

## Fate of oestrogenic compounds and identification of oestrogenicity in a wastewater treatment process

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**Abstract** Understanding of the fate of oestrogen and oestrogenic compounds is important in improving the removal efficiency for oestrogens in wastewater treatment plants (WWTPs). In this study an attempt was made to clarify the fate of oestrogen, oestrogen sulphates, and oestrogenic compounds (synthetic oestrogen, nonylphenol and its relatives) by an instrumental analysis, and the fate of oestrogenicity by an *in vitro* assay. The investigation was conducted in an activated sludge WWTP in winter and summer, focusing on identification of the primary substances that induce oestrogenicity. Wastewater samples were analysed by employing the silica-gel fractionation technique in conjunction with two-step column chromatography. The results revealed that, in winter, the WWTP efficiencies for the removal of nitrogen and oestrogens decreased and the oestrone level increased with the progress of the treatment. Oestrone and oestrogenic substances are likely to circulate between the aeration tank and the final sedimentation tank. In summer, however, these compounds were effectively removed in the WWTP. The results of the column chromatography coupled with the bioassay suggested that E1 and E2 are the predominant contributors to the oestrogenicity in the influent, return sludge and effluent of the WWTP. The measurement by the instrumental analysis supported these findings.

**Keywords** Endocrine disruptors; recombinant yeast screening assay; wastewater treatment

### Introduction

Much attention has been directed at the presence of oestrogens in an aquatic environment (Desbrow *et al.*, 1998) because of their endocrine disrupting properties. From the view point of the protection and conservation of aquatic organisms (Routledge *et al.*, 1998), oestrogenic compounds should be removed from wastewater in wastewater treatment plants (WWTPs). To improve the WWTP efficiency for the removal of oestrogens, understanding of the fate of oestrogen is important. An analytical method using GC/MS or LC/MS/MS is a sensitive, selective technique to measure oestrogens and oestrogenic compounds. However, this technique may not capture all oestrogenic compounds present in environmental samples. Because numerous compounds mingle with oestrogens and oestrogenic compounds in wastewater, interfering with accurate quantification of the target compounds, additional purification steps are necessary prior to instrumental analysis. The bioassay techniques that employ DNA recombinant yeast strain containing a human oestrogen receptor gene (Routledge and Sumpter, 1996) can determine total oestrogenicity in environmental samples. However, the oestrogenicity evaluated by this type of bioassay is often underestimated due to the presence of certain chemicals toxic to the yeast cells (Nakada *et al.*, 2004), or sometimes is overestimated due to the presence of linear alkylbenzene sulphonate (LAS) (Miyamoto *et al.*, 2002). LAS is not oestrogenic itself. The difference between the results obtained from the instrumental analysis and from the bioassay does not necessarily imply the presence of undetected oestrogenic

compound(s) in the samples. To identify the chemicals predominantly contributing to oestrogenicity, this study employed instrumental analysis in conjunction with the bioassay technique coupled with the fractionation technique. The fractionation method was established as a toxicity identification and evaluation (TIE) approach (Mount and Anderson-Carnahan, 1998), and was further modified to isolate and identify oestrogens (Desbrow *et al.*, 1998; Snyder *et al.*, 2001; Nakada *et al.*, 2004). The objectives of this study were to clarify the behaviour of oestrogenic compounds and oestrogenicity in a municipal, activated sludge WWTP, and to identify the chemicals predominantly contributing to the oestrogenicity using two-step column chromatography.

## Materials and methods

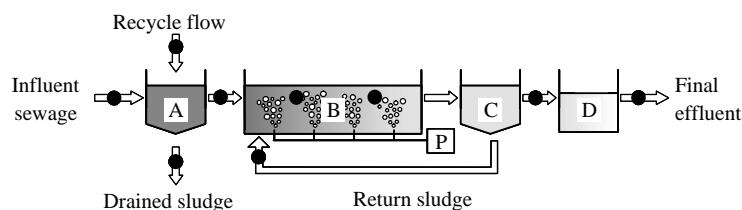
### Wastewater treatment plant and sampling procedure

The surveys were conducted in the activated sludge WWTP in Kanagawa prefecture, Japan, in winter (January) and summer (July) of 2004. The WWTP mainly receives domestic wastewater from the community of approximately 30,000 people and wastewater from local industries. The industries contribute approximately 10% of the total flow. The wastewater (12,300 and 13,400 m<sup>3</sup>/day in winter and summer, respectively) was subjected to biological treatment following primary treatment (sedimentation). The hydraulic retention times (HRT) of the primary sedimentation tank during the winter and summer survey periods were 12 and 10 h, respectively, and the solids retention times (SRT) were 8.2 and 6.0 d, respectively. The aeration tank is a long channel plug-flow reactor. The treated wastewater was chlorinated before discharging to the receiving water (Figure 1).

Twenty four-hour composite samples were collected from the nine sampling locations in the WWTP and were stored in a refrigerator or in a chest containing iced water until filtration. The aeration tank samples were collected at two locations: the mid-distance point (AT<sub>M</sub>) and end of the tank (AT<sub>E</sub>). A proportion of the composite samples was analysed for conventional water quality parameters. The remaining proportion was filtered using glass fibre filters (GF/B, pore size: 1.0 µm) within 24 h after collection. The filtrate was solid-phase extracted using an octadecyl silica cartridge (Sep-Pak tC18 long, Waters) at a rate of 10 mL/min and eluted by 15 mL of methanol. The filter residue was extracted three times with methanol in an ultra-sonic bath. The extract was subjected to instrumental analysis and yeast oestrogen screening (YES) assay.

### Instrumental analysis

The conventional water quality parameters (e.g. suspended solids (SS), nitrogens (NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, and NO<sub>2</sub><sup>-</sup>-N), biochemical oxygen demand (BOD) and chemical oxygen demand (COD)) were measured according to the standard method (Japanese Standards Association, 1998). Natural and synthetic oestrogens including oestrone (E1), 17β-oestradiol (E2), oestriol (E3), E1 sulphate (E1-3S), E2 sulphate (E2-mono S), and ethynyl



**Figure 1** Sampling location (indicated with a closed circle) in a municipal sewage treatment plant. A: Primary settling tank; B: Biological reaction tank; C: Final sedimentation tank; D: Chlorination tank; P: Aeration pump

oestradiol (EE2) were quantified using the LC/MS/MS method described elsewhere (Komori *et al.* 2004). Nonylphenol (NP), nonylphenol ethoxylates (NPEO) and nonylphenoxy acetates (NPEC) were also analysed using HPLC and LC/MS/MS (Komori *et al.*, 2002). NP, NPEO and NPEC are suspected endocrine disrupting chemicals. The relative oestrogenicity given in terms of E2-equivalent concentration (ng/L-E2 equivalent) is defined by  $EEQ_{INST} = (\text{Conc.}) \times (\text{REP})$ ; where  $EEQ_{INST}$  is the relative oestrogenicity (ng/L), Conc. is the concentration of given endocrine disruptors (ng/L) and REP is the relative oestrogenic potential (Yakou *et al.*, 1999).

#### Yeast oestrogen screening assay

Oestrogenicity was measured using the YES assay, which is described elsewhere (Yakou *et al.*, 1999). Each extract from the filtrates, filter residue and fractionated sample (described below) were dried under a gentle stream of nitrogen gas, redissolved in 1,000  $\mu\text{L}$  of methanol (stock solution). A part of the stock solution (200  $\mu\text{L}$ ) was dried by a nitrogen stream and redissolved in dimethyl sulphoxide (DMSO), and then subjected to the YES assay. The rest of the stock solution was stored below 4 °C until fractionation. The relative oestrogenicity given in terms of E2-equivalent concentration (ng/L-E2 equivalent) is defined by  $EEQ_{YES} = (\text{EC50 of E2})/(\text{EC50 of sample})$ ; where  $EEQ_{YES}$  is the relative oestrogenicity (ng/L), EC50 of sample E2 (ng) is estimated from the dose-response curve of E2 and EC50 of sample (L) is obtained from the dose-response curve of the test sample at a given concentration factor. E2 is the positive control. Both EC50 values were evaluated at the same absorbance.

#### Fractionation technique using normal-phase chromatography

For the winter survey samples, the remaining stock solution (see above) was dried under a gentle stream of nitrogen gas, redissolved in a hexane/dichloromethane (DCM) solution (1:1, v/v), and fractionated using a silica-gel open-column (Sep-Pak Si, 690 mg, Waters) into seven fractions. The procedure employed is based on previously reported methods (Nakada *et al.*, 1999; Miyamoto *et al.*, 2002; Nakada *et al.*, 2004) with minor modifications. In brief, the column was rinsed with 10 mL of hexane to remove organic contamination. Fractionation of the sample extracts was carried out by eluting the column with 5 mL each of hexane/DCM (50:50 v/v), hexane/DCM (30:70 v/v), DCM, DCM/acetone (70:30 v/v), DCM/acetone (30:70 v/v), acetone and methanol (fraction (Fr.) 1–7, Figure 4(a)). The composition and volumes of the solvents used in the column chromatography were optimised using the standard solutions for individual compounds. NP was eluted in Fr. 2; E1, E2 and EE2 in Fr. 4; E3, E1-3S, and E2-mono S in Fr. 5 as shown in Figure 4(a). Each fraction was dried under a gentle nitrogen stream and redissolved in 1,000  $\mu\text{L}$  of methanol (Si-fractionated stock solution). Part of the stock solution (200  $\mu\text{L}$ ) was dried by a stream of nitrogen gas and redissolved in DMSO, and then subjected to the YES assay. The remaining Si-fractionated stock solution was stored at 4 °C until used for the further fractionation.

#### Further fractionation technique using reverse-phase chromatography

The rest of the Si-fractionated stock solution, which had exhibited a positive oestrogenic response in the previous assay, was transferred into acetonitrile and made up to 300  $\mu\text{L}$ . The solution was then filtered using a membrane filter (pore size: 0.45  $\mu\text{m}$ ) and subjected to further fractionation using HPLC. This was performed using a HPLC model 131H1 (Gilson) equipped with a Hypersil Green ENV HPLC column (4.6  $\times$  250 mm, Thermo Hypersil-Keystone) fitted with a guard column (4.6  $\times$  50 mm) and ultra-violet and fluorescence detectors. HPLC-grade acetonitrile and water containing 0.01% of formic

acid were used as a mobile phase at a flow rate of 1.0 mL/min. The gradient pattern is described in Figure 5(a). Twenty microlitres of the acetonitrile solution were injected three times and then 48 2-mL fine fractions were collected (one every 2 min). The gradient pattern and interval of fractionation of the HPLC technique were optimised using standard solutions of individual compounds. E3 was eluted in Fr. 11, E2-mono S in Fr. 11–12, E1-3S in Fr. 13, E2 in Fr. 18, and E1 and EE2 in Fr. 20 as shown in Figure 5(b). Each fraction was transferred into DMSO and subjected to the YES assay.

## Results and discussion

### Fate of inorganic and organic compounds in the wastewater treatment processes

The conventional parameters observed in the WWTP are summarized in Table 1. The effective reduction of SS, DOC, BOD and COD occurred during the winter and summer survey periods. The reduction of  $\text{NH}_4^+$ -N was, however, relatively small with partial transformation of  $\text{NH}_4^+$ -N to  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N. The removal of total nitrogen was smaller in winter (26%) than in summer (60%), probably due to the effect of influent temperature on nitrification.

The concentrations of the oestrogenic compounds (NP, total NPEO, total NPEC, E2, E1, EE2, E3, E1-3S and E2-monoS) in the WWTP are presented in Table 2. The presented values are summations of the concentrations in the dissolved and suspended phases. NP, total NPEO and total NPEC in the plant influent and finally effluent exhibited the same levels as those reported by other investigators (Tanaka *et al.*, 2003). The concentrations of E2, E1 and E3 are also in similar ranges to those reported in the literature (Tanaka *et al.*, 2003; Komori *et al.*, 2004), except for E3 in the influent. EE2 was not detected in any of the samples analysed. Since 1999, the marketing of contraceptive pills containing EE2 has been permitted in Japan. Tanaka *et al.* (2003) studied EE2 in 20 WWTPs in 2000 and Komori *et al.* (2004) in 12 WWTPs in 2003, and reported that EE2 existed in wastewater, generally below their detection limits of 0.5 ng/L and 1.2 ng/L, respectively. As was reported by Tanaka *et al.* (2003) and Komori *et al.* (2004), the EE2 levels in wastewater in Japan are not as high as those in Europe and the US even though five years has passed since the use of EE2 was permitted in 1999.

The mass balance of E1 in the WWTP is shown in Figure 2. The E1 mass was computed based on the E1 concentration and wastewater flow at each sampling location. As seen, the difference in the E1 flux in the influent between the winter and summer survey periods is minimal. During the winter survey, considerably higher E1 fluxes were observed in the effluents of the primary settling tank and final sedimentation tank than those in the plant influent. The E1 flux increased markedly in the aeration tank.

Increases of E1 in WWTP processes were also observed in Spain (Carballa *et al.*, 2004) and Japan (Tanaka *et al.*, 2003). In the study by Carballa *et al.* (2004), the increase of E1 was thought to be due to the oxidation of E2 and the cleavage of glucuronides. Only a limited amount of data are available on conjugated oestrogen in wastewater. In the present study, two conjugates (sulphate) were analysed in addition to E1, E2, E3 and EE2 (Table 2). The conjugated oestrogen concentrations observed are somewhat lower than those reported by Komori *et al.* (2004). Changes in the concentrations of these compounds in the stages of the treatment processes cannot be fully explained based on the reactions proposed by Carballa *et al.* (2004). The concentrations of E2, E1-3S and E-2-monoA are not high enough to make up the observed increase in E1 in the WWTP. The results may suggest the presence of E1 precursors in raw sewage.

In both the winter and summer surveys, the sum of the E1 fluxes in the effluent of the final sedimentation tank and in the return sludge line is significantly higher than that in the aeration tank (Figure 2). The flux based on E1 in the dissolved phase is greater than that in

**Table 1** Characterization of wastewater samples along the treatment process

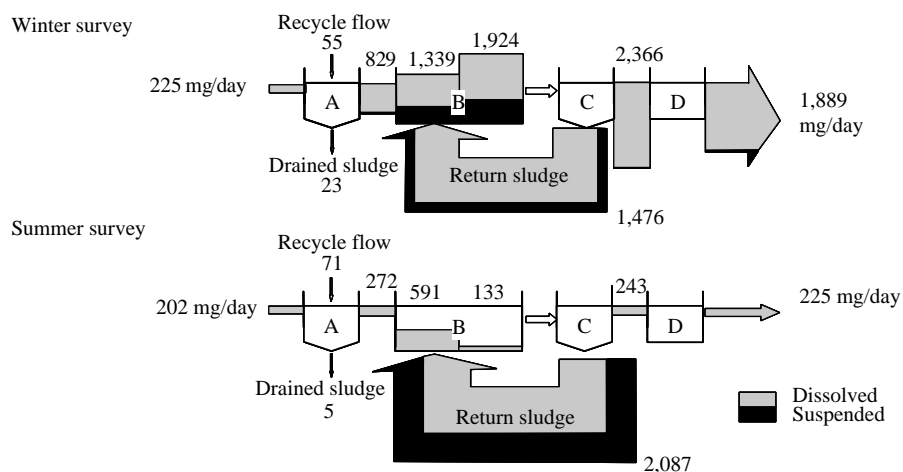
Month	Sample	T <sub>w</sub> (°C)	SS (mg/L)	VSS (mg/L)	DOC (mg/L)	NH <sub>4</sub> -N (mg/L)	NO <sub>2</sub> -N (mg/L)	NO <sub>3</sub> -N (mg/L)	BOD (mg/L)	COD <sub>Mn</sub> (mg/L)
January 2003 (Winter survey)	Influent	16.4	80.0	71	35.7	20.2	0.20	0.19	172	144
	Recycle flow	–	30.7	273	30.2	18.7	0.67	0.39	–	–
	Effluent of primary settling tank	–	72.0	64	30.3	21.6	0.00	ND	121	70
	Drained sludge	–	5,230	4,650	98.9	28.2	0.00	ND	–	–
	Return sludge	–	3,980	3,280	6.76	17.9	0.02	0.02	–	–
	Supernatant of aeration tank	–	1,440	1,200	6.98	19.2	0.13	0.07	–	–
	Supernatant of aeration tank	–	1,340	1,120	6.32	16.9	0.32	0.41	–	–
	Secondary effluent	–	4.5	3.7	6.79	17.2	0.46	0.45	8.7	8.6
	Final effluent	–	2.0	2.0	5.98	15.0	1.09	1.25	1.8	10
July 2003 (Summer survey)	Influent	26.7	160	138	34.1	18.7	0.00	ND	169	112
	Recycle flow	–	200	172	31.6	11.6	0.89	0.86	–	–
	Effluent of primary settling tank	–	68	59	31.8	15.7	0.18	ND	114	62
	Drained sludge	–	3,980	3,480	91.8	23.9	0.03	ND	–	–
	Return sludge	–	3,280	2,700	8.58	6.37	0.02	0.05	–	–
	Supernatant of aeration tank	–	1,160	965	8.33	10.8	0.38	0.42	–	–
	Supernatant of aeration tank	–	1,260	1,030	7.96	4.43	0.05	3.23	–	–
	Secondary effluent	–	3.0	2.0	7.07	4.72	0.33	5.88	9.0	11
	Final effluent	–	3.0	2.0	7.38	7.53	0.83	2.69	0.9	10

ND: not detected; –: not measured

**Table 2** Fates of nonylphenol (NP), total nonylphenol ethoxylates (SNPnEO), total nonylcarboxylates (SNPnEC), 17 $\beta$ -oestradiol (E2), oestrone (E1), oestriol (E3), sulphated E1 (E1-3S) and sulphated E2 (E2-monoS) along the wastewater treatment processes and limits of quantification (LOQ) of each compound

Month	Sample	NP ( $\mu\text{g/L}$ )	SNPnEO ( $\mu\text{g/L}$ )	SNPnEC ( $\mu\text{g/L}$ )	E2 (ng/L)	E1 (ng/L)	EE2 (ng/L)	E3 (ng/L)	E1-3S (ng/L)	E2-monoS (ng/L)
January 2003 (Winter survey)	Influent	3.4	106	11.1	23.4	18.2	ND	na	2.9	6.4
	Recycle flow	2.2	14.6	14.7	17.2	133	ND	11.4	1.8	4.1
	Effluent of primary settling tank	2.6	68.9	10.0	5.5	65.3	ND	72.5	2.8	5.6
	Drained sludge	67	435	18.6	59.4	85.6	ND	80.2	2.1	4.5
	Return sludge	13	97.6	50.3	17.4	181	ND	4.4	2.1	4.4
	Supernatant of aeration tank	7.5	55.8	29.4	13.8	65.4	ND	5.2	2.4	3.8
	Supernatant of aeration tank	8.2	58.8	32.3	13.3	94.0	ND	ND	1.7	2.9
	Secondary effluent	0.4	3.7	11.9	11.5	192	ND	ND	1.9	3.5
	Final effluent	0.4	3.0	12.7	7.0	154	ND	5.5	2.1	3.3
	July 2003 (Summer survey)	Influent	1.7	48.3	19.3	3.9	15.1	ND	na	1.1
Recycle flow		0.5	4.3	10.8	2.7	49.1	ND	8.4	2.8	5.8
Effluent of primary settling tank		1.0	36.7	18.1	3.8	19.3	ND	na	1.8	4.5
Drained sludge		24	197	33.3	17.2	20.3	ND	43.7	7.9	10.4
Return sludge		4.2	56.1	45.6	31.1	257	ND	24.8	15.0	16.5
Supernatant of aeration tank		2.4	37.8	25.3	8.1	26.6	ND	6.8	9.5	13.9
Supernatant of aeration tank		1.2	27.5	24.6	ND	6.0	ND	ND	7.9	18.0
Secondary effluent		ND	1.6	10.7	ND	15.3	ND	5.8	2.6	6.8
Final effluent		0.2	1.4	11.7	ND	22.2	ND	5.6	2.6	6.6
LOQ		0.1	1	0.01	1	2	2	2	0.1	0.4

ND: not detected; na: not available

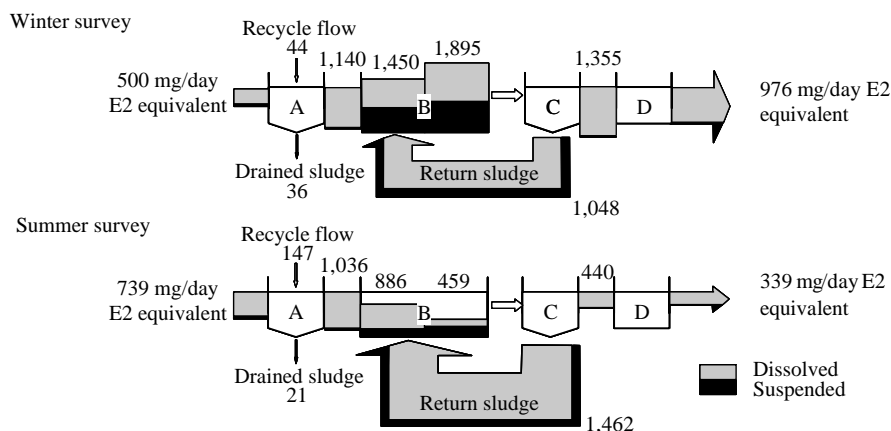


**Figure 2** Mass balance of oestrone (in mg/day) through physicochemical treatment (A and C), biological treatment (B) and chlorination process (D) during winter and summer in a municipal sewage treatment plant in Japan. A: Primary settling tank; B: Aeration tank; C: Final sedimentation tank; D: Chlorination tank

the suspended phase, suggesting the hydrophilic property of E1. E1 is effectively removed in the aeration tank in summer as compared with the results in winter. The E1 fluxes in the recycle flow from the sludge treatment process and in the sludge wasted from the primary sedimentation tank are insignificant in the mass balance in the WWTP process.

The relative mass fluxes computed based on the oestrogenicity (E2 equivalent) at the sampling locations are shown in Figure 3. The increase in oestrogenicity is apparent in the final sedimentation tank, and the elevated activity is evident in the return sludge in both winter and summer, as is the case for E1.

The removal efficiencies of the primary settling tank, aeration tank, final sedimentation tank and chlorination tank are summarised in Table 3. All target pollutants, except oestrogen sulphate, are effectively removed in summer. In particular, NP and NPEO are removed most effectively in both the biological and physicochemical treatment processes. It seems likely that the removal of free oestrogens (E1 and E2) is dependent on the



**Figure 3** Mass balance of oestrogenicity (in mg/day, E2 equivalent) through physicochemical treatment (A and C), biological treatment (B) and chlorination process (D) during winter and summer in a municipal sewage treatment plant in Japan. A: Primary settling tank; B: Aeration tank; C: Final sedimentation tank; D: Chlorination tank

**Table 3** Removal efficiency of each compound and oestrogenic activity in wastewater treatment processes

Month	Sample	NP (%)	SNPnEO (%)	SNPnEC (%)	E2 (%)	E1 (%)	E1-3S (%)	E2-monoS (%)	Oestrogenic activity (%)
January 2003 (Winter survey)	Primary settling tank	24	35	10	76	-259	4	13	-128
	Aeration tank and final settling tank	85	95	-18	-109	-194	33	38	-19
	Disinfection tank	0	18	-7	39	20	-11	6	28
	Overall	88	97	-13	70	-743	28	49	-95
July 2003 (Summer survey)	Primary settling tank	38	20	1	-3	-35	-72	-90	-40
	Aeration tank and final settling tank	95 < *	95	33	87 < *	11	-63	-70	57
	Disinfection tank	na	44	31	na	7	36	38	23
	Overall	91	98	54	87 < *	-11	-79	-100	54

na: not available because NP and E2 in influent of disinfection tank were undetectable

\*: half value of LOQ was used for calculation of removal efficiency



operating conditions of the aeration tank, and that these oestrogens circulate through the aeration tank and final sedimentation tank. The behaviour of free oestrogens is similar to that of substances exhibiting oestrogenicity. In contrast, oestrogen sulphates appear to follow an inverse trend, although this observation has not been fully verified due to the insufficient amount of data on oestrogen conjugates. Thus, more comprehensive studies are needed.

Saino *et al.* (2004) reported that an SRT of 12.5 d is required for E1 oxidation at water temperatures ranging from 11 to 14 °C. In the present study, the SRT is 8.2 d, which may not be sufficient for effective E1 removal. In summer, the effective reduction of E1 occurs with an SRT of 6 d. The high efficiency in summer can be attributed to the higher water temperature (27 °C), allowing rapid growth of the E1-oxidising microorganisms.

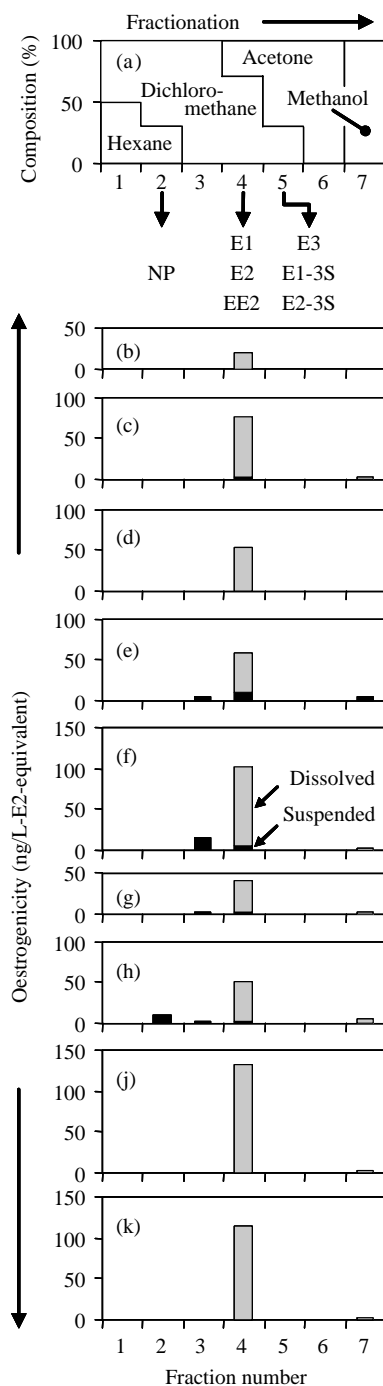
The fate of E1 was similar to that of the oestrogenicity in the WWTP. Furthermore,  $EEQ_{INST}$  corresponds to  $EEQ_{YES}$  (described below). Thus, E1 is probably the predominant compound that induces oestrogenicity. Since the YES assay is subjected to the compounds coexisting in wastewater, and often results in underestimation or overestimation of oestrogenic compounds, identification of the substances, which are unknown but dominant, contributing to the oestrogenicity is necessary. In this study, the fractionation technique was employed.

#### Identification of oestrogenicity (1)

The fractionation method using a silica-gel column was developed and applied to the WWTP samples collected in winter. Samples in both dissolved and suspended phases were fractionated into seven fractions (Figure 4(a)), which were further subjected to the YES assay. The greatest oestrogenicity was detected in the fourth fraction (Si-Fr. 4), which had been clarified to contain E1, E2 and EE2. Stronger activities are evident in the Si-Fr. 4 of the dissolved phase. The Si-Fr. 4 contributes 77–99% (average: 91%) of the total activity of each sample. Considerably smaller activities were detected in other fractions including Si-Fr. 2, 3 and 7.

#### Identification of the oestrogenicity (2)

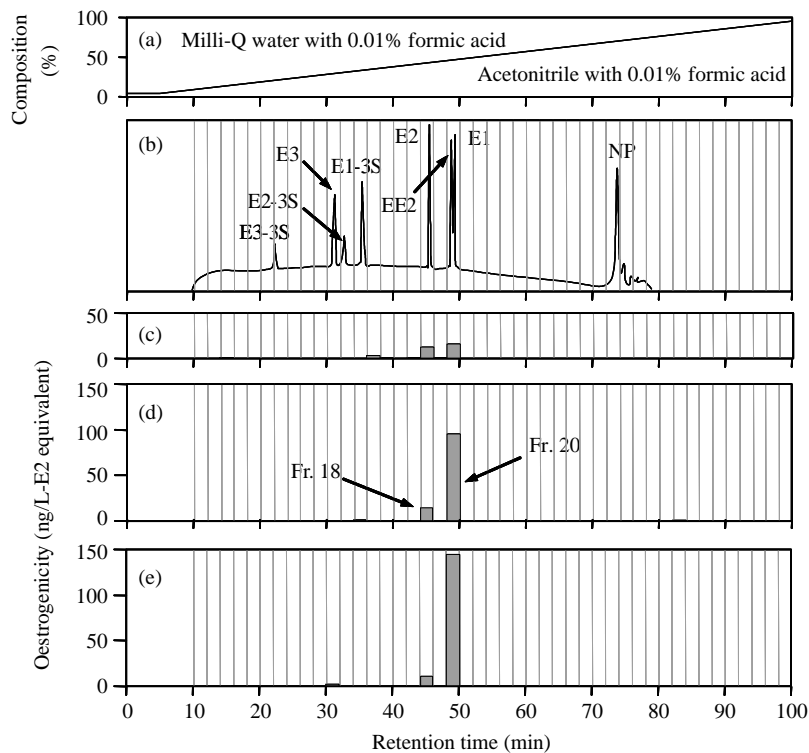
In order to ascertain the chemicals predominantly contributing to oestrogenicity, the oestrogenic active fractions (Si-Fr. 2, 4 and 7) extracted from the dissolved phase of the influent, return sludge and final effluent samples were further fractionated using HPLC followed by the YES assay. As seen in Figure 5, two fractions were detected between 45 and 50 min (corresponding to HPLC-Fr. 18 and 20) with the most significant activity in Si-Fr. 4. From the chromatogram of the standard mixture (Figure 5(b)), it can be conceived that E2, E1 and EE2 contribute to the high activity of the two fractions (HPLC-Fr. 18 and 20). Since EE2 was not detected in all the samples (limit of detection: 20 ng/L), E2 and E1 are probably the main species inducing oestrogenicity in HPLC-Fr. 18 and 20, respectively. The result of the instrumental analysis also supports the findings that E1 and E2 are the predominant chemicals contributing oestrogenicity in the WWTP. Although small activities were detected in a few fractions from each of Si-Fr. 4 (Figure 5), Si-Fr. 2 and 7 (data not shown), elution of specific compounds analysed in this study were not detected at these fractions. The contributions of the unknown activities to the total activity were estimated to be 19% for the influent, 6% for the return sludge and 6% for the final effluent. On the other hand, the percent contributions by NP, E1 and E2 were estimated to be 0, 45 and 36%, respectively, in the influent, 0.3, 8 and 13%, respectively, in the return sludge, and 0, 86 and 8%, respectively, in the final effluent.



**Figure 4** Solvent composition on Silica-gel fractionation with elution pattern of each compound (a), and profiles of oestrogenicity in each fraction of dissolved and suspended phases of influent (b), recycle flow (c), effluent of primary settling tank (d), drained sludge (e), return sludge (f), aeration tank,  $AT_M$  (g), aeration tank,  $AT_E$  (h), secondary effluent (j), final effluent (k)

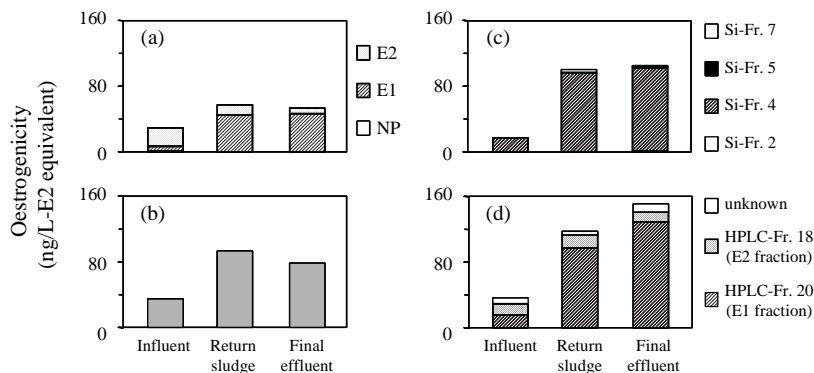
**Comparison of oestrogenicity**

Figure 6 shows the oestrogenicity values ( $EEQ_{INST}$  and  $EEQ_{YES}$ ) in the influent, return sludge and final effluent. The comparisons reveal that the  $EEQ_{YES}$  are somewhat larger



**Figure 5** Solvent composition on high performance liquid chromatography (a), chromatogram of target standards (b), oestrogenic activity profiles of each 4th fraction on the silica-gel chromatography of influent (c), return sludge (d) and final effluent (e). Filtration residuals of each sample were not measured oestrogenicity. Horizontal lines on parts b, c, d and e represent start and end time of collection periods, and then each collected fraction was subjected to YES assay

than  $EEQ_{INST}$ , especially in the return sludge and final effluent. These differences may suggest that the oestrogenic compounds unidentified by the instrumental analysis exist in the influent, return sludge and final effluent. With the Silica-gel column and HPLC technique, we could not detect oestrogenic fractions in rough and fine fractionations except the fractions which elute E1 and E2. The contribution by unknown substances to the oestrogenicity is estimated to be between 6 and 18% and their contribution is extremely



**Figure 6** Comparison of oestrogenicity between the instrumental analysis ( $EEQ_{INST}$ ) and the YES assay ( $EEQ_{YES}$ ): (a) the instrumental analysis, (b) the YES direct assay, (c) the YES after fractionation by the silica-gel column and (d) the YES after silica-gel/HPLC fractionation (see text for details)

small, as mentioned above. Thus, it can be concluded that the detected oestrogenicities were mainly induced by natural oestrogens, largely by E1.

### Conclusions

This field investigation and data analysis provided the following conclusions.

- (1) Reductions of E2, E1 and oestrogenicity in the WWTP were greater in summer than in winter. While SS, BOD and COD were removed effectively in both winter and summer, nitrogen was poorly removed in winter (i.e. 41% reduction in summer, 16% in winter).
- (2) Although E2 was removed effectively in both seasons (70% in winter and 87% in summer), E1 increased by 740% in winter and 10% in summer, overall.
- (3) Oestrogen sulphates are hardly degradable in the treatment processes. EE2 (synthetic oestrogen) existed below its detection level in all samples.
- (4) Overall, oestrogenicity was increased by 95 and 54% in winter and summer, respectively, in the WWTP studied. The substances that induce oestrogenicity appear to circulate through the aeration tank and the final sedimentation tank, as was observed for E1.
- (5) The oestrogenicity (in the influent, return sludge and final effluent) determined by the YES assay can be separated by the two-step fractionation technique. The  $EEQ_{YES}$  was compared with the  $EEQ_{INST}$ . Although there are some differences between the two EEQ values, the detected oestrogenicities are thought to be caused mainly by natural oestrogens.
- (6) Appropriate treatment and control measures are necessary in the WWTP to remove natural oestrogens, which consequently reduce the oestrogenicity in treated wastewater.

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