analytes in the 20 ml batch experiments (1 μg/ml). Always six point calibrations were used ranging from 1 pg/ml to 1.5 ng/ml for the 1 ng/ml batch experiments, and from 5 ng/ml to 1.5 μg/ml for the samples of the 1 μg/ml batch experiments. Detection limits allowing for the quantitation of analytes were set as the lowest or the second lowest calibration point of the linear correlation. Blank samples (ground water spiked with the surrogate standard 17β-estradiol-17-acetate) were included in every series of analysis.

2.2. Batch experiments with analyte concentrations of 1 μg/ml

A 2 ml suspension of activated sludge (mixed liquor suspended solids: 2.6 g/l) diluted in 18 ml drinking water was spiked with estrogens and 17β-estradiol glucuronide conjugates to obtain a nominal concentration of 1 μg/ml for the unconjugated analytes and 1.65 μg/ml for the conjugates (corresponding to 1 μg/l of the released 17β-estradiol). All natural estrogens (estrone, 17β-estradiol, 16α-hydroxyestrone), contraceptives (mestranol, 17α-ethinylestradiol) and glucuronides (17β-estradiol-17(β-D-glucuronide), 17β-estradiol-3(β-D-glucuronide)) were purchased from Sigma (Deisenhofen, Germany). The sludge was taken from a STP close to Frankfurt/Main and was immediately (1–2 h after sampling) utilized in batch experiments. In order to maintain a homogenous suspension and aerobic conditions, the slurry was horizontally shaken over the whole investigation period (up to 72 h). At the beginning of the experiment the concentration of oxygen was 9 mg/l and this declined down to 8 mg/l after 72 h. At appropriate time intervals (1 min, 2 min, 5 min, 15 min, 0.5 h, 1 h, 3 h, 5 h, 24 h, 28 h, 48 h, 72 h), 1 ml aliquots of the homogenized suspension were taken and were immediately inactivated by addition of 20 μl of 0.5% hydrochloride acid (pH 3). The acidic slurry (pH 3) was evaporated to dryness at room temperature by a nitrogen stream. 1 ml of hexane was then added to the residue and was evaporated by nitrogen to 200 μl. Then, before using the silica gel clean-up, the surrogate standard 17β-estradiol-17-acetate was spiked. For clean-up the extracts were quantitatively transferred to the top of the prepared 1 g silica gel column. The glass vessels were rinsed twice with 2 ml of the elution mixture hexane/acetone (65:35, v/v) to assure that the whole extract including most of the solid matter were transferred to the column. The elution in the clean-up step and the GC/MS/MS detection was performed as described in Ternes et al. (1999). By this procedure the portion of analytes which were adsorbed to sludge particles should be mainly desorbed and hence are included into the mass balance. All batch experiments were performed in triplicate.

2.3. Batch experiments with analyte concentrations of 1 ng/ml

A 200 ml suspension of activated sludge (mixed liquor suspended solids: 2.6 g/l) diluted in 800 ml drinking water was spiked with 17β-estradiol, 17α-ethinylestradiol or 17β-estradiol-17(β-D-glucuronide) to obtain a nominal concentration of 1 ng/ml for the unconjugated estrogen and 1.65 ng/ml for the conjugates (corresponding to 1 ng/l of the released 17β-estradiol), in order to assure that the results obtained in the μg/ml-range were transferable to environmental concentrations in the ng/ml-range. The sludge was taken from the STP close to Frankfurt/Main and was immediately (2 h after sampling) utilized in the batch experiments. In order to maintain aerobic conditions and a homogenous suspension, the slurry was stirred over the whole investigation period (up to 72 h). At appropriate time intervals (5 min, 15 min, 0.5 h, 1 h, 3 h, 5 h, 24 h, 28 h, 48 h), 110 ml of the homogenized suspension were collected and the microorganisms were inactivated by addition of 200 μl of 0.5% hydrochloride acid to obtain a pH of 3. After glass fibre filtration (< 1 μm), 100 ml of the sample was spiked with 17β-estradiol-17-acetate and was extracted by SPE, treated by silica gel clean-up and detected by GC/MS/MS (Ternes et al., 1999) for native samples. All batch experiments were performed in triplicate.

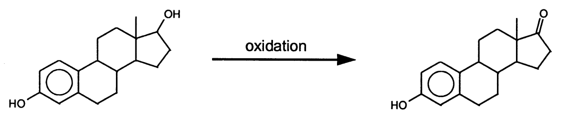
2.4. Determination of glucuronidase activity in the activated sludge solution

A 10 ml-suspension of activated sludge (mixed liquor suspended solids: 2.6 g/l) diluted in 990 ml Milli-Q-water was spiked with a 3 ml-solution (c_0: 3.2 μmol/ml = 1.14 mg/ml) of 4-methylumbelliferryl-β-D-glucuronide (MUF-β-glucuronide) from Sigma (Deisenhofen, Germany) to obtain a nominal concentration of 9.6 μmol/l. The solution was covered with aluminum foil and stirred over the whole investigation period. At appropriate time intervals 1 ml of the homogenized solution was collected and stabilized by addition of 1 ml of buffer (4.4 g/l glycine, 3.5 g/l sodium chloride and 0.1 mol/l sodium hydroxide until pH 10 is adjusted). For quantitation an eleven point calibration of MUF ranging from 0.01 to 1 μmol/l was performed. The detection was carried out using an excitation wavelength of 354 nm and a fluorescence emission wavelength of 441 nm.

3. Results and discussion

3.1. Behaviour of 17β-estradiol, 16α-hydroxyestrone and estrone in batch experiments

The concentration of 17β-estradiol was immediately reduced in the batch experiments at both spiking levels of 1 μg/ml (Fig. 1a) and 1 ng/ml (Fig. 1b). After a period of 1–3 h, more than 95% of the unconjugated 17β-estradiol had vanished, whereas the concentration of estrone increased up to 95% with regard to the initial concentration of 17β-estradiol. 17β-estradiol was almost quantitatively oxidized to estrone.



17β-estradiol estrone

Qualitatively a similar behaviour for 17β-estradiol and the metabolite estrone was observed, using both initial concentrations of 17β-estradiol. However, at a spiking level of 1 ng/ml the removal of 17β-estradiol and the formation and elimination of estrone was accelerated. After 5 h neither 17β-estradiol nor estrone were to be found above the detection limits (shown in Fig. 1b as $c/co = 0.01$). Hence, the results of the 1 μg/ml batch experiments can be transferred only qualitatively to the ng/ml-range, whereas at lower concentration the turnover rates were even accelerated.

Estrone at a spiking level of 1 μg/ml exhibited a similar behaviour (Fig. 2a) as in the batch experiments spiked with 17β-estradiol (Fig. 1a). The concentrations of estrone declined continuously (approx. 50% after 24 h) without detection of any degradation products. No further degradation products were detected in the scan spectra of the GC/MS using the previously described analytical method.

16α-Hydroxyestrone, one of the possible human metabolites of estrone, was not identified as a degradation product of estrone in contact with activated sludge, but it was immediately removed in the batch experiments after spiking (Fig. 2b). After 5 h approximately 60% of 16α-hydroxyestrone was eliminated, whereas the degradation was incomplete after 72 h (approx. 10% remained). Degradation products could not be identified.

In all cases loss by sorption effects are rather unlikely. Firstly, in the 1 μg/ml batch experiments sorbed portions are mainly desorbed by the performed transfer of solid matter to the silica gel column for clean up and secondly the estrogens were almost quantitatively recovered at the beginning of the experiments either unchanged or as metabolites.

3.2. Behaviour of 17β-estradiol glucuronides in batch experiments

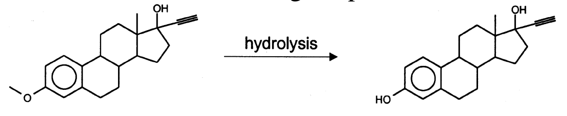
In order to investigate the release of estrogens from glucuronide conjugates in a municipal STP, 17β-estradiol-17(β-D-glucuronide) and 17β-estradiol-3(β-D-glucuronide) were spiked into the activated sludge slurries at a concentration level of 1.65 μg/l (corresponding to 1 μg/l unconjugated 17β-estradiol). After only 15 min both 17β-estradiol and estrone were detected (Fig. 1c,d). Approximately 70% of the conjugated 17β-estradiol was found for both glucuronides in the oxidized form (estrone) at the maximum concentration (20–30 h). Therefore most portions of the glucuronides were cleaved after 20 h. However, up to 28 h 17β-estradiol was detected in small amounts of approximately 3% indicating that the cleavage of the glucuronides was not complete.

Using 1.65 ng/ml of 17β-estradiol-17(β-D-glucuronide) as initial concentration (corresponding to 1 ng/l unconjugated 17β-estradiol), the results of the μg/ml level could be qualitatively confirmed (Table 1). The glucuronide conjugate was cleaved, the released 17β-estradiol was oxidized to estrone and approximately 80% of the initial amounts were recovered as the sum of the two estrogens. However, the turnover rates were faster compared to those obtained in the batch experiments with a spiking level of 1 ng/ml of 17β-estradiol (Fig. 1b). After 5 min, approximately 50% were detected as estrone and after 24 h neither 17β-estradiol nor estrone were present above the detection limits.

3.3. Behaviour of 17α-ethinylestradiol and mestranol in batch experiments

The concentration of 17α-ethinylestradiol was not appreciably reduced during the batch experiments (Fig. 2c). Only approximately 20% of the initial amount could not be accounted for after 24 h at an initial concentration of 1 ng/ml and after 48 h at 1 μg/ml, but the reduction was not statistically significant. Even though 17α-ethinylestradiol has a log octanol/water partition coefficient of 4.2 (personal communication, Schweinfurth/Schering AG, Berlin), sorption effects do not appear to be a factor in the batch experiments. Further investigations are necessary to analyse the conjugates together with the unconjugated estrogen, in order to confirm these results and to distinguish between sorption and degradation behaviour. However, 17α-ethinylestradiol appears to be mainly stable under the present aerobic conditions of an activated sludge process.

In another experiment, mestranol was degraded after a lag-phase of approximately 5 h and yielded a turnover rate of approximately 80% after 24 h (Fig. 2d). As metabolite only 17α-ethinylestradiol was identified, which was formed by 7% with regard to the initial concentration of mestranol. However, the portion of 17α-ethinylestradiol did not change during the time period from 24 to 72 h indicating its persistence.

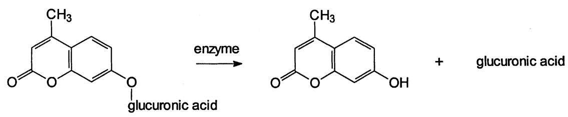


mestranol 17α-ethinylestradiol

Similar results were reported for 17α-ethinylestradiol by Norpoth et al. (1973), who observed no elimination of this contraceptive in contact with activated sludge. However, in contrast to our results in their batch experiments mestranol persisted over 120 h.

3.4. Glucuronidase activity in the activated sludge solution

In order to confirm the presence of the glucuronidase activity of the activated sludge the cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUF-β-glucuronide) using fluorescence detection was explored. As shown in Fig. 3 the concentration of MUF increased linearly with the investigation time. After 6.7 h approximately 19% of the glucuronide conjugates were cleaved, while the turnover rate obtained was approximately steady state, with a turnover rate of 0.1 μmol/l for the released MUF.



MUF-β-glucuronide 4-methylumbelliferone

Glucuronide conjugates of estrogens and other organic compounds can obviously be cleaved in contact with activated sludge by microorganisms. Sorption effects can be mainly ruled out due to the small amounts of solid matter in the batch experiment spiked with MUF-β-glucuronide. As the activated sludge was taken from a real municipal STP and immediately used in the batch ex-

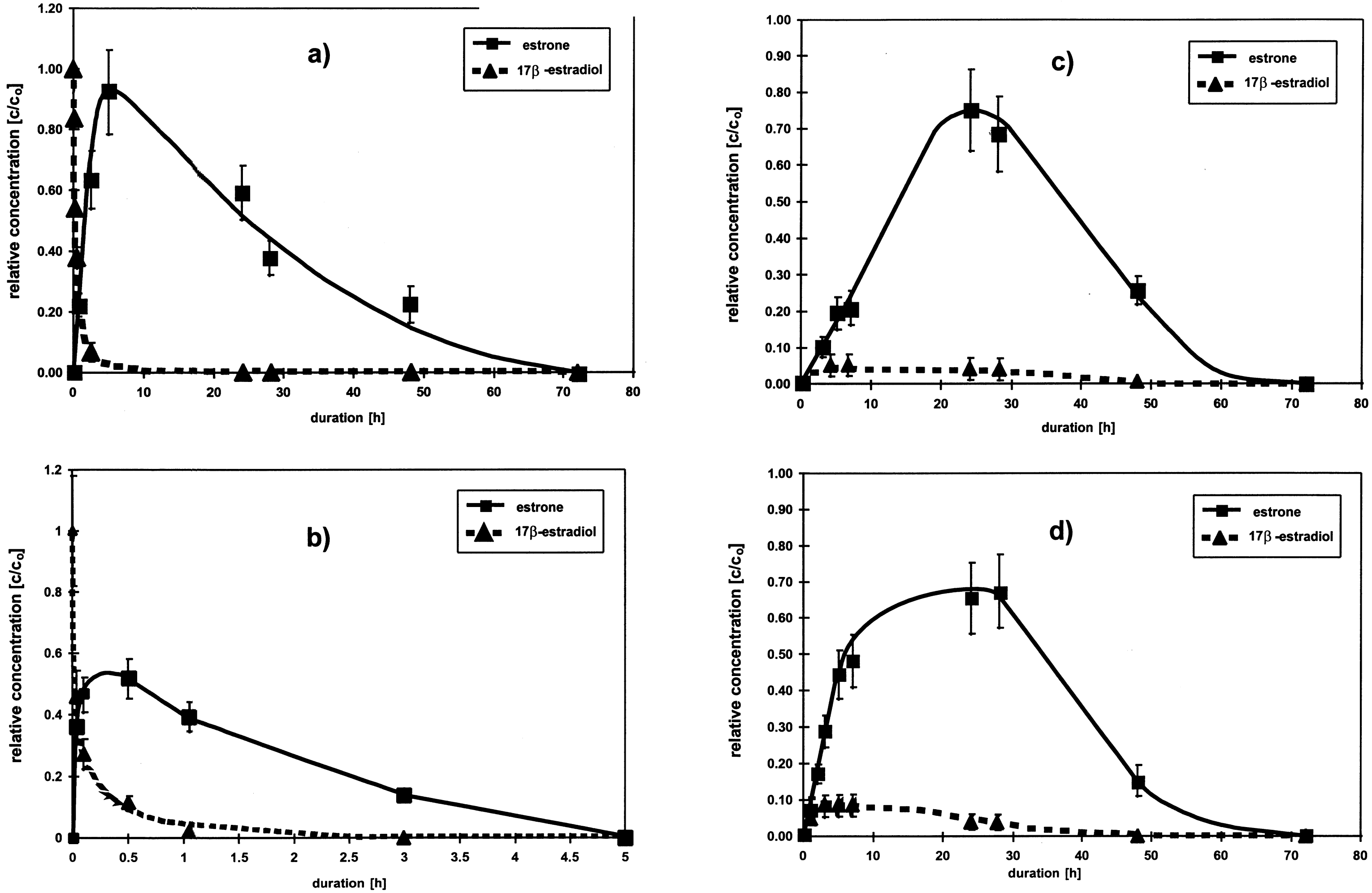


Fig. 1. Relative concentrations of natural estrogens and released estrogens from glucuronide conjugates. Batch experiments with activated sludge solutions (diluted 1:10) spiked with different estrogens and conjugates (a) 17β-estradiol at 1 μg/ml, (b) 17β-estradiol at 1 ng/ml, (c) 17β-estradiol-17(β-D-glucuronide) at 1 μg/ml, (d) 17β-estradiol-3(β-D-glucuronide) at 1 μg/ml.

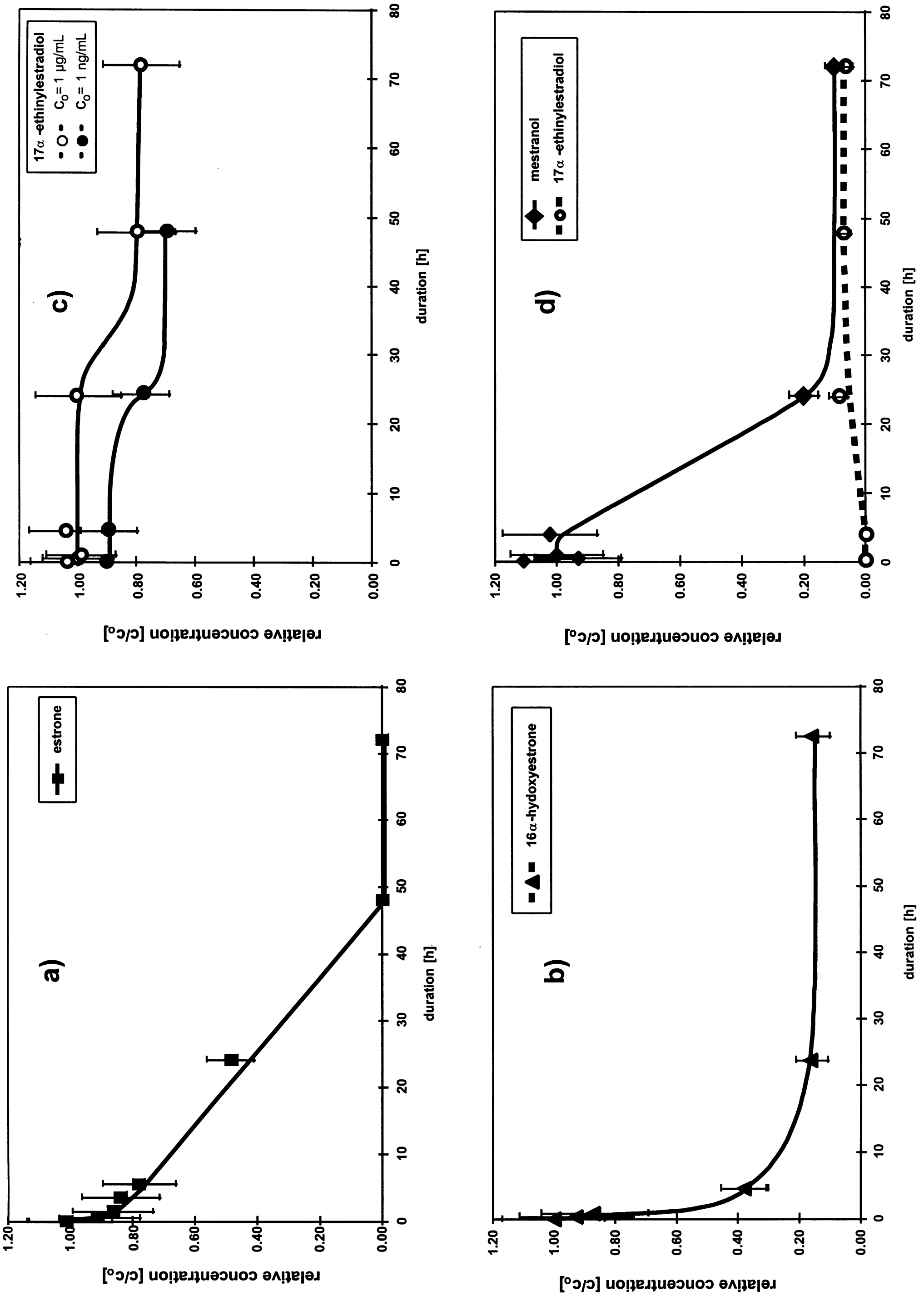


Fig. 2. Relative concentrations of natural estrogens and contraceptives. Batch experiments with activated sludge solutions (diluted 1:10 with water) spiked with different estrogens (a) estrone at 1 μg/ml, (b) 16α-hydroxyestrone at 1 μg/ml, (c) 17α-ethinylestradiol at 1 μg/ml and 1 ng/ml, (d) mestranol at 1 μg/ml.

Table 1
Batch experiments with activated sludge solutions spiked with 17β-estradiol-17(β-D-glucuronide) at 1 ng/ml

Time	17β-estradiol $[c/c_0]$	Estrone $[c/c_0]$	Recovery (sum of estrone and 17β-estradiol) [%]
0	n.d.	n.d.	n.d.
5 min	0.30	0.50	80
3 h	n.d.	0.15	15
24 h	n.d.	n.d.	0

periments, the principal results are presumably transferable to the real sewage treatment. Basically, no lag phase has been observed and the glucuronides were cleaved immediately, indicating that the microorganisms present have the enzymes to deconjugate estrogen glucuronides. However, whether the retention time of a real STP (mainly below 20 h) is sufficient to cleave all of the glucuronide conjugates present, can not be concluded. In a real STP the concentrations of the estrogens are in the ng/l-range, a multitude of other substrates are present in high concentrations and the TOC is at least one order of magnitude higher than those in the batch experiments. Glucuronide conjugates are one of the principal excreted metabolites for organic compounds by humans. Microorganisms which gain energy by cleaving and degrading glucuronides are therefore expected to be present in the STP. Hence, it is likely that estrogen glucuronides were cleaved in the STP, even though their concentrations are relatively low. In the batch experiments the concentrations of glucuronides in the ng/ml-range were cleaved and eliminated even faster than in the experiments spiked with higher concentrations (μg/ml-range). Nevertheless, to prove the hypothesis that glucuronides are cleaved in the municipal STP, the development of an analytical method which allows for the determination of estrogen glucuronide conjugates in sewage and STP discharges at low concentrations is essential.

4. Conclusions

The batch experiments showed that various turnovers of estrogens occur in municipal STPs.

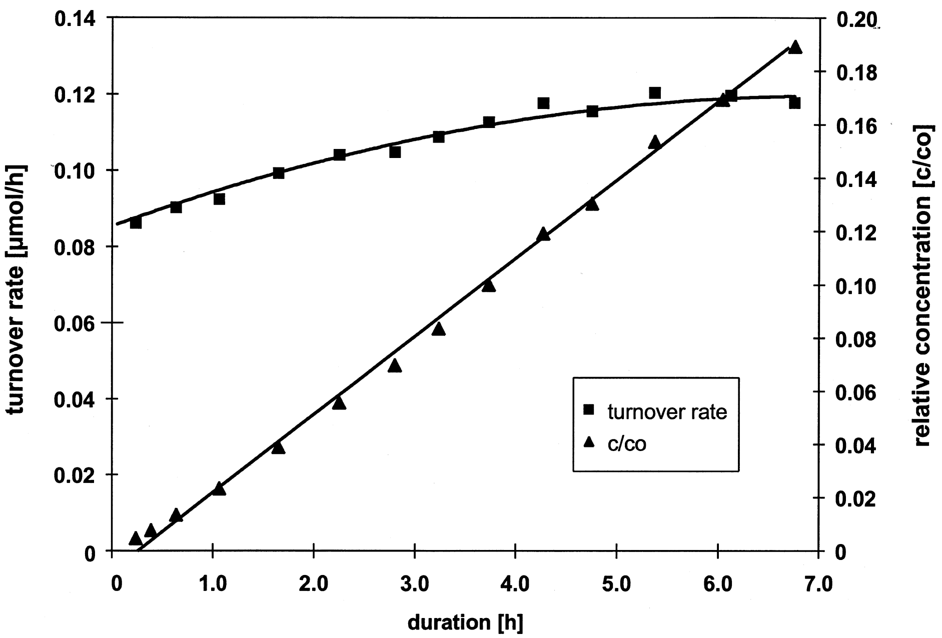


Fig. 3. Relative concentrations of 4-methylumbelliferone (MUF) and turnover rate of MUF-β-glucuronide. Batch experiments with activated sludge solutions (diluted 1:100) spiked with 4-methylumbelliferryl-β-D-glucuronide (MUF-β-glucuronide) to adjust a nominal concentration of 9.6 μmol/l.

For instance, the predominant presence of estrone in STP effluents and rivers is presumably a result of the relative high stability of estrone within the STP, the cleavage of glucuronide conjugates from both estrone and 17β-estradiol and the oxidation of 17β-estradiol to estrone. In the batch experiments 17β-estradiol, mestranol and the metabolite 16α-hydroxyestrone were eliminated relatively fast. Besides oxidation and demethylation reactions, glucuronide conjugates can be cleaved in contact with activated sludge. Thus, for assessing the relevant environmental concentration, the conjugates have to be included into the mass balance of the potential endocrine active compounds. Although the annual prescriptions of 17α-ethinylestradiol in Germany amount only to approximately 50 kg, the contraceptive was detected sometimes in comparable concentrations to the natural estrogens in STP effluents. This indicates its higher stability under environmental conditions, as underlined by the persistence in the batch experiments, and the cleavage of the principal excreted glucuronide conjugates. In any case, further investigations are necessary to analyse the conjugates together with free estrogens and metabolites in real STPs, in order to confirm the described results under the real conditions of waste water treatment and to distinguish between sorption and degradation behaviour.

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