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Behavior of Endocrine Disrupting Chemicals in Johkasou Improved Septic Tank in Japan

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Abstract The behavior of estrogens (estrone: E1, 17β-estradiol: E2, estriol: E3 and ethinylestradiol: EE2) and an androgen (testosterone) in the water and sludge from Johkasou in Japan was investigated. The concentrations of E1, E2, E3 and testosterone in water samples from the Johkasou were 33–500, N.D. ~150, N.D. ~6,700 and 500 ng/L, respectively. In sludge samples, the concentrations of E1, E2, E3, and testostrerone were N.D. ~39, N.D. ~6.7, N.D. ~60 and 0.2–9.0 ng/L, respectively. EE2 was not detected in all samples. The removal rates of E1, E2, E3 and testosterone in Johkasou were 45%–91%, 66%–100%, 90%–100%, and about 90%, respectively.

Keywords Estrogen · Androgen · EDCs · Johkasou

In recent years, social concerns have risen about estrogenic adverse effects for aquatic organisms caused by endocrine disrupting chemicals (EDCs). There are steroid hormones in the environment such as estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethinylestradiol (EE2) which is used as a birth control medicine. Recent research reports suggested that these steroid compounds may be the main contributive sources of estrogenicity in many municipal sewage treatment plants (STPs) (Andersen et al. 2003; Ankley and Johnson 2004; Hashimoto et al. 2004; Jobling et al. 2002). In general, wastewater is treated by activated sludge in STPs (Hashimoto et al. 2004; Holbrock et al. 2002; Shi et al. 2004). However, since the source of all natural estrogen excreted from animals (including humans) cannot be eliminated, specific treatment processes in STPs must be optimized. To date, it is known that municipal STPs reduce steroid estrogen to some extent. However, STPs cannot reduce concentrations to levels safe enough for aquatic life (Jobling et al. 2002).

Johkasou is a type of wastewater treatment plant (WTP) and it is an improved traditional septic tank. Septic tanks are used worldwide for the treatment of human and household wastewater. However, that construction is simple and has an anaerobic or an aerobic biological treatment system only. On the other hand, Johkasou has a series of anaerobic, aerobic and chlorination treatment systems. Basic treatment systems of Johkasou are similar to those of STPs, which use microorganisms. Johkasou is as prevalent as STPs in Japan now. Johkasou are set up easily at a lower cost than STPs, because the construction of Johkasou is so compact that it can be used underground each house. Moreover, the capability to remove organic materials of Johkasou is as good as that of STPs. Therefore, Johkasou is a practical wastewater treatment plant. However, there is little research on the behavior of steroid hormones in Johkasou. The aim of this study is to investigate the concentrations of E1, E2, E3, EE2 and testosterone, and removal efficiencies in each treatment procedure of three Johkasou (Table 1).

Materials and Methods

The samples used for this study were collected from each Johkasou (Johk. 1 in Kumamoto city; Johk. 2 and 3 in Aso city) in Kumamoto Prefecture, Japan (Fig. 1). The schematic diagram of the Johkasou is shown in Fig. 2. The

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Table 1 Information of family composition

Sorts	Johkasou 1		Johkasou 2		Johkasou 3	
	Ages	Number	Ages	Number	Ages	Number
Male adult	30s	1	30s	1	50s	1
Female adult	30s	1	30s	1	50s	1
Male child	Elementary school students	1	Kindergarten children	2	Junior and high school students	2
Female child	Elementary school students	1	–	–	–	–

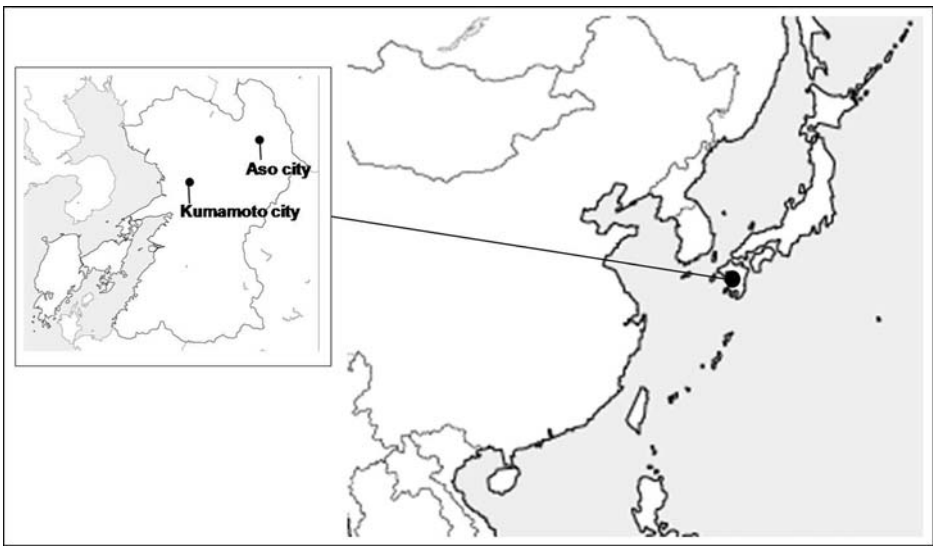

Fig. 1 Sampling sites

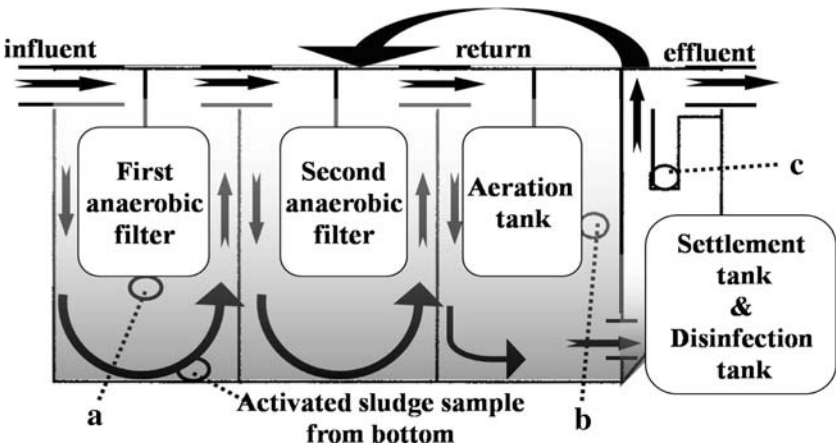

Fig. 2 Schmetic of the Johkasou; *a*, *b*, *c* sampling spot

sampling spots were decided (a) first anaerobic filter, (b) aeration tank, and (c) disinfection tank in each Johkasou. These Johkasou samples were collected at Johkasou 1, Johkasou 2 and Johkasou 3 during July to August 2006. There were two kinds of the sludge samples, which were mixed liquor suspended solids (floating sludge: FS) from Johkasou 1, and activated sludge at the bottom (sedimentation sludge: SS) from Johkasou 2 and 3. These samples were centrifuged (3,000 rpm, 20 min), and separated into water and solids. The separated water was stored at 5°C until extraction after adding 0.01% NaN_3 for prevention of biodegradation.

The separated water sample (1 L) was treated by a solid-phase extraction using an XAD-2 column at pH 3 adjusted with CH_3COOH. The extract with XAD-2 was washed with 0.1 N $NaHCO_3$ and eluted by 50 mL of methanol. The eluent was concentrated with a rotary evaporator and the residue was dissolved in 1 mL of ethyl acetate. The ethyl

acetate phase was diluted with 20 mL of hexane and cleaned up by silica-gel chromatography. The first fraction was eluted for E1, E2 and EE2 with hexane/acetone (30 mL, 65:35 v/v), and the second fraction was eluted for E3 with ethyl acetate/methanol (20 mL, 70:30, v/v). Every fraction was concentrated to 300 μL of volume with a rotary evaporator and transferred to screw vials to which 100 μL of bisphenol A-$^{13}C_3$ (1.0 mg/L) was added as an internal standard for LC/MS analysis.

The solid sludge samples (2 g) were treated by an ultrasonic extraction for 30 min with 40 mL of acetic acid/methanol. The extract was dried with a rotary evaporator and the residue was diluted with 200 mL of milli-Q water. This sample was treated by solid-phase extraction in the same way as the treatment method for water samples.

To analyze testosterone by GC/MS, the sample was dried with a gentle flow of nitrogen at 60°C and the residue was derivatized with 40 μL of MSTFA [*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide] : TMIS (Iodotrimethylsilane) : DTE (1, 4-Dithioerythritol) (1,000:2:2, v/v/w, 80°C, 15 min). This derivatized sample was dissolved into 80 μL of benzene that contained 80 μL of the internal standard decafluorobiphenyl (1.0 mg/L).

The estrogens (E1, E2, E3 and EE2) were analyzed with a Shimazu high-performance liquid chromatograph (HPLC) combined with a mass JEOL LC mate spectrometer attached with atmospheric pressure chemical ionization (APCI) source. As a column for the LC/MS analysis a GL Science Inertsil ODS-3 V column (250 × 4.6 mm, 5 μm) was used. The mobile phase was programmed from 50% methanol at 0 min to 100% methanol for 60 min at a flow rate of 1 mL/min. Testosterone was analyzed with an Aglient 6890GC series gas chromatography (GC) system combined with a mass spectrometer (JEOL JMS-700) with a DB-5 (30 m × 0.25 mm, 0.1 μm). The limit of detection of E1, E2, E3, EE2 and testosterone were 60, 60, 300, 300, and 0.2 pg based on a ratio of 3S/N, respectively.

The estrogen agonist activity was measured by a yeast two-hybrid estrogen receptor assay. The yeast two-hybrid assay system using yeast cells (*Saccharomyces cerevisiae* Y190) was produced by introduction of the medaka fish (*Oryzias latipes*) estrogen α-receptor (medERα) and the coactivator TIF2 (pFAAD424-TIF-2). The medERα assay was adapted to a chemiluminescent receptor gene (for *β*-galactosidase) method employing a 96-well culture plate (Sumilon, Sumitomo Bakelite, Japan) (Shiraishi et al. 2000).

Results and Discussion

The concentrations of E1, E2, E3 and testosterone in treated water from Johkasou were 33–500, N.D. ~150, N.D. ~6,700 and 500 ng/L, respectively (Fig. 3). In sludge samples, the concentration of E1, E2, E3, and testosterone were N.D. ~39, N.D. ~6.7, N.D. ~60 and 0.2–9.0 ng/L, respectively (Fig. 4). EE2 could not be detected in Johkasou. These recoveries were effectively corrected with every surrogate standard (E1-d_4, E2-$^{13}C_4$, E3-$^{13}C_4$, EE2-d_2 and testosterone-d_3).

E1 was detected in all treated water from Johkasou and was a dominant estrogen in all samples (it accounts for 80%). The concentration of E1 was higher than E2, because the E2 was converted to E1 by oxidation of activated sludge (Ternes et al. 1999). In some studies, concentrations of E1 and E2 and/or estrogenic activity after treatment by activated sludge increased as compared to the influent (Hashimoto et al. 2004; Ternes et al. 1999). The concentrations of estrogen in Johkasou 1 and 2 were higher than that of in Johkasou 3. Johkasou 1 and 2 were used by a young family (husband and wife: about 30 years old), Johkasou 3 was used by an elder family (husband and wife: 50 years old). A high concentration of E3 was detected in Johkasou 2. The quantity of E3 excretion from a woman is related to physiological conditions such as pregnancy. The quantity of E3 excreted in Johkasou 2 was 500–1,000 times of a normal condition. It was estimated that the estrogen concentration of E3 in Johkasou 2 was affected by excretion from the pregnant woman. EE2, a synthetic compound for the birth control, was not detected in all treated water.

Testosterone was detected from all treated water samples, same as E1. Testosterone was an important androgen which is a male steroid hormone and controls male body's condition. It is said that the excretion quantity of estrogen from females depends on age, while that of androgen from males does not depend on age. The excretion quantity of testosterone from males is twenty times of the excretion quantity from females. Compared with the first anaerobic tank in Johkasou 2 and Johkasou 3, the ratios of testosterone/(E1 + E2) were 0.09 and 0.44, respectively. The high ratio of testosterone to E1 + E2 reveals that the family consisted of mainly males.

Regarding the behavior of steroid hormones in Johkasou, estrogen and androgen in water samples decreased through the treatment processes in Johkasou. The removal rates of E1, E2, E3 and testosterone in Johkasou were 80, 90, 80 and 95%, respectively. These removal rates of steroid hormones in Johkasou were comparable levels in STPs (Hashimoto et al. 2004). This was followed by concentration levels of estrogen and androgen from sludge samples in Johkasou. Estrogen was not detected in the FS, however, was detected in the SS. In this result, it is estimated that steroid hormones were removed from wastewater by adsorption to activated sludge. Therefore, these steroids remained in activated sludge in Johkasou. Similar results to the elimination of steroid hormones in activated sludge

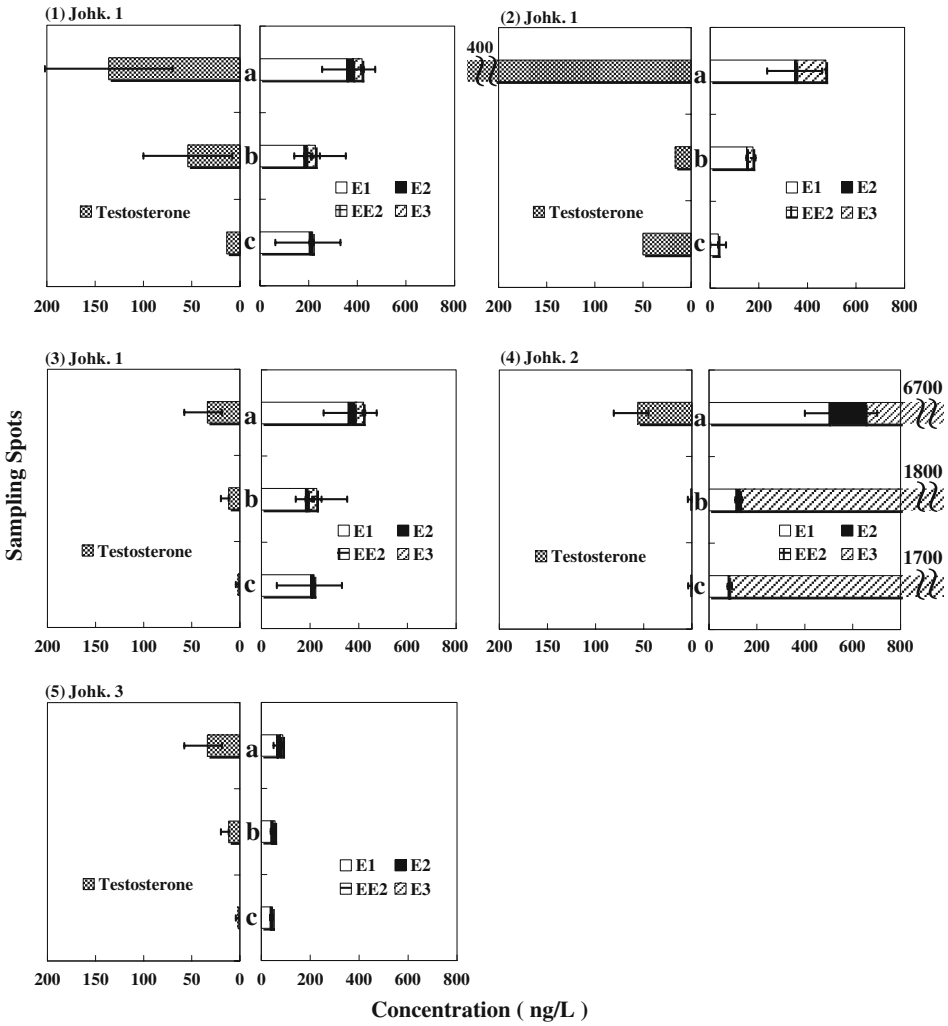

Fig. 3 Concentration of testosterone and estrogens in water sample in each Johkasou. (*1*)–(*3*) Water samples were collected in Johkasou 1. (*4*) Water samples were collected in Johkasou 2. (*5*) Water samples were collected in Johkasou 3

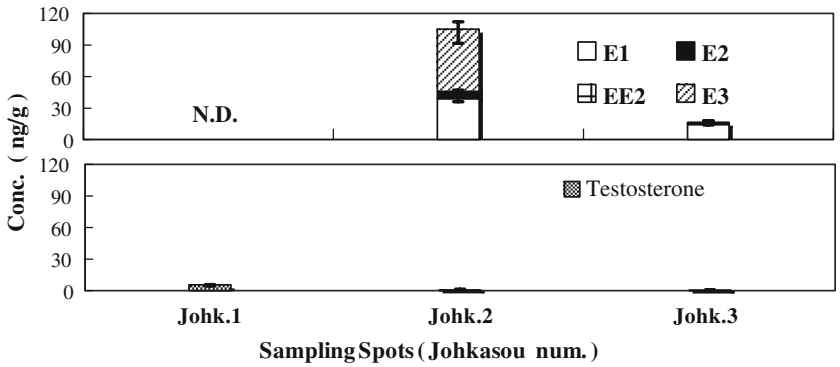

Fig. 4 Concentration of testosterone and estrogens in sludge samples from each Johkasou. *N.D.* Less than the minimum limit of detection

systems were found by some reports (Hashimoto et al. 2004; Suzuki and Maruyama 2006). Consequently, it is estimated that some parts of estrogenic compounds were discharged to the environment, but the estrogenic compounds still remaining at bottom in Johkasou.

To survey the endocrine disrupting effect for organisms, the estrogenic activity was tested. These quantities were calculated by EEqs (17β-estradiol equivalence quantities). An estrogenic effect for medERα was detected in water samples from Johkasou 2 and 3. The concentration of estrogenic activity of (E1 + E2) was N.D. $\sim$57 ng-E2/L (Fig. 5). Estrogenic activity of E1 is 0.17 times as much as that of E2 (E2 = 1.0), and that of E3 is only 0.0027 times as much as E2 (Kurabayashi and Ninomiya 2005). Estrogenic activity of E3 was very weaker than E1 and E2, the concentration of estrogenic activity of E3 was ignored.

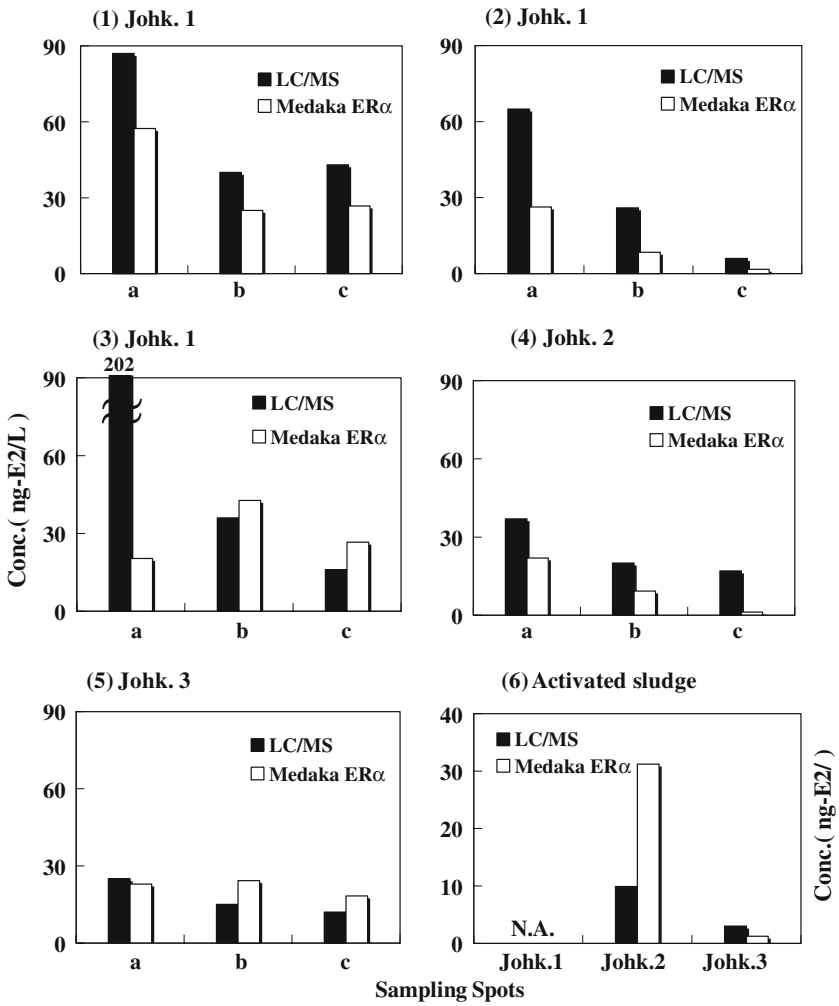

Fig. 5 Estrogenic activities from each Johkasou. (*1*)–(*3*) Estrogenic activities of water sample in Johkasou 1. (*4*) Estrogenic activities of water sample in Johkasou 2. (*5*) Estrogenic activities of water sample in Johkasou 3. (*6*) Estrogenic activities of sludge sample in Johkasou 1–3. *N.A.* Not activity ng-E2/L, ng-E2/g: E2 equivalence concentration

In this study, the result by an instrumental analysis (LC/APCI/MS) was supported by the results of yeast two-hybrid assay. Because environmental samples as Johkasou samples contained many matrixes, there was observed dispersion of results which was caused by the matrixes. E1, E2, and E3 were not detected in mixed liquor suspended solid, however, the estrogenic effect for medERα was detected in sludge samples at the bottom. The concentration of estrogenic activity of (E1 + E2) was N.D. ~31 ng-E2/g/activated sludge (Fig. 5). This case was similar to the results by the instrumental analysis. However, the quantity by the Two-hybrid assay tended to be higher than that by the instrumental analysis. However, the contributing rate of estrogenic activity compounds was not satisfied with only natural estrogen. It is assumed that there were unknown estrogenic compounds except natural estrogens in activated sludge in Johkasou (Terasaki et al. 2005). When the contributing rate of estrogenic activity of natural estrogens in Johkasou was compared to STPs, that rate in Johkasou was higher than in STPs. Many types of synthetic estrogenic compounds such as bisphenol-A, nonylphenol and so on influence may exist in STPs. Both treated water and sediments had estrogenic activity for medERα. This result was the same as municipal STPs.

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