The fate and behaviour of human estrogens in a night soil treatment process

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Abstract A night soil treatment plant which collects a high amount of human urine and excreta, is a very unique system to Japan and the object of this investigation on the fate and behavior of the human estrogens in the water treatment process. Water and sludge samples at different treatment phases were taken at a night soil treatment plant. Their hydrophobic fractions were assayed using an *in vitro* yeast assay (yeast estrogen screen) and an enzyme immunoassay (ELISA) employed for the detection of overall estrogenic activity and 17β-estradiol (E2), respectively. Estrogenic activity observed by the yeast assay was quantified as E2 concentration equivalent, which was comparable with E2 concentration measured by the ELISA, in order to estimate the contribution of E2 to the total activity. Based on the ELISA results, a sketch of the mass balance of E2 in the treatment system was drawn and treatability of E2 was discussed.

Keywords Estrogen; 17β-estradiol; night soil treatment; ELISA; yeast estrogen screen

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Introduction

Steroidal hormones, such as 17β-estradiol (E2) and estrone, the most effective estrogens can cause the strongest endocrine disrupting effect especially on aquatic organisms when existing in the aqueous environment. We have reported the findings from the analysis of sewage treatment process waters that E2 excreted from human is the main causative substance in terms of the estrogenic activity of sewage and its treated effluents (Matsui *et al.*, 2000). In Japan, sewage treatment water has been increasingly contributing to the volume of urban river water where the remaining E2 exists in the ng/L range (Japanese Ministry of Construction, 1998). Some researches indicate that such environmentally relevant concentrations of E2 are sufficient to account for the levels of vitellogenin (VTG) synthesis observed in caged male fish in river waters (Routledge *et al.*, 1998). However, little is known about the movement or degradation characteristics of E2 in the aqueous environment. In order to investigate the fate and behavior of human estrogens, a night soil treatment plant, which is very unique in Japan and accepts quite a high amount of human estrogens, was focused on in this research.

The following were our special concerns and were investigated.

1. Concentration of human estrogens (E2) receiving as night soil (influent).
2. Treatability of human estrogens (E2) through each treatment step.
3. Mass balance of human estrogens (E2) through the treatment system.

Materials and method

Description of the investigated night soil treatment plant process

The investigated plant serves the north-eastern parts of Shiga prefecture, Japan. The plant is designed for a night soil and septic tank sludge flow of 255 m^3 /day which corresponds to the population of 150,000. The biological stage is operated as a nitrified liquor recycled nitrification/denitrification process which has a total volume of 4,600 m^3. The biologically

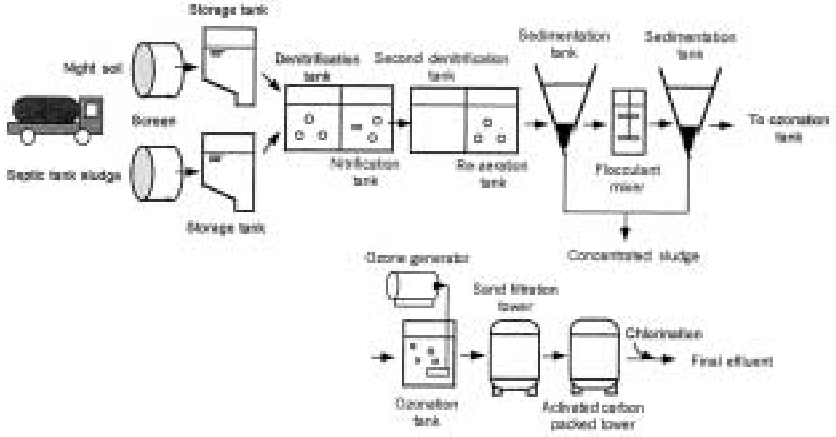

Figure 1 Schematics of the investigated night soil treatment plant

treated water is then led to the advanced treatment process (i.e., ozonation, sand filtration and GAC treatment). A flow diagram of this process is shown in Figure 1.

Sample collection and pretreatment

Water samples were taken at twelve different phases (i.e., raw night soil, septic tank sludge, first denitrification tank, nitrification tank, second denitrification tank, first sedimentation tank, second sedimentation tank after flocculation, ozonation tank, sand filtration tower, activated carbon packed tower, final effluent, dewatering filtrate) from the night soil treatment plant. A solid-phase extraction and concentration method using C18 cartridges was adopted for the fractionation and concentration of estrogenic substances in the aqueous samples. 50–2,000 mL of sample waters was filtered through a GF/C glass microfiber filter and then passed through the two preconditioned Sep-pak® C18 (plus environmental) cartridges in tandem to prevent breakthrough. The pH values of water samples were in the range of 7.02–7.73 and no pH adjustment was conducted. The adsorbed substances were eluted with 10 mL of methanol per cartridge. Eluent was then evaporated to dryness. The residue was finally redissolved into the appropriate volume (1–2 mL) of dimethylsulfoxide (DMSO) and applied as the analytical sample. After this procedure, the final volumetrical concentration factor of samples was 30–1,500.

Sludge samples were taken at six phases (i.e., night soil, septic tank sludge, each activated sludge from the denitrification tank, the nitrification tank, the second denitrification tank, and concentrated sludge). Sludge samples were centrifuged and their precipitates were mildly heated to dryness. Then the residual mass was broken up with a pestle in a mortar and ground to a fine powder. Extraction of organic substances from the sludge powder (5g-dry weight) was conducted by using the accelerated solvent extraction (ASE) device (Dionex, ASE-200) with 40 mL of methanol in the optimized conditions (oven temperature: 200ºC, pressure: 100 atm, static time: 5 min after 9 min pre-heat equilibration, flush volume: 60% of the cell volume, nitrogen purge: 90 sec at 150 psi, and static cycles: 2). Methanol extracts were evaporated and redissolved into the same volume of DMSO. Both extracts of the aqueous and sludge samples were applied to the following two bioassays.

Determination of extraction efficiencies

Extraction efficiencies were calculated by measuring the spiked E2 concentrations for the aqueous and sludge phase samples as follows:

Aqueous phase. Environmental water sample (lake water; E2 concentration was a few ng/L) was spiked with E2, so that a final concentration of 10 μg/L of E2 was present in the sample. 5 mL of this solution was passed through the C18 cartridge. The cartridge was eluted with 5 mL of methanol. The concentration of E2 in the eluent was analyzed by HPLC equipped with a fluorescence detector at excitation and emission wavelengths of 283 and 308 nm, respectively.

Sludge phase. 5 g of sludge samples (the sludge from the first denitrification tank and the concentrated sludge) were respectively spiked with 7.6 μg of E2 which was much higher than the amount originally present in the sample. The extract after the ASE was analyzed by HPLC equipped with a fluorescence detector. The E2 value in the unspiked sample was extracted from that in the spiked one to calculate the recovery of E2.

Bioassays

Yeast estrogen screen. The yeast estrogen screen (YES) was selected for the detection of estrogenic activity. A recombinant yeast strain was obtained from Prof J.P. Sumpter (Brunel University, Uxbridge, UK). In this assay system, human estrogen receptor (hER) is expressed in yeast in a form capable of activating transcription of a promoter carrying ERE in an estrogen-dependent manner. Enzyme activity of β-galactosidase (β-gal), an expression product of ERE-regulated *lacZ* reporter gene was adopted as the endpoint of this assay. It was measured by using a chromogenic substrate (Chlorophenol red-β-D-galactopyranoside) at an absorbance of 540nm. This bioassay can detect xenoestrogens, such as alkylphenolic compounds, Bisphenol A, phytoestrogens as well as natural estrogens, and produce quantitative measurements of estrogenic activity without identification of chemicals (Routledge and Sumpter, 1996). Therefore, the YES has been successfully employed for the evaluation of environmental complex mixtures. The assay was carried out using a previously established 96-well microtiter plate method (Routledge and Sumpter, 1996). Positive control (E2) and samples were assayed in triplicate. The assay results were evaluated quantitatively by a simple index. The E2 equivalent was introduced to evaluate the estrogenic strength in the samples. Four parameter logistic regression was applied to formulate the standard curve, using Microplate manager III/Macintosh software (Bio-Rad Lab., Cal., USA). Based on the dose-response curves obtained for E2 and test samples, the E2 concentration equivalent in each tested sample was calculated by comparing its EC50 value with that of E2.

E2 enzyme immunoassay. Enzyme immunoassay was selected for the analysis of E2 in the samples because of its high selectivity and sensitivity. The quantitative measurement of E2 was conducted using the Correlate-EIA E2 kit (Assay Designs, Inc. Mich, USA). This kit applies a polyclonal antibody to E2 to bind E2 in a target sample or an alkaline phosphatase conjugate which has E2 covalently attached to it, in a competitive manner. The data obtained from the standard were handled by a microplate analysis software package, Microplate Manager III (Bio-Rad Lab., Cal., USA) utilizing a four-parameter logistic curve fitting program. The concentration of E2 in the unknown was determined by interpolation. Duplicate assays were performed for each sample.

Results and discussion

Determination of extraction recovery efficiencies

The solid phase extraction recovery efficiency of E2 from the aqueous phase was 100%. The ASE extraction recovery efficiencies of E2 from the sludge phase were 75.2% for the first denitrification tank sludge and 69.7% for the concentrated sludge. No recovery

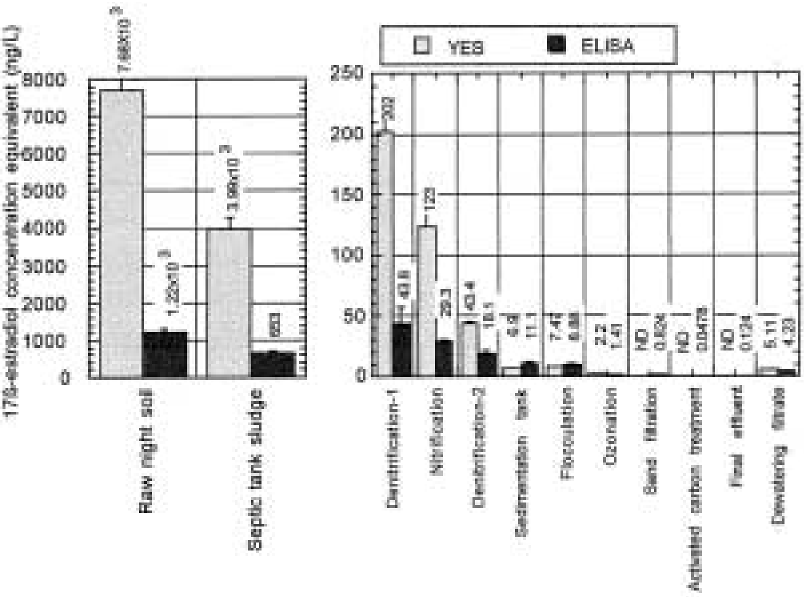

Figure 2 17 β-estradiol concentration equivalent (ng/L) detected in the aqueous samples of the investigated night soil treatment plant. Vertical bars indicate SD

correction was made except the mass balance calculation of E2 in the treatment system where recovery efficiency of E2 from sludge samples was all assumed as 70%.

Quantification results of estrogen assayed by YES and ELISA

Aqueous sample. E2 concentration equivalent (YES results) and actual E2 concentration (ELISA results) in each aqueous sample were shown together in Figure 2. The raw night soil and the septic tank sludge contained approximately 8,000 and 4,000 ng/L of E2 equivalent, respectively. During the biological treatment, the value decreased and the overflow from the sedimentation tank showed the value less than 1/1,000 of the raw night soil. After the subsequent physico-chemical treatment, the value was under the detection limit (approximately 2 ng/L) in the final effluent. A similar tendency with the results of the yeast was observed in the ELISA results. The concentrations of E2 in the raw night soil and the septic tank sludge showed high values (1,200 and 650 ng/L, respectively) which were reduced remarkably in the first denitrification process and finally indicated 0.12 ng/L of E2 in the final effluent. The E2 equivalent concentrations in test samples were compared with the E2 concentrations measured by the ELISA to evaluate the contribution of E2 to the whole estrogenic activity. In the raw night soil and the septic tank sludge, E2 occupied 16% of the whole estrogenic activity. The calculated contribution of E2 became higher during the treatment despite the decrease in concentration. This suggests that E2 is relatively recalcitrant (or deconjugated from its conjugated form) and tends to remain in the aqueous phase compared with other estrogenic substances.

Sludge sample. The E2 concentration equivalent and the actual E2 concentration in six sludge samples were shown in Figure 3. The septic tank sludge showed the highest value of E2 equivalent (1,120 ng/g-dry weight), twice as high as the value of the raw night soil (624 ng/g). The E2 concentration equivalent ranged from 159 to 322 ng/g in the activated sludge samples. The concentrations of E2 in the raw night soil and the septic tank sludge were respectively 303 and 274 ng/g. The content of E2 in the activated sludge was almost

Table 1 Comparison of estrogenic activity of various substances in terms of EC_{50} (mol/L) measured by the YES. The ratio of substance's activity relative to that of E2 (0.25 nmol/L) is shown below

Substances	Relative ratio of estrogenic activity
Natural estrogen and its derivatives	
17β-estradiol (E2)	1
Estrone	0.21
Estriol	1.3×10^{-3}
17α-estradiol	0.01
17β-estradiol 3-sulfate	5.3×10^{-5}
β-estradiol 3,17-disulfate	ND
Estrone 3-sulfate	9.6×10^{-5}
β-estradiol 17-(β-D-glucuronide)	5.9×10^{-7}
β-estradiol 3-(β-D-glucuronide)	3.1×10^{-5}
Estrone β-D-glucuronide	1.0×10^{-6}
Estriol 17-(β-D-glucuronide)	ND
Estriol 3-(β-D-glucuronide)	ND
17β-estradiol 3-sulfate 17- glucuronide	ND
2-hydroxyestradiol	6.1×10^{-3}
2-hydroxyestrone	1.1×10^{-3}
2-hydroxyestriol	1.9×10^{-6}
Artificial compounds	
Nonylphenol	1.0×10^{-3}
Bisphenol A	2.7×10^{-4}
Phytoestrogen	
Genistein	$1.\ 1\times10^{-4}$

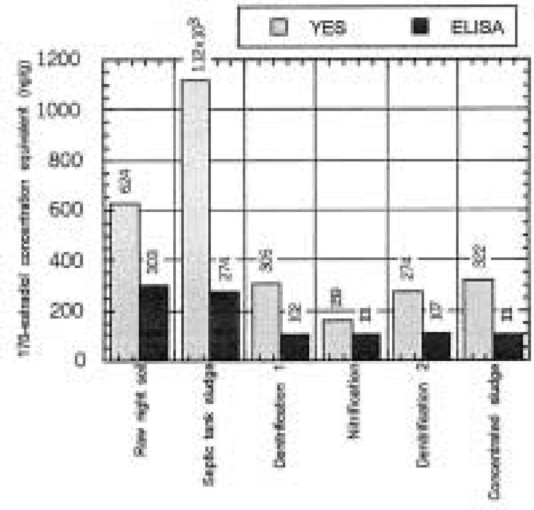

Figure 3 17 β-estradiol concentration equivalent (ng/g) detected in the sludge samples of the investigated night soil treatment plant

constant (100 ng/g) at four sampling points. The ratio of E2 to the total estrogenic activity in the sludge samples tended to be higher (24.5–63.5%) compared with the ratio of the aqueous samples.

Human excretion of natural estrogens and their metabolites

Human estrogens are excreted into urine and feces on the order of μg per day. They are eliminated as conjugated or unconjugated forms (Turan, 1996). Human daily estrogen discharge into the urine (0.9–1.2 L/day) is reported to be 1.0–3.0 μg of E2, 3.9–8.0 μg of estrone, 1.0–4.8 μg of estriol and a larger quantity of inactive form such as glucuronide and sulfate conjugates (Fotsis *et al.*, 1980; Fotsis and Adlercreutz, 1987). The conjugates are more polar and water-soluble than free estrogens. Estrogenic activity decreases remarkably with the addition of conjugate groups (Table 1). On the other hand, feces (70–140 g/person/day) mainly contain free estrogens which have undergone deconjugation by enteric bacteria and are out of the enterohepatic circulation. The assay results for the raw night soil and the septic tank sludge corresponds well with the above-mentioned information in that their E2 and E2 equivalent concentrations were on the order of μg/L, and the ratio of E2 to the total estrogenic activity was higher in sludge than in water. The sample analyses of estrone, estriol and other positive estrogen metabolites were not conducted in this research. It is necessary to survey those compounds including artificial estrogenic substances that have a possibility to flow into the plant.

Mass balance of E2 in the treatment system

Based on the ELISA results and knowing the flow rate and the SS concentration in each treatment step on the sampling day, mass balance of E2 in the treatment system was calculated as shown in Figure 4. In this calculation, recovery correction of the results for sludge samples was made. The total daily loading rate of E2 from the storage tanks of the raw night soil and the septic tank sludge was 970 mg. It was estimated that 670 mg of E2, 70% of the total daily load was degraded in the first denitrification tank.

However, about 8,000–9,000 mg of E2, which was eight to nine times higher than the daily load, was already accumulated in the activated sludge and circulated through the biological tanks. The concentrated sludge containing 300 mg of E2 was pulled out from the sedimentation tank and treated daily. In this plant, the excess sludge has been dewatered and incinerated. Consequently, the overflow from the sedimentation tank which contained 10 mg of E2, 1/100 of the initial load, was directed to the subsequent treatment processes.

From this plant, final effluent and excess sludge go out of the system. The values of E2 equivalent and E2 of the final effluent were below all the reported and our investigated values of sewage treatment works effluents (Matsui *et al.*, 2000; Desbrow *et al.*, 1998). In male fish, the threshold concentration for VTG elevation in blood plasma to E2 has been reported to be between one and ten ng/L (Routledge *et al.*, 1998), which the value of the final

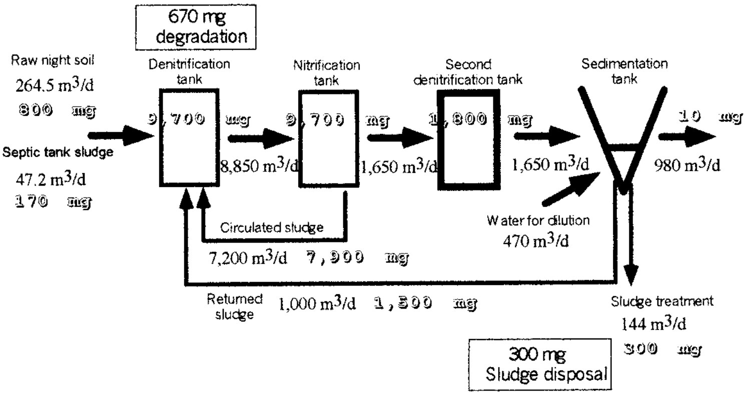

Figure 4 Estimated mass balance (per day) of 17β-estradiol in the investigated night soil treatment plant

effluent (0.12 ng/L) did not exceed in the investigated plant. Biological treatment can lower the estrogen concentration in the aqueous phase remarkably indeed, but only to the "gray" level of several to tens of ng/L which can enhance the VTG production. Combination of the applicable physico-chemical treatments with biological treatment is an important key to control estrogens as low as possible. On the other hand, the mass balance calculation suggests a transport of E2 onto activated sludge. Attention should be paid to sludge disposal with proper treatment.

Conclusions

Water and sludge samples at different treatment phases of a night soil treatment plant were assayed by the *in vitro* yeast estrogen screen and the ELISA for E2. The raw night soil and the septic tank sludge respectively contained approximately 8,000 and 4,000 ng/L of E2 equivalent as estrogenic activity. E2 accounted for 16% of their total estrogenic activity. Estrogenic activity in the aqueous phase decreased by less than 1/1,000 during biological treatment, and the concentration of E2 indicated 0.12 ng/L in the final effluent. Transport of estrogens onto activated sludge was suggested from the analyses of sludge samples.

The E2 equivalent ranged from 159–322 ng/g in four activated sludge samples, where the contents of E2 were almost constant (100 ng/g) and E2 explained 31.4–63.5% of the total activity. Estrogens were considered to be accumulated in the activated sludge and circulated through the biological tanks.

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