Evaluation of estrogen-like activity on sewage treatment processes using recombinant yeast

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Abstract Four sewage treatment plants based on an activated sludge process and a pilot scale plant for advanced sewage treatment located in Japan were evaluated for removal of estrogenic substances using in vitro recombinant yeast assay and chemical analysis. The results indicated that 17ß-estradiol (E2) significantly contributed to estrogen-like activity analyzed by yeast assay especially in secondary treated effluents. On the other hand, batch study showed that estrogen-like activity of spiked E2 was easily decreased by an activated sludge treatment. This result suggested that E2 concentrations measured by enzyme immunoassay (EIA) were interpreted as false positives in effluents, and that unknown estrogenic substances other than E2 might have contributed to estrogen-like activity in the secondary treated effluents. Further, in the pilot scale study, advanced sewage treatment processes such as a biological aerated filtration (BAF) process, an advanced oxidation process (AOP), were effective for the removal of those estrogenic activities contributed by unknown estrogenic substances in sewage secondary treated effluent.

Keywords Endocrine disruptors; 17ß-estradiol; estrogen-like activity; sewage treatment

Introduction
It is known that many natural and synthetic chemicals are estrogenic (Bitman and Cecil, 1970; McLachlan, 1980; Soto et al., 1991). Fish in river water containing discharges from sewage treatment plants (STPs) in the UK have been observed to have sexual abnormalities attributed to environmental estrogen (Purdom et al., 1994; Harries et al., 1996). Extracts of some STP effluents have been shown by use of in vitro assays to be estrogenic due to the presence of natural and synthetic estrogens (Desbrow et al., 1998; Takigami et al., 1998). Synthetic chemicals such as nonylphenol (NP) and bis-phenol A (BPA), which are estrogenic, and E2, which is naturally excreted by humans and beasts, have been detected above the detection limit in effluents of many STPs in Japan (Nasu et al., 2001). These substances such as E2 are influential at a concentration of low ng/l for some kinds of fish (Purdom et al., 1994). It is considered that advanced treatment processes of secondary treated effluent of sewage will be necessary to remove these substances sufficiently from treated sewage effluent.

In this study, estrogenic substances from STPs in Japan were evaluated using in vitro recombinant yeast assay and chemical analysis. For confirmation of treatability of E2 with activated sludge process, batch study of an activated sludge treatment for spiked E2 was carried out. Further, in a pilot scale study of advanced sewage treatment processes, the efficiencies of a BAF process, an ozonation process, an AOP (O3/H2O2), and a biological activated carbon (BAC) process were examined for removal of estrogen-like activities from treated sewage effluents.

Materials and methods
Sampling and extraction
In December 1998, samples were collected from four STPs in the environs of Tokyo that
utilized the activated sludge process. During December 1998 to September 1999, samples of a pilot scale sewage treatment plant were collected for evaluation of estrogen-like activities and for chemical analysis of each synthetic substance found to be estrogentic.

All water samples for yeast assay were filtered through glass fiber filter. Solid-phase extraction (SPE) of the filtrates was performed with Sep-pak C18 plus EMV cartridges (Waters). The elutes with methanol were evaporated under nitrogen to dryness and the residue was dissolved in 0.5 ml of dimethylsulfoxide (DMSO).

**Recombinant yeast assay**
Assay was carried out in the manner described by Routledge and Sumpter (1996), with slight modification. The extracted samples were serially diluted with DMSO. 5 ml of each dilution was added to wells on a microtiter plate, and 200 µl of yeast seeded medium containing chlorophenol red-ß-D-galactopyranoside was added to each well. After incubation at 32°C for 4 days, the supernatants of the incubated mediums were transferred to a new plate, and were read at OD 540 nm. Total estrogen-like activities in the extracts were assessed as E2 equivalent by comparing the EC50 of the sample with that of the positive control E2.

**Quantitative analysis of substances**
E2 in the extracted samples was measured by enzyme immunoassay using Correlate-EIA 17ß-Estradiol Kit (Assay Designs, Inc.). E2 of some samples in the batch study was measured by LC/MS/MS after clean-up by SPE and liquid extraction. 4-t-octylphenol (OP), NP, and BPA were measured by GC/MS. Nonylphenol ethoxylates (NPEs) were measured by HPLC with fluorescence detector.

**Batch study of activated sludge treatment for E2**
Treatability of E2 with an activated sludge process was evaluated by batch study. An activated sludge (MLSS 3000mg/l) from an experimental plant in which artificial sewage was treated was used for this study in order to exclude E2 from the sewage. The activated sludge was spiked with E2 to obtain a nominal concentration of 1 µg/l. The activated sludge was maintained at 20°C with forced aeration. At appropriate time intervals, one litre of the suspension was taken and centrifuged. Estrogen-like activity and E2 concentration in the supernatants of centrifugation were measured by the foregoing procedure. The precipitates of centrifugation from 40ml of suspensions were extracted by methanol/1M acetate buffer (9:1). The extracts were evaporated to 5ml, and dissolved in water. The dissolved samples were subjected to SPE in the same manner as the supernatants.

**Pilot scale sewage treatment plant**
A flow diagram of a pilot scale sewage treatment plant is shown in Figure 1. Municipal sewage pretreated by sedimentation was supplied to the pilot scale plant. The influent was first treated with an activated sludge process. Then the activated sludge treated effluent was supplied to advanced treatment processes (Flow A and Flow B). The experimental conditions and apparatus of the pilot scale plant are shown in Table 1.

**Results and discussion**
**Evaluation of estrogen-like activity on influents and effluents of STPs**
The extracted samples from influents and effluents in the STPs showed dose-dependent activities with this yeast assay. A summary of estrogen-like activities (E2 equivalent) and E2 concentrations by EIA in influents and effluents of STPs is shown in Table 2. Expressed in E2 equivalent, total estrogen-like activities in influents of the four STPs were found in
the range of 35.5 to 72.0 ng E2/l. E2 in influents was found in the range of 17.9 to 41.0 ng/l. The contributions of E2 to total estrogen-like activity in influents were estimated to be nearly 50% in influents. In effluents, E2 equivalents were found in the range of 3.9 to 35.3 ng E2/l. E2 concentrations were obtained in the range of 6.9 to 21.4 ng/l. The contribution of E2 to estrogen-like activity in the effluents was higher than that in the influents. Removal rates for estrogen-like activity during treatment varied from 0.6 to 90.7%.

Estrogenic substances in sewage samples

In September 1999, influent and secondary treated water of the pilot scale sewage treatment plant were sampled for determination of E2 and some estrogenic substances (OP, NP, BPA, and NPEs). In the sewage influent, NP, BPA, and NPEs (≤ tetramer) were found at concentration of 8.6, 0.43 and 58.0 µg/l, respectively. OP was not observed (Table 3). E2 concentration by EIA and E2 equivalent with yeast assay was 36 ng/l and 66 ng E2/l, respectively. The theoretical contributions to estrogen-like activity in influent were estimated to be 6.5% for NP, 54.6% for E2 and 61.1% for the total. Because not every oligomer of NPEs in this sample was identified, the contribution of NPEs to estrogen-like activity

Table 1  Experimental conditions and apparatus of the pilot scale plant

<table>
<thead>
<tr>
<th>Process</th>
<th>Apparatus and conditions</th>
</tr>
</thead>
</table>
| BAF     | Filtration media: granular media  
Bed volume: 251 l  
Linear velocity: 132 m/day |
| AOP     | Effective volume: 139 l  
Ozone consumption: 10 mg/l  
H₂O₂ dosage: 2.0 mg/l |
| Ozonation | Effective volume: 139 l  
Ozone consumption: 10 mg/l |
| BAC     | Bed volume: 251 l  
Linear velocity: 144 m/day |

Table 2  Estrogen-like activities and E2 concentrations in influents and effluents of STPs

<table>
<thead>
<tr>
<th>Sites</th>
<th>E2 Equivalent (ng E2/l)</th>
<th>E2 Concentration (ng/l)</th>
<th>Contribution (E2 conc./E2 equ.) (%)</th>
<th>Removal rate of E2 equ. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STP A</td>
<td>35.5 ± 1.7</td>
<td>18.9 ± 3.2</td>
<td>53.2</td>
<td>60.6</td>
</tr>
<tr>
<td>STP B</td>
<td>41.9 ± 1.5</td>
<td>19.7 ± 1.6</td>
<td>47.0</td>
<td>176.9</td>
</tr>
<tr>
<td>STP C</td>
<td>72.0 ± 6.4</td>
<td>41.0 ± 3.6</td>
<td>56.9</td>
<td>66.8</td>
</tr>
<tr>
<td>STP D</td>
<td>39.9 ± 0.9</td>
<td>17.9 ± 1.2</td>
<td>44.9</td>
<td>54.1</td>
</tr>
</tbody>
</table>

Each value represents the mean and standard deviation of three replicated assays.
could not be estimated. This suggests that some active substances other than those substances existed in the influents. In secondary treated water, E2 equivalent with yeast assay was 2.9 ng E2/l. The synthetic estrogenic substances other than NPEs were too low to be detected. Synthetic estrogenic substances in secondary treated water made substantially no contribution to estrogen-like activity. The results indicated that E2 significantly contributed to estrogen-like activity in secondary treated water.

**Correlation between estrogen-like activity and E2 in pilot scale sewage treatment plant**

Figure 2 shows the correlation between E2 equivalent and E2 in influent and secondary treated water of the pilot scale sewage treatment plant. Throughout the investigation period, E2 equivalent and E2 in treated water were considerably lower than those in influent. It seemed that the activated sludge process worked successfully over this period. But in some cases, E2 equivalent was found at concentration of 20 ng/l and higher. E2 concentration was roughly proportional to E2 equivalent, but E2 concentration exceeded the E2 equivalent in the treated water samples showing lower activity. The cause was presumed to be that the EIA used for the determination of E2 in this study recognized other non-estrogenic substances as E2, and also that anti-estrogenic substances in the samples interfered with yeast assay.

**Batch study of activated sludge treatment for E2**

E2 in influent sewage was at times not thoroughly reduced during activated sludge process. For confirmation of treatablity of E2 with activated sludge process, a batch study of an

<table>
<thead>
<tr>
<th>Substances</th>
<th>Relative potency</th>
<th>Influent Concentration (µg/l)</th>
<th>E2 equivalent (µg E2/l)</th>
<th>Effluent Concentration (µg/l)</th>
<th>E2 equivalent (µg E2/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>100</td>
<td>0.0360</td>
<td>0.0360</td>
<td>0.0070</td>
<td>0.0070</td>
</tr>
<tr>
<td>4-t-OP</td>
<td>0.1</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NP</td>
<td>0.05</td>
<td>8.60</td>
<td>0.0043</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>0.005</td>
<td>0.43</td>
<td>0.00002</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NPEs</td>
<td>58.0</td>
<td>58.0</td>
<td>–</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td></td>
<td>0.0403</td>
<td></td>
<td>0.0070</td>
<td></td>
</tr>
<tr>
<td>yeast assay</td>
<td></td>
<td>0.0660</td>
<td></td>
<td>0.0029</td>
<td></td>
</tr>
<tr>
<td>total/yeast assay %</td>
<td>61.1</td>
<td>241.4</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

n.d. = not detected

**Table 3** Comparison of chemical analysis and estrogen-like activities in yeast assay for sewage

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**Figure 2** Correlation between estrogen-like activity and E2 in the pilot scale sewage treatment plant
activated sludge treatment for spiked E2 was carried out. An activated sludge from an experimental reactor in which artificial sewage was treated was used for this study in order to exclude E2 from the sewage. As shown in Figure 3, E2 equivalent in the control before spiking was not found, but E2 was found at concentration of 6.0 ng/l in supernatant and 8.4 ng/l in precipitate, respectively. Just after spiking of E2, E2 equivalents were found to be 213.0 ngE2/l in supernatant and 86.3 ngE2/l in precipitate, respectively. E2 was also found to be 122.6 ng/l in supernatant and 25.8 ng/l in precipitate, respectively. This theoretical recovery rate to spiked E2 was approximately 30% for E2 equivalent and 15% for E2. This result suggests that spiked E2 was immediately degraded weaker estrogens. After 3 hours, E2 equivalent in supernatant was reduced to 2.1 ngE2/l, but E2 remained 7.4 ng/l. E2 equivalent and E2 after 3 hours were similar to the concentrations of the control before spiking. E2 was not present in the activated sludge used for this study theoretically, and LC/MS/MS analysis did not detect E2 above the detection limit (≤ 1 ng/l) in the supernatant of the control sludge. Also in the artificial sewage used for the experimental reactor, no estrogen-like activity or E2 concentration was detected by EIA or LC/MS/MS measurement. The results suggest that EIA falsely recognized non- or weak estrogenic substances other than E2 which were produced during activated sludge treatment. The fact that E2 and E2 equivalent in the precipitant exhibited similar behavior to those in the supernatant was seen as evidence that E2 and its by-products did not migrate to the activated sludge.

The results of the current study indicate that the conclusion of earlier reports that most estrogen-like activity in treated sewage is attributable to E2 (Takigami, et al., 1998) can be traced to false recognition of E2 by EIA. The results of the batch study suggest the possibility of thoroughly removing E2 present in the influent by activated sludge treatment. Ternes et al. reported that in E2 biodegradation experiments using diluted activated sludge the E2 rapidly oxidized to remain as estrone (Ternes et al., 1999). Although estrone was not measured in the present study, the probability that low-activity by-products like estrone were produced is considered high in light of the slow initial reduction rate of E2 equivalent compared with reduction of E2 concentration. Moreover, the ensuing rapid decline in activity suggests that the by-products were also rapidly degraded. As shown in Figure 2, relatively high estrogen-like activity and correlation with E2 were observed in a number of the secondary treated waters. One explanation for this may be that E2 remained in the treated water owing to a problem with the activated sludge processing conditions or to conversion of hormone conjugates to E2. It is also possible that synthetic estrogenic substances and natural hormones other than E2 contributed to the estrogen-like activity of the secondary

Figure 3 Profiles of E2 and estrogen-like activity in activated sludge in batch

![Graph showing E2 equivalent and concentration over time](https://iwaponline.com/wst/article-pdf/46/11-12/367/426134/367.pdf)
treated water. Further research is therefore necessary to ascertain the behavior of these substances in the activated sludge process.

**Evaluation of estrogen-like activity in pilot scale advanced sewage treatment plant**

As shown in Figure 2, it is difficult to remove the estrogen-like activities in sewage completely with an activated sludge process only. It is considered that advanced treatment processes of secondary treated effluent of sewage are necessary to completely remove these unknown estrogenic substances from secondary treated sewage effluent. Figure 4 shows E2 equivalent in each treated water from the pilot scale advanced sewage treatment plant. E2 equivalent with the yeast assay was 74.0 ng E2/l in the influent for the pilot scale plant and 30.6 ng E2/l in secondary treated water. Removal rates for E2 equivalent during secondary treatment were found to be 58.6%. In contrast to this result, the removal rate during the BAF process in Flow A was found to be 96.8%. The following AOP was effective for activity removal too. In Flow B, the ozonation process followed by BAC was as effective as Flow A. Estrogen-like activities showed conspicuously higher removal rate than dissolved TOC during each treatment. The pilot scale plant study showed that advanced sewage treatment such as a BAF process, AOP, and ozonation-BAC process were effective for estrogen-like activity removal from sewage secondary treated effluent.

**Conclusions**

Four sewage treatment plants based on an activated sludge process and a pilot scale plant for advanced sewage treatment located in Japan were evaluated for removal of estrogenic substances using *in vitro* recombinant yeast assay and chemical analysis. The results indicated that 17ß-estradiol (E2) significantly contributed to estrogen-like activity especially in secondary treated effluents. In some cases, it was difficult to sufficiently remove the estrogen-like activities in sewage with an activated sludge process only.

Batch study showed that estrogen-like activity of spiked E2 was easily decreased by an activated sludge treatment. This result suggested that E2 concentrations measured by EIA were interpreted as false positives in effluents, and that unknown estrogenic substances other than E2 might contribute to estrogen-like activity in the secondary treated effluents.

In the pilot scale study, advanced sewage treatment processes such as a biological aerated filtration (BAF) process, an advanced oxidation process (AOP), were effective for the removal of those estrogenic activities contributed by unknown estrogenic substances in sewage secondary treated effluent.
Acknowledgement

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References


