Determination of Estrogenic Substances in the Water of Muko River Using *in Vitro* Assays, and the Degradation of Natural Estrogens by Aquatic Bacteria

Sumiko Matsuoka,* Misa Kikuchi, Sachi Kimura, Yuko Kurokawa, and Shin'ichiro Kawai

School of Human Sciences, Kobe College, Okadayama 4–1, Nishinomiya, Hyogo 662–8505, Japan

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In order to determine the level of estrogenic substances in river water and to evaluate the degradation of estrogen by aquatic bacteria, three *in vitro* assays, E-Screen, Ishikawa cell-alkaline phosphatase (Ishikawa cell-ALP) assay and yeast estrogen screen (YES) assay were used. Water samples were collected throughout one year at upstream, midstream and downstream locations on the Muko River, Hyogo Prefecture, Japan. Estrogenic substances in the water were extracted by solid phase extraction using Sep-pak C18 cartridges. The levels of estrogenic substances in river water changed daily, weekly and monthly. The highest 17β-estradiol equivalent (E2 equivalent) was obtained as 32.9 ng/l at the midstream in July of 2002. Although some differences in E2 equivalent were observed in some water samples between the E-Screen and Ishikawa cell-ALP assays, the levels measured by these assays were generally similar and ranged from not detected (ND) to 32.9 ng/l. E2 equivalent levels assayed by YES were low or below the detection limit. Degradability of estrogen by aquatic bacteria was investigated in the summer and winter. E2 and estrone (E1) were degraded completely within five days in the summer, and within seven days in winter. Bacterial degradation of a synthetic estrogen, 17α-ethynylestradiol (EE2), was much lower compared to that of E2 or E1.

Key words —— estrogenic substance, river water, E-Screen, Ishikawa cell-alkaline phosphatase assay, yeast estrogen screen, biodegradation

INTRODUCTION

Various kinds of chemicals such as pesticides, medicines and detergents are frequently discharged into the environment after being used, especially into the aquatic environment, and considerable attention has been paid to the adverse effects of these chemicals on wildlife species. Endocrine-disrupting chemicals (EDCs) have been receiving increasing attention in recent years, and many studies have been carried out to determine the effects of EDCs on terrestrial and aquatic organisms. Among EDCs, estrogenic substances including natural and synthetic estrogens in addition to synthetic estrogenic chemicals have been much investigated because of their reproductive effects. However, only a few causal relationships between these substances and endocrine disruption have yet been identified. Therefore, it is important to investigate the level and distribution of EDCs in the aquatic environment and to examine their influence on various organisms. In many countries, water pollution has been caused by both domestic and industrial chemicals, and many estrogenic substances have been loaded into rivers through sewage treatment plants.[1)]

In the present study, three types of *in vitro* assay [E-Screen,[2–4)] Ishikawa cell-alkaline phosphatase (Ishikawa cell-ALP),[5,6)] and yeast estrogen screen (YES) assay[7)]] were used to examine the estrogenic substances in an aquatic environment. The human breast cancer cell T-47D has an estrogen receptor and proliferates in the presence of estrogenic substances in an E-Screen assay. Ishikawa cells were established from human endometrial cancer cells having an estrogen receptor; ALP activity in these cells increases in the presence of estrogen. In the YES assay, yeast cells transformed with plasmids

*To whom correspondence should be addressed: School of Human Sciences, Kobe College, Okadayama 4–1, Nishinomiya, Hyogo 662–8505, Japan. Tel. & Fax: +81-798-51-8422; E-mail: fwnh3644@mb.infoweb.ne.jp

encoding the human estrogen receptor, and an estrogen responsive promoter linked to a reporter gene were used. The sensitivity of E-Screen and Ishikawa cell-ALP assays to estrogens are almost same, and the YES assay showed 100 to 1000-fold lower sensitivity than those of other two assays. These *in vitro* assays are useful for detecting total estrogenic activities in an aquatic environment, and can determine the total estrogenic activities of a number of samples within a short period of time; they are, therefore, useful in the investigation of the potential influence of EDCs on wildlife.

It is not sufficient to use only one *in vitro* assay for assessing the levels of estrogenic substances in the aquatic environment. E-Screen and YES assays have been used extensively for the determination of estrogenic substances in the environment in many laboratories, whereas determinations of estrogenic substances in the aquatic environment using Ishikawa cell-ALP assay have not been carried out except in our report. Thus, these three *in vitro* assays which have different endpoints, respectively, were used, and were compared the difference in the sensitivity to estrogenic substances in the aquatic environment.

Levels of estrogenic substances in the Muko River water were determined using these *in vitro* assays and, because most estrogenic substances are comprised of natural estrogens such as 17β-estradiol (E2) and estrone (E1), followed by a synthetic estrogen, 17α-ethynylestradiol (EE2) showing potent estrogenic activity, the degradability of both natural and synthetic estrogens by aquatic bacteria was also investigated.

MATERIALS AND METHODS

Cultured Cells and Chemicals —— Human breast cancer estrogen-sensitive T-47D cells were obtained from the American Type Culture Collection. Human endometrial cancer cells, specifically Ishikawa 3H-12 cells, were kindly provided by Dr. Masato Nishida, Tsukuba University, Ibaraki, Japan, and recombinant yeast for the YES assay was kindly provided by Dr. J. P. Sumpter of Brunel University, Uxbridge, U.K. Fetal bovine serum (FBS), 0.25% trypsin-EDTA, Dulbecco's Modified Eagle's Medium (DMEM), E2, E1 and EE2 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Sampling of Water and Extraction of Estrogenic Substances —— Water samples were collected from sites upstream (St. 1), midstream (St. 2) and downstream (St. 3) of the Muko River in the southern part of Hyogo Prefecture (Fig. 1). River water was collected primarily at the downstream site, and occasionally at the midstream and upstream sites. The sampling was performed thirty times from June 2002 to July 2003. For each sample, approximately 3.5 l of river water was collected in glass bottles previously rinsed with acetone and hexane. The water samples were then filtered using a glass fiber filter paper. Estrogenic substances in the filtrated water samples were extracted using a Sep-pak C18 cartridge (Waters Co., Ltd., Milford, Massachusetts, U.S.A.) previously rinsed with 7 ml of diethyl ether, 7 ml of methanol and 10 ml of ultrapure water in that order (3 cartridges/3.5 l). Adsorbed estrogenic substances in the cartridge were extracted with 7 ml of methanol followed by 7 ml of diethyl ether. Extracts in the methanol and diethyl ether solution were concentrated by a rotary evaporator, and were then dissolved in 2 ml of ethanol. One ml of the extracts was evaporated completely under a gentle stream of nitrogen gas and dissolved in 1 ml of dimethyl sulfoxide (DMSO). Recovery rates of estrogens by Sep-pak C18 cartridge were approximately 90%.

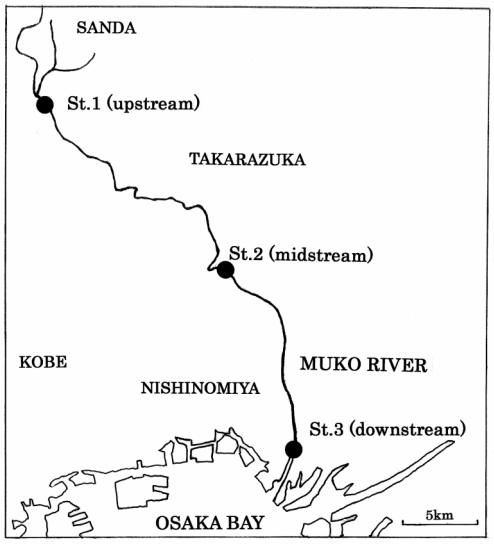

Fig. 1. Sampling Sites on the Muko River

***In Vitro* Assays for Estrogenic Substances** ——

E-Screen Assay: E-Screen assay by T-47D cells was carried out following the method described by Soto *et al*. with some modifications.[2,8] T-47D human breast cancer cells were cultivated at 37°C in 75 cm^2 flasks in DMEM supplemented with 5% FBS and 5% CO_2/95% air. T-47D cells were trypsinized

and plated in 24-well plates at an initial concentration of 4–6 × 10^4 cells/ml in 5% FBS in DMEM. After 24 hr incubation, the medium was exchanged for a phenol red-free experimental medium containing 5% charcoal dextran-treated fetal bovine serum (CD-FBS) and a 0.5% volume of the samples dissolved in DMSO. Six days later, cell growth was measured spectrophotometrically using sulforhodamine B (SRB) assay[9] as follows. Cells were fixed with 10% trichloroacetic acid, washed with pure water, stained with a 0.4% solution of SRB in 1% acetic acid, and washed with 1% acetic acid. The dye was dissolved in 1 ml of 10 mM Tris buffer (pH 10.5) per well, and extinction at 492 nm was measured with a microplate reader by transferring aliquots of 200 μl into cavities of a 96-well plate. E2 standard solution in six concentrations ranging from 0.5 pM to 50 nM was the internal positive control in each assay.

Ishikawa Cell-ALP Assay: Ishikawa 3H-12 cells,[6,8] human endometrial cancer cells, were cultured at 37°C in 75 cm^2 flasks in DMEM supplemented with 15% FBS and 5% CO_2/95% air. Ishikawa cells were trypsinized and plated in 24-well plates at an initial concentration of 10 × 10^4 cells/ml in 15% FBS in DMEM. After 24 hr incubation, the medium was exchanged for a phenol red-free experimental medium containing 15% CD-FBS and a 0.5% volume of the samples dissolved in DMSO. Three days later, the medium was removed and washed with phosphate buffered saline (PBS), and then stored at –80°C for 1 hr. One ml of substrate solution containing 5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$ and 1 M diethanolamine were added to each well, and incubated for 1 hr at 30°C in a shaker. Yellow color due to the formation of *p*-nitrophenol was measured spectrophotometrically at 405 nm.

YES Assay: The YES assay was conducted following the procedures described by Routledge and Sumpter.[7] Yeast cells were grown in the growth medium for 24 hr at 28°C in a shaker. E2 and samples diluted in ethanol were evaporated to dryness in a 96-well plate. The assay medium was prepared by adding chlorophenol red-β-D-galactopyranoside (CPRG) to the growth medium. After the ethanol was evaporated, the assay medium was seeded with yeast from a 24 hr yeast culture to the assay plate, and the absorbance at 540 nm was measured. The assay plate was further incubated for 65 hr at 30°C. After 65 hr, the chlorophenol red produced in the medium was measured spectrophotometrically at 540 nm, and the E2 equivalent was calculated from the sigmoid curve for E2 standard.

Biodegradation of Natural and Synthetic Estrogens by Aquatic Bacteria: Water from the Muko River was collected at St. 3 in August and December 2002, and 100 ml of river water was dispensed into 100 ml Erlenmeyer flasks (10 flasks for each chemical and control). One hundred μl of E2 and E1 (10 μM) were added to each flask to give a nominal concentration of 10 nM of E2 and E1, and each flask was incubated in a shaker at 28°C in the summer and at 15°C in the winter under dark conditions. Autoclaved river water of each sampling site was used as a sterile control. Biodegradation of EE2 was also measured under the same experimental conditions (incubated at 28°C in the winter). The water sample in each flask of E2 or E1 was filtered through a glass fiber filter paper, and the estrogen in the filtrated water was extracted by solid phase extraction using a Sep-pak C18 cartridge every day in the case of E2 and E1, and every week in the case of EE2. Organic solvents in each extract were evaporated and dissolved in 1 ml ethanol. The concentrations of estrogens in the extracts were measured by YES assay, and the E2 equivalent was calculated from the sigmoid curve for E2 standard solution.

RESULTS AND DISCUSSION

Estrogenic Substances in River Water Collected from Muko River

Figures 2–4 show the E2 equivalents assayed by E-Screen and Ishikawa cell-ALP assay in the Muko River water collected from June 2002 to July 2003. Although the YES assay for river water was carried out, the E2 equivalents were below the detection limit. Each assay was carried out twice or three times for each sample. Almost all samples were collected at St. 3 (downstream), because St. 3 is located in easy place to collect river water, and downstream in a river is most vulnerable to pollution. Sampling of water was performed thirty times to assess daily, weekly, and monthly changes in estrogenic substances in the river water. In all samples, the E2 equivalents of the E-Screen and Ishikawa cell-ALP assays were between 0 and 32.9 ng/l. The highest estrogenic activity was observed at St. 2 (midstream) on July 15, 2002, with E2 equivalents in the extracts of 32.9 and 32 ng/l in the E-Screen and Ishikawa cell-ALP assays, respectively (Fig. 4).

Figure 2(a) shows the daily variations in E2

equivalents through one week, and Fig. 2(b) shows weekly variations. E2 equivalents were observed daily, but levels were generally low. A high E2 equivalent was noticed on July 22, 2002, by E-Screen. However, it was low in the Ishikawa cell-ALP assay [Fig. 2(b)]. Figure 3 shows monthly changes in the E2 equivalent from July of 2002 to July of 2003. Although the levels of E2 equivalent in the water samples collected on September 2, 2002, and assayed by E-Screen were very high (31.9 ng/l), the levels of E2 equivalent in other samples were low. The E2 equivalent of water samples collected on September 2 and measured by Ishikawa cell-ALP assay was much lower than that measured by E-Screen due to differences in the endpoint of each *in vitro* assay and to differences in the sensitivity of each bioassay to an extract containing various organic chemicals.

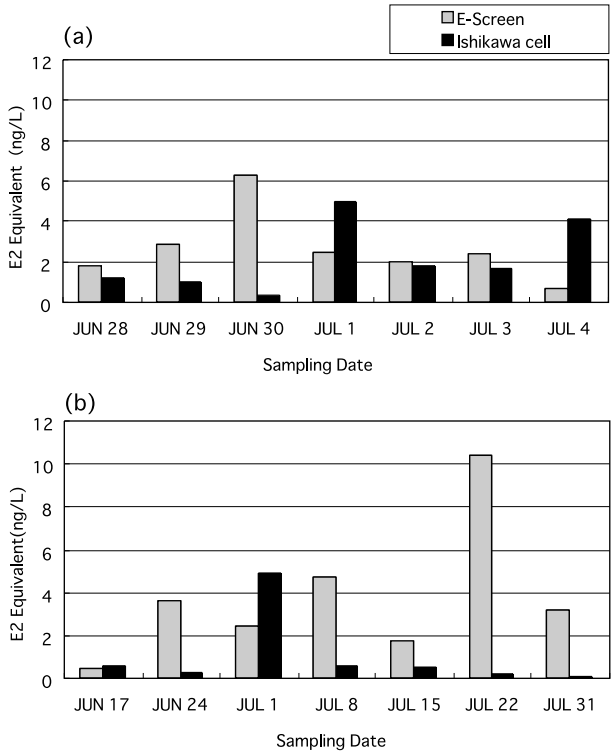

Fig. 2. (a) Daily Changes in the E2 Equivalent of Water Samples Collected at St. 3 Measured by E-Screen and Ishikawa cell-ALP Assays for One week (ng/l)
(b) Weekly changes in the E2 equivalent of water samples collected at St. 3 measured by E-Screen and Ishikawa cell-ALP assays (ng/l).

Figure 4(a) shows variations in the E2 equivalent in river water collected at St. 3 three times in one day, and Fig. 4(b) shows those at Sts. 1–3 on the same day. Although the highest level of E2 equivalent was observed in the morning, the overall level was very low. Comparing E2 equivalents at the three sites, estrogenic substances were hardly detected upstream (St. 1), and the highest concentration was noticed at midstream (St. 2). A sample was collected again at St. 2 in 2003, at which time estrogenic activity in the extracts was much lower than in the sample from 2002 (data not shown). St. 2 is located close to a sewage treatment plant where there might be temporary surges of estrogenic substances into the water, and considerably more rainfall was observed on the sampling day in 2002. However, no primary cause of the high levels of estrogenic substances at St. 2 has been identified. In our previous study of estrogenic substances in another river in Japan, E2 equivalents in the river water measured by the same *in vitro* assays were very high

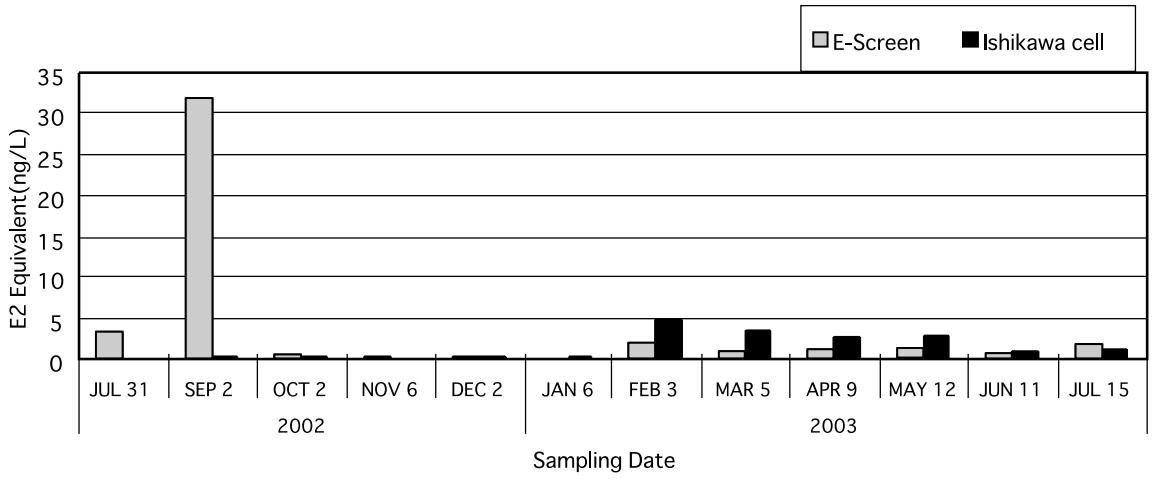

Fig. 3. Monthly Changes in the E2 Equivalent of River Water Collected at St. 3 for One year Measured by E-Screen and Ishikawa Cell-ALP Assays (ng/l)

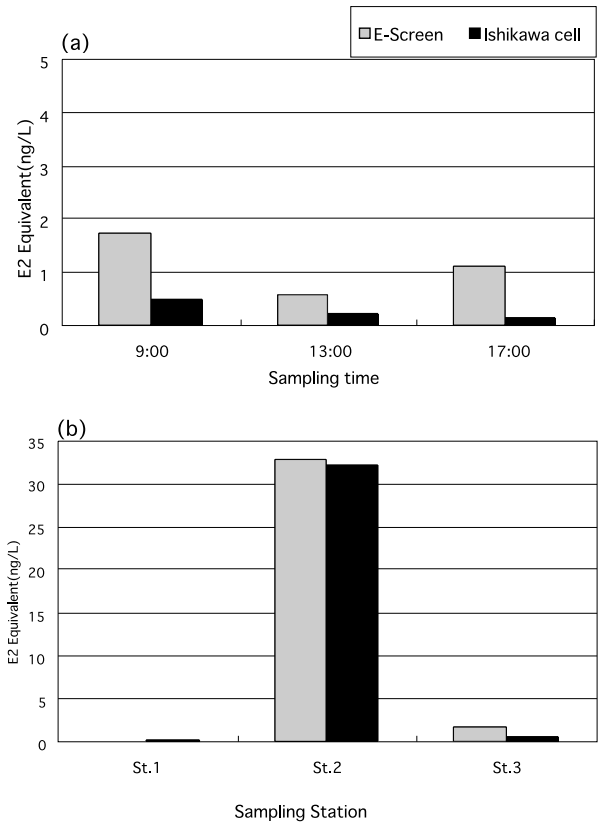

Fig. 4. (a) E2 Equivalents of River Water Collected Three Times in One day at St. 3 Measured by E-Screen and Ishikawa Cell-ALP Assays (ng/l)

(b) E2 equivalents of river water collected at sites upstream (St. 1), midstream (St. 2) and downstream (St. 3) on the same day measured by E-Screen and Ishikawa cell-ALP assays (ng/l). Sampling Date: July, 15, 2002.

at the sampling sites close to the sewage treatment plant.[10] Though water quality data such as BOD and SS were not shown here, E2 equivalent levels corresponded to the values of BOD and SS of river water.

In a study conducted by the Environment Ministry of Japan in 2001, in which the levels of estrogenic substances in an aquatic environment were measured by gas chromatography/mass spectrometry (GC/MS), E2 was detected in 43.5% of 124 river water samples at levels ranging from 0 to 7.2 ng/l, and EE2 was detected in 4% of the samples at levels from 0 to 1.6 ng/l.[11] In the other reports, the concentrations of E2 ranged between 0.51 and 1.41 ng/l measured by enzyme-linked immunosorbent assay (ELISA) in river water in Japan from 2002 to 2003,[12] and approximately 12 ng/l of E2 equivalent was detected in river water measured by YES assay in southern Germany.[13] Based on these findings, the levels of natural estrogen in the Muko River are considered to be similar to those at other sites, except in the case of a few samples. In our previous paper, we determined that E2 equivalents were primarily comprised of E1.[10] Therefore, chemical analysis of E1 is important for the evaluation of estrogenic activity in an aquatic environment. We also conclude in the present study that the levels of estrogenic substances in Muko River water differ from day to day, and thus also from week to week and from month to month; clearly, continuous monitoring is necessary in the future as well.

Estrogenic substances were not detected by YES assay in this study, because yeast has cell walls and estrogen permeability of yeast is inferior to those of cells of E-Screen and Ishikawa cell-ALP assays. Therefore, it is better to use the YES assay for samples containing high levels of estrogens. E-Screen and Ishikawa cell-ALP assays show high sensitivity to estrogenic substances. Cells of these two assays can respond to the presence of 10 pM E2, and the sensitivity of cells to E1 is 10-fold lower than that of E2. However, the sensitivity of cells sometimes changes in E-Screen. And, the sensitivity of cells decreases after 25 passages in the Ishikawa cell-ALP assay. Therefore, appropriate maintenance of a cell is required for the reliable determination of estrogenic substances.

Biodegradation of Estrogen in River Water

Figure 5 shows the results of degradation of natural estrogens such as E2 and E1 by aquatic bacteria in Muko River water. The levels of estrogens were determined using YES assay, because this is most suitable when there are many samples. Non-biological degradation of estrogen was not observed in our sterile controls, though the values of E2 equivalent fluctuated. E2 and E1 were completely degraded within five days in the summer, and within seven days in the winter. It is likely that the difference in the number of days required for complete degradation is due to differences in incubation temperature and to the seasonal change of bacterial flora from summer to winter. Figure 6 shows the degradation of EE2, which is a major constituent of oral contraceptive pills, by aquatic bacteria. The half-life of biodegradation for the synthetic estrogen EE2 is two weeks or more, and no complete degradation was observed, suggesting that the synthetic estrogen EE2 is less degradable than natural estrogens. The half-life of E2 has been reported to be 1 to 2 days, and EE2 was found to degrade within 85 days (half-life 17 days) in an English river,[14] showing tendencies

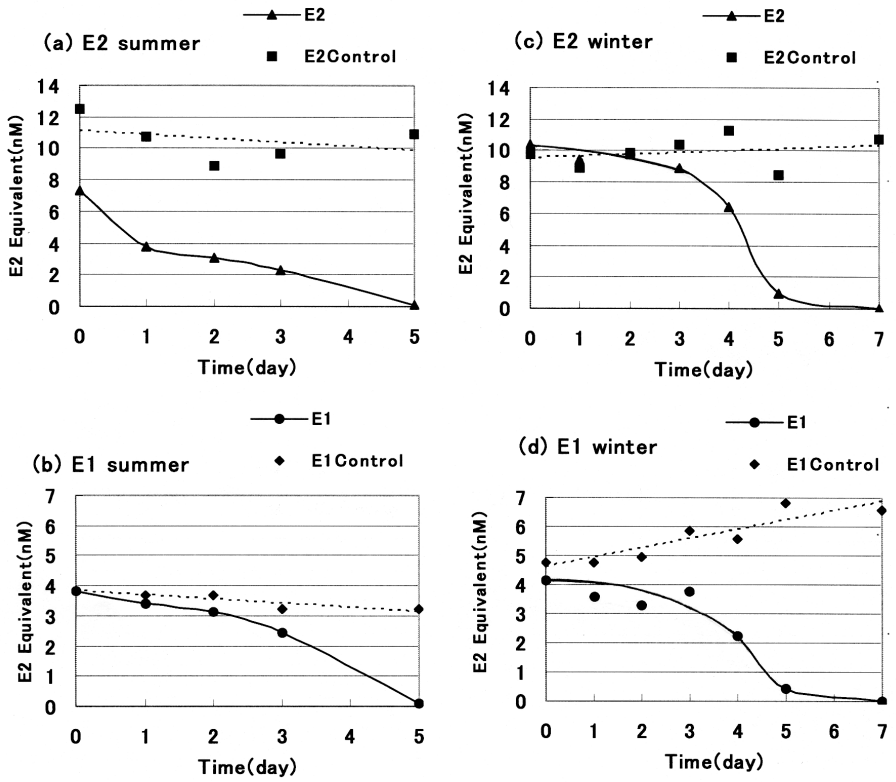

Fig. 5. The Degradation of E2 and E1 by Aquatic Bacteria in River Water
E2 equivalent was measured by YES assay. (a) E2, summer; (b) E1, summer; (c) E2, winter; (d) E1, winter.

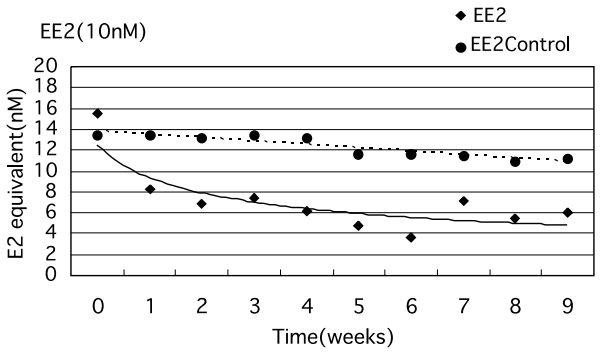

Fig. 6. The Degradation of EE2 by Aquatic Bacteria
E2 equivalent was measured by YES assay.

similar to those found in the present study. Contraceptive pills have been used in Japan since 1999, and the concentration of EE2 may increase hereafter in the aquatic environment. Since EE2 has as strong estrogenic activity as E2, continuous observation of the levels of EE2 in the aquatic environment is essential, and further research should also be conducted on whether these levels produce adverse effects on wildlife, and especially on fish.

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